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Screening of parasitic weeds for natural compounds with biotechnological value

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Abstract

The parasitic plant species of *Cuscuta* have a long history of traditional medicinal and nutraceutical applications, but hardly any investigations have taken on comparisons of species or different parts of the plant, or put their results in relation to respective host plants. The aim of this thesis was to evaluate and compare stem samples of three different *Cuscuta* species: *Cuscuta reflexa*, *Cuscuta platyloba* and *Cuscuta campestris*. Moreover, the flowers of *C. campestris* and the host plant *P. zonale* where included in the evaluation to compare compound composition in different tissues and search for similarities between parasite and host. Methanol extracts of the samples were fractionated and evaluated for their activities in different bioassays. Additionally samples were analyzed via mass spectrometry to compare compound composition and identify active compounds. Results showed unresolved anti-bacterial activity, as well as anti-cancer activity that may partly be connected to a compound named quercetin known already for its anti-cancer potential. Biofilm inhibiting activity as well as enzyme inhibition against protein tyrosine phosphatase 1B (PTP1B), an enzyme linked to diabetes mellitus type 2, could not be observed here. Mass spectrometric data as well as differences in the cell viability assay point to differences in compound composition among the different *Cuscuta* species. Comparison of stem and flower tissues of *C. campestris* accounted for a higher potential of cytotoxic activity in flower extracts. However, in contrast to the two other species that were evaluated, the cytotoxicity was equal against cancerous and non-cancerous cell lines. Activities in *C. reflexa*- and *C. platyloba*-extracts were higher against the human melanoma cell line A2058 than the human lung fibroblast cell line MRC5 and might therefore be specific against cancer cells. Overall, this study shows that a comparative approach is worthwhile, and in the context of host/parasite interactions, the inclusion of host species in the study design is particularly relevant.

Kurzfassung

Die parasitische Pflanzenspezies *Cuscuta* wird schon seit langem in der traditionellen Medizin verschiedener Völker für die Heilung von Krankheiten und zur Aufwertung von Nahrungsmitteln verwendet. Es gibt kaum Untersuchungen, die Vergleiche zwischen den verschiedenen Arten oder unterschiedlichen Teilen der Pflanze herstellen und ihre Ergebnisse auch auf die Wirtspflanze bezogen betrachten. In dieser Arbeit wurden Proben vom Stiel dreier verschiedener *Cuscuta* Arten, *Cuscuta reflexa*, *Cuscuta platyloba* und *Cuscuta campestris,* untersucht und verglichen. Außerdem wurden die Blüten von *C. campestris* und die Wirtspflanze *Pelargonium zonale* in die Untersuchungen mit eingebunden, um die Zusammensetzung von unterschiedlichen pflanzlichen Geweben zu vergleichen sowie nach Gemeinsamkeiten in Parasit und Wirt zu suchen. Methanolextrakte der Proben wurden fraktioniert und auf ihre Wirksamkeit in verschiedenen Bioassays untersucht. Massenspektroskopische Analysen wurden verwendet um ihre chemische Zusammensetzung zu untersuchen und aktive Substanzen zu identifizieren. Eine Wirksamkeit der Proben sowohl gegen bakterielles Wachstum, als auch gegen das Wachstum von Krebszellen wurde festgestellt. Während im Fall der Hemmung von bakteriellem Wachstum keine bestimmte aktive Substanz festgestellt werden konnte, wurde die Wirkung der Proben gegenüber Krebszellen teilweise mit Quercetin, einer Substanz, die bereits für ihre Wirksamkeit gegen Krebs bekannt ist , in Verbindung gebracht. Außerdem wurden die Proben auf ihre Wirksamkeit gegen die Bildung von Biofilmen und auf ihre hemmende Wirkung in Bezug auf das Enzym *Protein Tyrosin Phosphatase 1B* (PTP1B), das in Verbindung mit Diabetes Mellitus Typ 2 steht, hin untersucht. In beiden Fällen konnte allerdings kein Effekt der Proben festgestellt werden. Die Ergebnisse aus Massenspektrometrie und Viabilitätstest weisen auf eine unterschiedliche Zusammensetzung der Extrakte der verschiedenen *Cuscuta* Arten hin. Vergleiche der Stiel- und Blütenproben von *C. campestris* zeigten eine größere Zytotoxizität in den Blütenproben. Im Gegensatz zu den anderen beiden untersuchten Arten war hier die zelltötende Wirkung gegen die humane Melanomzelllinie A2058 und humane Lungenfibroblasten (MRC5) allerdings gleich. *C. reflexa*- und *C. platyloba*-Extrakte dagegen, zeigten eine höhere Wirksamkeit gegen die Krebszelllinie, was für eine spezifische Wirkung spricht. Insgesamt zeigt diese Studie, dass ein vergleichender Ansatz in Bezug auf unterschiedliche *Cuscuta* Arten durchaus lohnenswert ist und im Zusammenhang mit Wirt-Parasit-Interaktionen die Wirtspflanze in entsprechende Studien mit eingebunden werden sollte.

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Abbreviations

AQOS	Aqueous One Solution	**MS**	Mass Spectrometry
BPI	Base Peak Intensity	**NMR**	Nuclear Magnetic Resonance
CFU	Colony Forming Units	**OD**	Optical Density
CNS	Central Nervous System	**ONC**	Overnight culture
DiFMUP	6,8-difluoro-4-methylumbelliferylphosphat	**PCA**	Principle Component Analysis
DMEM	Dulbecco's Modified Eagle Medium	**PDA**	Photodiode Array Detector
DMSO	Dimethyl-Sulfoxide	**PTP1B**	Protein Tyrosine Phosphatase 1B
DWF	Dry Weight of Fractions	**Rcf**	Relative Centrifugal Force
EDTA	Ethylenediaminetetraacetic Acid	**RPMI**	Roswell Park Memorial Institute
EMEM	Eagle's Minimum Essential Medium	**TC-PTP**	T-Cell Protein Tyrosine Phosphatase
ESI	Electrospray Ionization	**TIC**	Total Ion Count
FBS	Fetal Bovine Serum	**ToF**	Time of Flight
HPLC	High Performance Liquid Chromatography	**TSB**	Tryptic Soy Broth
LB	Lysogeny Broth	**UHPLC**	Ultra High Performance Liquid Chromatography
LC	Liquid Chromatography	**UiT**	Universitetet i Tromsø
m/z	Mass to Charge Ratio	**UNN**	Universitetssykehuset Nord-Norge
MIC	Minimum Inhibition Concentration	**UV**	Ultraviolet
MQ water	Milli-Q Water		

1. Introduction

1.1 Traditional Use of Plants

Human evolution has been connected to plants from the very beginning. Wherever men lived, they found a large variety of plants. Thus, mankind has a long history in exploring the various possible utilizations of plants.

Soon early humans must have discovered that plants were not only edible but that certain types also affected their bodies and minds in a special way. They made them feel sick or even killed them, they had the ability to alter their perception and some of them helped with recovery when people were injured or ill. As this was useful to them, humans tested and experimented with all kinds of plants and learned much about the properties of their contents. Ultimately, they were able to use that knowledge to their advantage and the respective plants were integrated in their daily life for tasks like healing or hunting, hygiene, preservation and spiritual rituals (Etkin & Ross, 1982; Hill, 1982). This vast knowledge about the uses of plants still exists today in native peoples (Arnason *et al.*, 1981; Hutchings, 1989) and is collected in ethnobotany. Today, we can use it as a lead and analyze it further with the new methods that we have now at our disposal.

However, there is a certain kind of specialists within the plant kingdom: parasitic plants. They have been used for the same applications as other plants for just as long, but they differ from them in many other aspects.

1.1.1 Parasitic Plants

Parasitic lifestyle can be found throughout all kingdoms of life and is highly successful. Parasitic plants are represented by about 4000 species around the world. About 1% of Angiosperm species are considered parasites and within those the parasitic lifestyle has developed independently 12 to 13 times (Poulin & Morand, 2000). Thus, the spectrum of parasitic plants is quite diverse and spread over different families and orders. The most familiar parasitic plants in Europe are mistletoes, which grow high up in trees and have had a special meaning in healing dating back until the early days of the druids.

The Unique Lifestyle of Parasitic Plants

Parasitic plants are generally defined as plants that derive their nutrients from other plants. They obtain all or part of them with the help of specialized feeding organs called haustoria (from the Latin *haurire*, to drink) (Hibberd & Jeschke, 2001; Smith, 2013). They can be classified according to certain attributes of their lifestyle. The presence or absence of functional

chloroplasts and therefore the parasites' ability or disability to perform photosynthesis (Musselmann, 1995) in a way that they are able to sustain themselves defines their status as hemiparasites or holoparasites. Hemiparasites can be facultative, only opportunistically parasitizing, or obligate parasites; holoparasites are not able to survive on their own but depend on their host and are therefore always obligate parasites (Westwood *et al.*, 2010). The site of attachment further classifies parasitic plants as shoot or root parasites, specificity in host choice distinguishes them into generalists and specialists. Parasitic plants are also able to infect multiple host plants or even host plant species at the same time (Poulin, 2011). As parasitic plants interact intimately with other plants in their surroundings, it is also consistent that they influence growth, phenotype and reproduction of other plants, leading to a chain of effects on many other features of the ecosystem, like community structure, the zonation of vegetation, herbivores, pollinators and distribution of seeds. They can even have influence on physical parameters like temperature, water and nutrients (Press & Phoenix, 2005). While under natural conditions, parasitic plants are well integrated and an important part of a working ecosystem, they can cause serious problems in agriculture as they can completely destroy harvests (Smith, 2013).

Parasites differ in how they acquire solutes from their hosts. Using haustoria, the parasite connects to the solute fluxes of the host plant and draws nutrients out. These are amino acids, organic acids, ions and water in the host xylem or sugars, ions and amino acids of the host phloem. The nature of contact can vary from direct links and continuity between the conducting cells, to the involvement of transfer cells between host and parasite (Hibberd & Jeschke, 2001). The connection to phloem or xylem, or both, and the extent of solute flow varies depending on the parasite and the regulation. The exact mode and selectivity of exchange however, are still not entirely known. Possible modes of connection might be open channels between xylems or phloems of parasite and host but also through specialized cell-to-cell connections. For example, *Cuscuta* species establish contact through interspecific plasmodesmata (Birschwilks *et al.*, 2006).

The Parasitic Genus *Cuscuta* and Its Occurrence

Cuscuta species, commonly referred to as "dodder" are part of the *Convolvulacea*, or morning glory, plant family. They are distributed all over the world, with an exception of the arctic regions (Figure 1), with about 200 known species (Garcia *et al.*, 2014). They are perennial parasitic plants in the form of vines (Press & Phoenix, 2005) that wrap around their host plant and draw out water as well as nutrients. Their appearance is special for a plant in that the root

degrades shortly after attachment to a host and that leaves are reduced to rudimentary scales so the plant seems to be comprised of stem only (Kim & Westwood, 2015). *Cuscuta* attack the aerial parts of a wide range of host plants whereby host attachment and intrusion of host tissue are mediated by haustoria. They are still able to perform photosynthesis to a certain degree but not to live completely autotrophic. Consequently *Cuscuta* can be classified as holoparasitic generalist stem parasites (Patel *et al.*, 2012).

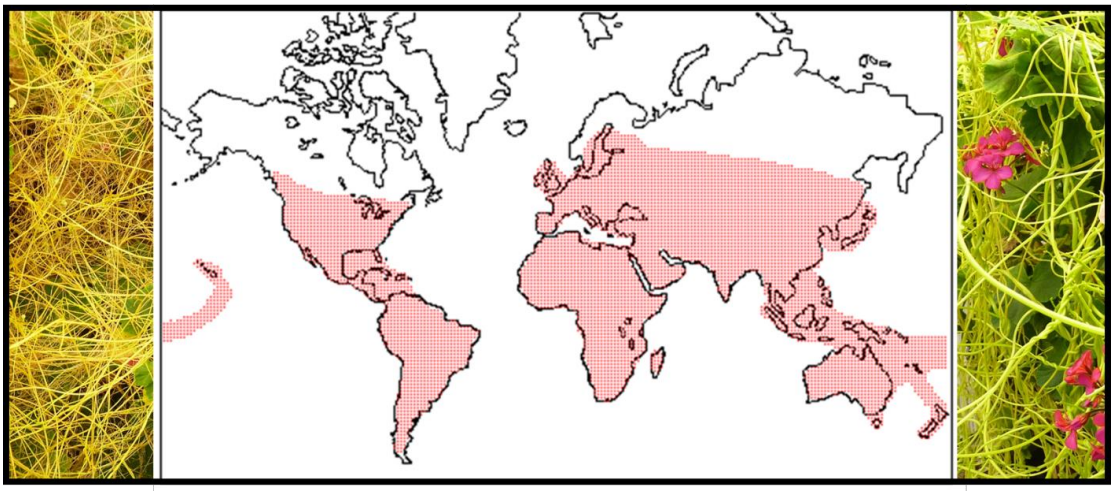


Figure 1 – Distribution of *Cuscuta* species throughout the world. *C. campestris* (left side, Phytotron at UiT Tromsø) and *C. reflexa* (right side, Phytotron at UiT Tromsø) (http://parasiticplants.siu.edu/Cuscutaceae/index.html, 21/03/2017)

It is known from the species *Cuscuta reflexa* that 99% of the carbon it uses is taken up from the host and that most of the solutes are taken up come from the host phloem (Hibberd & Jeschke, 2001). Plasmodesmata serve as direct portals to obtain resources from both, host xylem and phloem (Roney *et al.*, 2007; Smith, 2013). The degree of selectivity in solute transfer is highly discussed in parasitic plant literature, but it seems that at least in *Cuscuta*, various sugars, amino acids and hormones move unselectively (Birschwilks *et al.*, 2006). It is known that other classes of macromolecules including mRNAs, proteins and viruses are also transferred between host and parasite (Kim & Westwood, 2015; Roney *et al.*, 2007). This would require very large portals, which would also accommodate unspecific transport of smaller molecules like phytohormones and other secondary metabolites. Those might then also influence development and defense mechanisms of the parasite (Smith, 2013). Ultimately, it seems that *Cuscuta* not only obtains many secondary metabolites from the host but also metabolizes those (Behbahani, 2014). When applying *Cuscuta* in a biotechnological or medicinal context, it is therefore actually the combination of parasite and host that has to be evaluated.

1.1.2 Medicinal Use of Cuscuta

Cuscuta species have long been used in traditional Asian medicine as additives to food and beverages where they enriched the daily diet with minerals and proteins but also benefitted health with their antioxidative properties (Saqib *et al.*, 2014; Yen *et al.*, 2008). Additionally they have been used as remedies for severe illnesses that include for example diabetes mellitus (Rahmatullah *et al.*, 2012; Tag *et al.*, 2012) (Table 1).

C. reflexa is the *Cuscuta* species that has been studied most extensively so far. It is prevalent in the regions of Malaysia, Thailand, India, Nepal and Ceylon as well as Afghanistan and Pakistan. It is an extensive climber and attacks a large variety of host plants in various families (Patel *et al.*, 2012). Its stems are relatively green compared to other *Cuscuta* species, thick, and sturdy; therefore, it is commonly referred to as "giant dodder". As this species is widespread and has been investigated thoroughly, there are many applications of its traditional medical use known to modern science. Some of them are worth further investigation while others seem rather arbitrary (Table 1).

Figure 2 – *Cuscuta reflexa* parasitizing on *Pelargonium zonale* (Phytotron UiT Tromsø)

Table 1 – Ethno medical use of *Cuscuta reflexa* in different cultures ([1](Patel *et al.*, 2012); [2](Tag *et al.*, 2012); [3](Choudhury *et al.*, 2015); [4](Rahmatullah *et al.*, 2012); [5](Saqib *et al.*, 2014))

	India	Bangladesh	China	Pakistan
whole plant	jaundice (all yellowish pigmentation of the skin)[1]			jaundice[5]
	rheumatism[1]			paralysis[5]
	gout[1]	-	-	purgative[5]
	headache[1]			
	diabetes mellitus[2]			
	digestive system disorders (DSD) [3]			
plant juice	retention of urine (internally)[1]	-	-	-
	skin itches (externally)[1]			
stem	bilious disorders[1]	diabetes mellitus[4]		
	protracted fevers (internally)[1]			
	body pain[1]			
	itchy skin[1]			
	constipation[1]	-	-	-
	flatulence[1]			
	liver complaints[1]			
	bilious affections[1]			
	hair growth promoter[1]			
fruit	fever[1]	-	-	-
	cough[1]			
seeds	sedative[1]		liver[1]	
	emmenagouge (promotes menstrual discharge)[1]		kidney[1]	
	alterative (alters favorably the course of an ailment) [1]		yin and yang deficiencies[1]	
	anti-helmintic[1]		aphrodisiac[1]	
	flatulence [1]		longevity[1]	
	diuretic in liver and spleen diseases[1]		impotence[1]	
	chronic fevers[1]	-	premature ejaculation[1]	-
	griping [1]		frequent urination	
	hiccough[1]		ringing in the ears[1]	
	tonic[1]		lower back pain[1]	
	diaphoretic (sudatory)[1]		leucorrhea (vaginal discharge)[1]	
	analgesic [1]		dry eyes[1]	
	blood purification[1]		blurred vision[1]	
			tired eyes[1]	
more	purgative[1]			
	melancholy[1]	-	-	-
	insanity[1]			
	wash for sores[1]			

However, the possible medicinal uses of *Cuscuta* are not only limited to traditional applications that have not been examined scientifically. Especially the thoroughly investigated *Cuscuta reflexa*, but also other species, like *Cuscuta campestris*, have been subject to several studies concerning their pharmacological activities (Table 2). *Cuscuta campestris* is a yellow climber with much thinner stems than *C. reflexa* and bears the common name "golden dodder" or "yellow dodder". It is spread throughout the world but is mainly found in North America and Europe as well as South Africa and Eastern Australia.

Figure 3 – *Cuscuta campestris* parasitizing on *Pelargonium zonale* (Phytotron at UiT Tromsø)

Table 2 – Pharmacological activities of *Cuscuta reflexa* and *Cuscuta campestris* collected from various studies. [1](Patel *et al.*, 2012), [2](D. K. Pal *et al.*, 2006), [3](Suresh *et al.*, 2011), [4](Rahmatullah *et al.*, 2010), [5](Raza *et al.*, 2015), [6](Pandit *et al.*, 2008; Patel *et al.*, 2014; Roy *et al.*, 2007) , [7](Mazumder *et al.*, 2003), [8](D. Pal *et al.*, 2003) [9](Agha *et al.*, 1996), [10](Lee *et al.*, 2011), [11](Behbahani & Mohabatkar, 2013).

	Pharmacological activity	
Plant part	***C. reflexa***	***C. campestris***
whole plant	hypoglycaemic effect[1,4]	analgesic action[9]
		hypothermic activity[9]
		CNS-depressant activity[9]
		anti-inflammatory activity[9]
stem	relaxant and spasmolytic action[1]	
	anti-steroidogenic activity (antifertility)[1]	-
	anti-convulsant activity[1]	
seeds	-	anti-proliferative activity[10]
		anti-inflammatory activity[10]
non-specified	effect on blood pressure[1]	anti-HIV activity[11]
	hair growth activity[1,6]	
	hepatoprotective activity[1]	
	diuretic activity[1]	
	anti-inflammatory and anticancer activity[1,3]	
	anti-bacterial [2]	
	anti-oxidative properties[5]	
	effect on kidney and hematological parameters[7]	
	psychopharmacological activities[8]	

1.2 Bioactive Compounds

A bioactive compound is broadly defined as a natural compound influencing living cells or tissues. The term is often used for compounds that are part of the human diet and in applications to improve health (Biesalski *et al.*, 2009).

1.2.1 Plants Produce – We Use

As plants are sessile organisms, unlike animals they cannot rely on the typical fight or flight response. Instead, they employ, among other defense strategies, an arsenal of chemicals to deal with their enemies. These so-called secondary metabolites are compounds that are not produced for growth and development of the plant but rather for its specific defense mechanisms (Azmir *et al.*, 2013). Their chemical composition differs greatly between different taxonomic groups.

But how can these chemicals be useful for human health? This can be explained with the different kinds of adversaries a plant has to fight: plant defense is often directed against bacteria, fungi or other pathogens, which are also a menace for us. In this case, we can use the plant's defense mechanism to protect us from similar hazards. However, there are also mammals like ourselves intending to eat the plant. In that case, the plants' protective mechanism most likely also harms us.

Bioactive Secondary Plant Metabolites

The major groups in bioactive secondary plant metabolites, also called natural products, are divided into three major groups based on their biosynthetic origins: terpenoids, alkaloids and phenylpropanoids plus related phenolic compounds.

Terpenoids (Figure 4) are derived from the five-carbon precursor isopentenyl diphosphate (IPP) and synthesized via the acetate/mevalonate pathway or the glyceraldehyde 3-phosphate/pyruvate pathway. Alkaloids (Figure 4) are biosynthesized principally from amino acids and many possess pharmacologically important activity. Phenolic compounds are formed by way of either the shikimic acid pathway or the malonate/acetate pathway. (Croteau *et al.*, 2000; Das *et al.*, 2010)

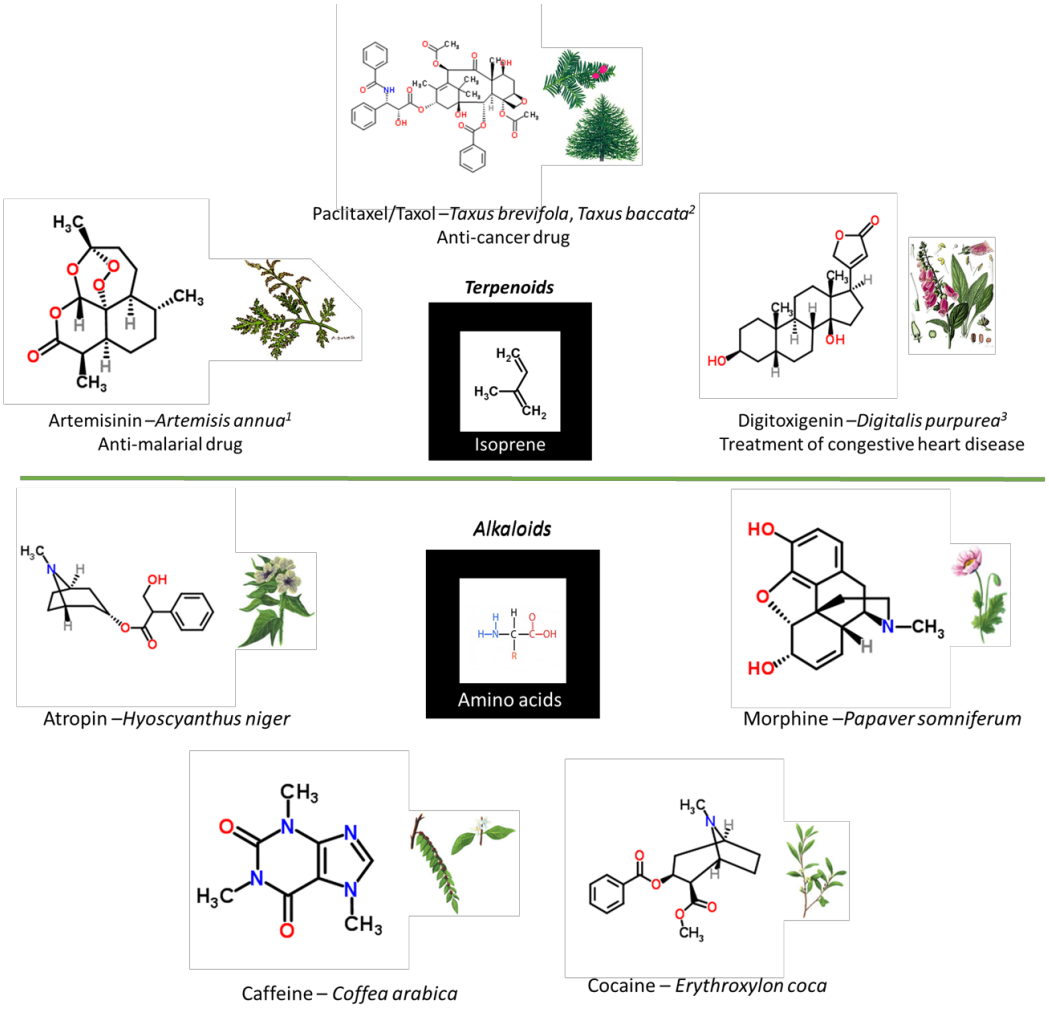


Figure 4 – Examples for terpenoids with pharmacological applications and bioactive alkaloids. Plant drawings (21/03/2017): [1] https://www.mskcc.org/cancer-care/integrative-medicine/herbs/artemisia-annua, [2]https://snl.no/barlind, [3]http://www.pfaf.org/user/Plant.aspx?LatinName=Digitalis+purpurea, the rest by Croteau *et al.* (2000))

The array of phenolic compounds within a plant is remarkably diverse (Figure 5). Phenolic compounds include flavonoids, among which the anthocyanins serve as pigments, proanthocyanidins or condensed tannins as feeding deterrents and wood protectants, and isoflavonoids as defensive products and signaling molecules. Lignans strengthen the cell wall mechanically, while the structurally closely related lignins aid defense. Other subgroups are coumarins, furanocoumarins and stilbenes that protect against bacterial and fungal pathogens and other phenolic compounds that are responsible for characteristic tastes and odors. (Croteau *et al.*, 2000)

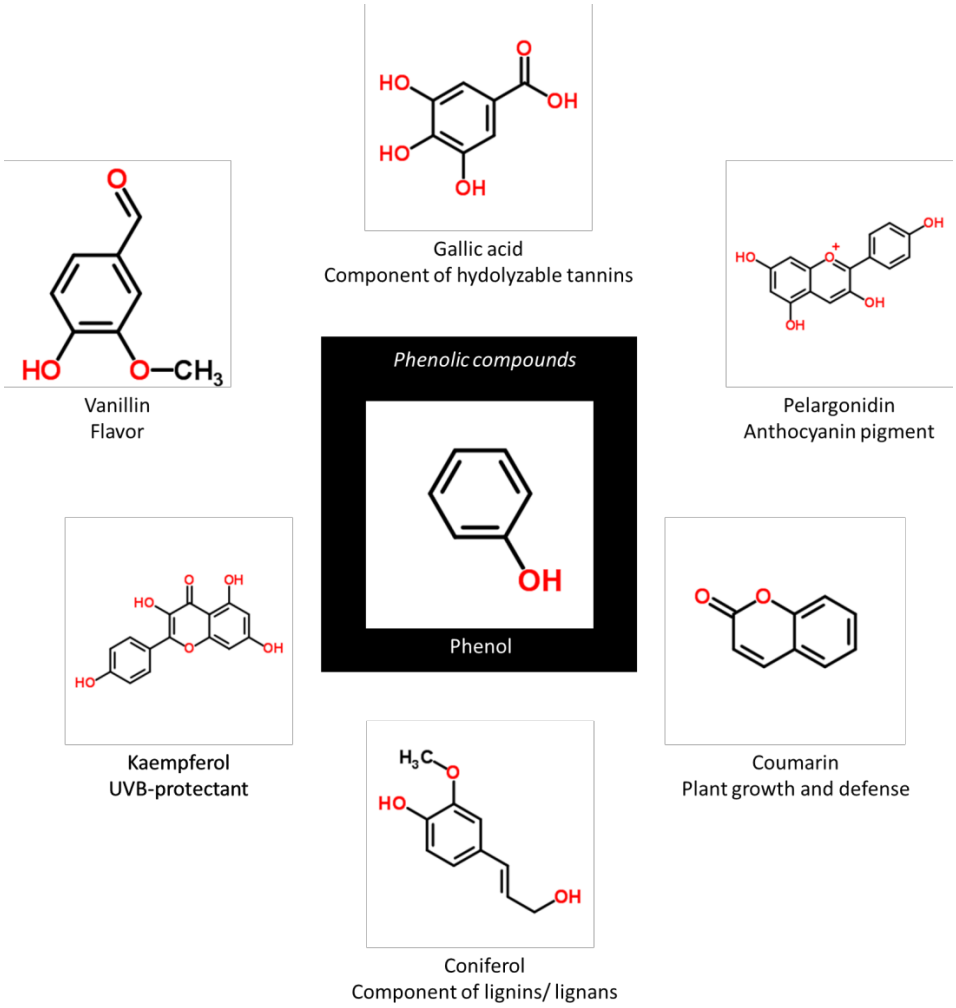


Figure 5 – Examples of phenolic compounds exhibiting bioactivity and the functions they adopt in plants (Croteau *et al.*, 2000)

Compounds Found in *Cuscuta* Species

As described in 1.1.2 *Cuscuta* shows various medicinal applications and effects. To learn about the causes of those effects, many natural compounds have already been extracted from different *Cuscuta* species, which were grown on various hosts (Patel *et al.*, 2012). Compounds found in *Cuscuta reflexa* include many sugars like dulcitol and mannitol and carotenoids, among them some that led to the findings of a specialized xanthophyll cycle for photoprotection in *Cuscuta* (Kruk & Szymanska, 2008). Other compounds are already known for various biotechnologically applicable activities like quercetin, kaempferol, myricetin, cuscutin and eugenol (Patel *et al.*, 2012). Some of these compounds have been studied more closely, usually

in the context of medicinal or nutraceutical value and applications. The flavonoid quercetin, for example, was linked to anti-proliferative activity against different cancer cell lines (Lee *et al.*, 2011); lupeol, a triterpene, was suggested to show high anti-inflammatory effects (Saleem, 2009) and lutein, lupeol and eugenol as well as their epoxidized forms where attributed anti-oxidant and cytotoxic activity in breast cancer cells (Behbahani, 2014). Overall, a wide spectrum of the substances found in *Cuscuta* might be of medical or biotechnological interest.

1.2.2 Pipelines to Discover Bioactive Compounds

Approximately 42% of newly approved drugs from 1981-2014 were natural products or their derivatives. Another 21% were synthetic mimics of natural products and 4% were synthetic drugs derived from a natural product pharmacophore, the active part of the drug. In 2014 the respective percentages were around 28%, 25% and 2% (Newman & Cragg, 2016). These statistics show that natural products are still of utmost importance in drug development.

Plants are not the only ones that produce possibly interesting compounds for biotechnological and medicinal applications. Therefore, the amount of samples reaches a number that requires a fast screening method, a high throughput platform. Such pipelines have established protocols, workflows and bioassays to test for activities and can produce results by high-throughput screening. Though it is true that by application of high throughput methods and automation, which is usually the case in those pipelines, some of the low-concentration but high-affinity compounds are missed. To avoid this, screening data is usually kept and can be reconsidered at a later time point, after the compounds that are easiest to work with have been investigated (Svenson, 2013) (Figure 6).

1.2.3 Workflow of a Discovery Pipeline

Marbio is an analytical platform located at UiT, a pipeline for discovery of natural products with interesting biological activities. It is usually specialized on marine organisms but was in this case used to screen plant samples. The three stages of work are bioactivity screening, identification, and purification (see Figure 6). This thesis is settled within the stages of bioactivity screening and identification. The methods used in this thesis for sample extraction, fractionation and screening for bioactivities were chosen considering the experiences and established protocols at the Marbio platform.

Figure 6 illustrates the usual workflow at Marbio. A crude extract is prefractionated using flash fractionation. The resulting fractions are then tested for various activities in a set of bioactivity assays. Inactive fractions are discarded while active fractions are analyzed via mass

spectrometry. Promising compounds are singled out and evaluated. While already known compounds with established activities are discarded, novel compounds and compounds showing new activities are retained to be further evaluated in a process called dereplication. Target compounds are then isolated and purified so their structure can be elucidated via NMR (Nuclear Magnetic Resonance). Finally, the purified compound is used for a bioactivity profiling, repeating the same bioactivity assays that were used before isolation.

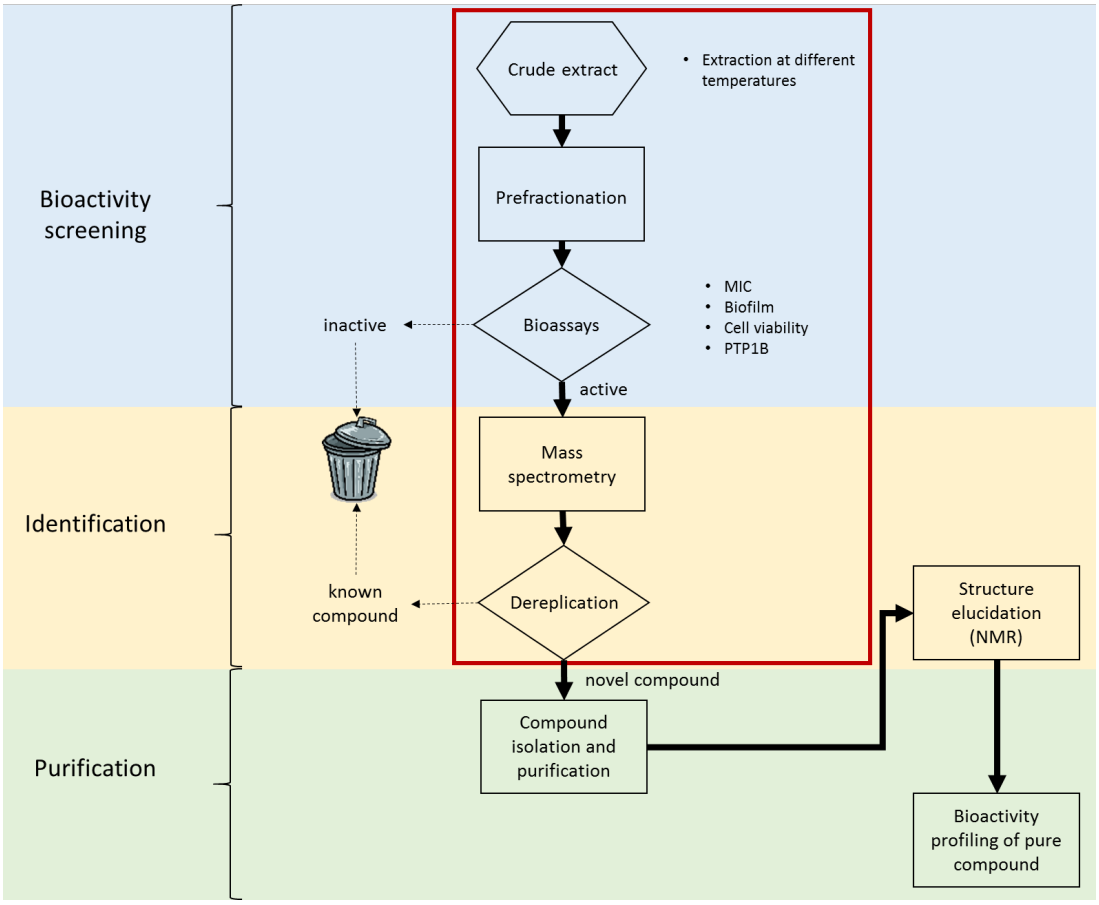


Figure 6 – Workflow at Marbio. Content that was covered during this thesis is marked by a red box.

1.3 Aim of the Thesis

To identify bioactive compounds in the parasitic plant *Cuscuta*, a high throughput screening was performed. Extracts of three different species of the parasitic genus *Cuscuta* (*C. reflexa, C. campestris* and *C. platyloba*) as well as their host plant *Pelargonium zonale* were evaluated. Among them *C. reflexa* is well investigated, genomic sequences of *C. campestris* are available in the working group of Prof. K. Krause and *C. platyloba* is an uncommon species that has virtually not been researched so far.

The aims of this thesis were

1) To compare different species of the parasitic plant *Cuscuta* grown under the same conditions and on the same host plant by using bioassays testing for bioactivity against bacterial pathogens, biofilm formation, cancer and diabetes.

2) To compare two different tissues, stem and flower, of the same *Cuscuta* species in bioassays testing against bacterial pathogens, biofilm formation, cancer and diabetes.

3) To compare different species of *Cuscuta* to its host plant *Pelargonium zonale* in terms of bioactivity against bacterial pathogens, biofilm formation, cancer and diabetes.

4) To evaluate the influence of different extraction temperatures on the activity observed in assays testing against bacterial pathogens, biofilm formation, cancer and diabetes.

5) To identify candidate compounds that are responsible for the observed bioactivities via mass spectrometry and subsequent dereplication.

In the long term, compound candidates showing biological activities could be developed into new drugs to fight diseases the human society is struggling with today.

2. Materials and Methods

2.1 Plant Material

Equipment:

Freeze dryer	Heto Power PL9000, Heto HSC 500
Stone mortar and pestle	
Centrifuge tubes, 50 ml	VWR International

Cuscuta reflexa, *Cuscuta platyloba* and *Cuscuta campestris* were routinely grown on *Pelargonium zonale* hosts in a greenhouse of the Phytotron at the University of Tromsø at 24 h of light and approximately 21 °C.

Stems of all three *Cuscuta* species and flowers of *Cuscuta campestris* were sampled in spring and first stored frozen at -80 °C. *Pelargonium zonale* samples were taken in the beginning of September and also frozen and stored at -80 °C until further handling. All samples were taken in duplicates. Samples were then freeze-dried without thawing them in between and dry samples stored at 6 °C in plastic zip-bags before being ground into a fine powder using a stone mortar. The weight of sample powder was recorded and samples then stored at 4 °C.

2.2 Extraction

The first step of the analysis of compounds from plant material is their extraction. There are traditional techniques as well as several relatively newly developed approaches, but still there is no standardized method to extract natural bioactive compounds from plants (Azmir *et al.*, 2013). The defining parameters of an extraction are solvent, temperature, pressure and time, as well as the properties of the extraction material (Azmir *et al.*, 2013). The choice of the solvents' polarity influences the possibility to extract hydrophilic and hydrophobic compounds.

Maceration, the extraction method used here, is a traditional method: The plant is ground to increase surface area, then the desired solvent is added to a closed container. After a certain time the solid residues are filtered and pressed to recover the extraction solvent.

Equipment:

Buechner Funnels	
Filter papers Whatman™, Grade 1, 85 mm	Springfield Mill, England
Rotary evaporator	IKA-DEST
Round flasks different volumes	Duran, Germany

Reagents:
Methanol (gradient grade for liquid chromatography) Merck, Germany

For extraction, the ground plant powder was incubated under slight shaking with 10 ml 80% (v/v) aqueous methanol per g of sample in a conical flask. The solution was extracted twice, using fresh 80% methanol in the second round and storing the first extraction at 6 °C. The first round of extraction lasted 2.5 hours, the second 22 hours. All extractions were done in darkness.

To evaluate temperature-dependency, the temperature was kept at 6 °C for the first set of the duplicates (A) and at 27 °C for the second set of duplicates (B) (Table 3).

After each round of extraction, a filtering step with Buechner funnels using Whatman filter papers and applied vacuum was performed. The two extracts (2.5 hours and 22 hours extraction) were pooled for each sample and immediately dried on a rotary evaporator (40 °C, reduced pressure). As a control for the extraction 80% methanol was exposed to the same procedures as the actual samples.

When adding 100% methanol to dissolve the dried extracts, a white layer that did not dissolve formed on top of the sample shortly after methanol was added. Sample material, which was located below that layer and was not in contact with the methanol, did not change color and was not affected visibly. The solvent, 100% methanol, adapted to a certain extent the color of the dried extract. As dried extracts could not be dissolved in 100% methanol, MQ water was added until a final concentration of 80% methanol and 20% water, which correlated with the solvent that had been used for the original extraction. Most of the visible part of the dried extracts could be dissolved in this solvent at room temperature.

Table 3 – Plant samples used in this study and respective abbreviations

Sample plant	Plant part sampled	Extraction temperature [°C]	Sample label
Pelargonium zonale	Stem	6	PA
Pelargonium zonale	Stem	27	PB
Cuscuta reflexa	Stem	6	CrA
Cuscuta reflexa	Stem	27	CrB
Cuscuta platyloba	Stem	6	CpA
Cuscuta platyloba	Stem	27	CpB
Cuscuta campestris	Stem	6	CcA
Cuscuta campestris	Stem	27	CcB
Cuscuta campestris	Flowers	6	CcfA
Cuscuta campestris	Flowers	27	CcfB

2.3 Flash Chromatography

The word "chromatography" is derived from the greek words "chroma" for "color" and "graphein" for "to write". It is a group of methods used for the separation of mixtures. The chromatographic setup in liquid chromatography (LC) consists of two phases, one solid and stationary, the other liquid and mobile. One of the phases is hydrophobic, the other hydrophilic. The components of a mixture distribute in the two phases according to their charge and relative solubility. In normal phase separation, the stationary phase is hydrophilic and the mobile phase hydrophobic, while reversed phase separation has a hydrophobic stationary phase and a hydrophilic mobile phase. Here polar compounds elute first.

In flash chromatography, or flash fractionation, the solvent flow is enhanced by pressure, it is therefore also known as "medium pressure chromatography". The advantages over chromatography methods that are applied without pressure are a faster separation ("like a flash") and a higher chromatographic resolution. Flash chromatography is used in a preparative way, to separate large quantities (grams) of a complex sample material into several fractions (Ayare *et al.*, 2014).

Equipment:

Rotary evaporator	Laborata 4002, Heidolph Germany
Round flasks of different volumes	Duran, Germany
Cartridge	Biotage SNAP
Flash	Biotage SP4 System
Polyvap	Buechi Syncore

Reagents:

Column material Supelco Diaion® HP-20SS	Sigma Aldrich, USA, 13615-U
Methanol (Chromasolv® for HPLC, ≥ 99.9%)	Sigma Aldrich, Germany, 34960
Acetone (Chromasolv® for HPLC, ≥ 99.8%)	Sigma Aldrich, Germany, 34850
Dimethyl sulfoxide (DMSO ≥99.5% (GC))	Sigma Aldrich, Germany, D4540
MQ Water (Milli-Q® Gradient 10)	Millipore, Billerica, USA

Columns for flash fractionation were prepared in advance and stored at 4 °C (for up to one week) until use. To prepare a column, 10 g of column material was weighed in a flask, about 100 ml of 100% methanol added and incubated for 20 minutes. Methanol was poured back and reused for the same purpose. MQ water was added to transfer the material to a cartridge. The column was rinsed with MQ water several times without letting it run dry to remove remaining methanol.

The extracts were dried on a rotary evaporator, dissolved in 10 to 20 ml 90% methanol and 2 g (sample PA) or 3 g (rest of samples) column material added depending on the weight of the respective sample.

The extract was dried with the column material and loaded on a second column that was set on top of the packed column (setup see Figure 7).

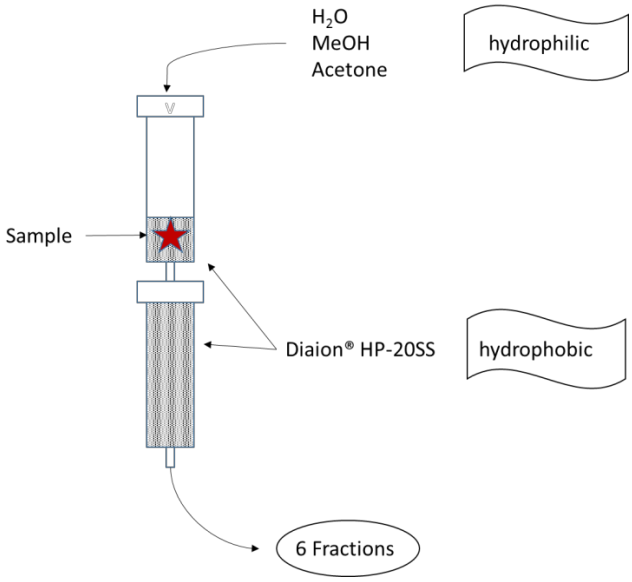


Figure 7 – Flash fractionation setup. Here reversed phase chromatography was used: The more hydrophilic a substance is, the earlier it elutes.

27 vials were collected along a stepwise gradient; each fraction contained 24 ml and was collected within 2 minutes. For the following procedures those vials were pooled into 6 fractions according to Table 4.

Table 4 – Stepwise elution gradient in flash fractionation combining water, methanol and acetone. The fractions numbered 1-6 collect the respective elutes. For fraction 6, 12 vials were pooled, while for every other fraction 3 vials were pooled respectively.

Fraction name	H_2O [%]	Methanol [%]	Acetone [%]	Time [min]
1	95	5	0	6
2	75	25	0	6
3	50	50	0	6
4	25	75	0	6
5	5	95	0	6
6	0	100	0	12
6	0	50	50	4
6	0	0	100	13

After flash fractionation sample fractions were dried in a polyvap, weighed to obtain the dry weight of the fractions (DWF), dissolved in DMSO yielding concentrations of 40 or 80 mg/ml (DWF) and stored at -20 °C until further use.

2.4 Bioassays

A bioassay is an established setup producing a signal to identify possibly interesting fractions for desired effects. It can be used to quantify a biological process (Lilly, 2007). Many different types of bioassays exist, each specific for a certain activity. Depending on the goal, respective types can be chosen. The bioassays chosen here focus on medical uses. Growth inhibition assay and biofilm assay are prokaryotic assays, the cell viability assay is a eukaryotic assay and the diabetes assay is an enzymatic assay.

The bioassays used in this study were chosen from a set of available assays within Marbio. Fractions obtained during flash chromatography as well as the total extracts were tested in prokaryotic (growth inhibition), biofilm), eukaryotic (cell viability) and enzymatic (PTP1B) assays.

Equipment:	
96 Deepwell plate	VWR International, 81372425
Freeze dryer	Heto
Reagents:	
Dimethyl sulfoxide (DMSO ≥99.5% (GC))	Sigma Aldrich, Germany D4540
MQ Water (Milli-Q® Gradient 10)	Millipore, Billerica, MA, USA

A 96 deep well plate containing 1 mg of sample (DWF) per well was prepared. Proper amounts of the frozen DMSO stock fractions as well as from the total extracts, which were stored in 80% methanol, were added separately to each well and the whole plate was freeze-dried for 72 hours. Samples were again dissolved in a mixture of 2.5% DMSO and 97.5% MQ water, by first incubating the dried samples on a shaker with the DMSO for 30 minutes and then adding the water. The plate was used as a stock for different bioassays. It was stored at 4 °C for up to one week or otherwise frozen in between assays at -20 °C.

Due to solubility issues for some of the fractions, the DMSO concentration was raised to 20% for performing the cell viability assay.

2.4.1 Bacterial Growth Inhibition

Bacterial infections become increasingly prominent as more pathogens and facultative pathogens are gaining antibiotic resistances that hinder treatment. Finding new antimicrobial compounds is therefore crucial. Minimum inhibitory concentration (MIC) is the lowest concentration of an antimicrobial substance that visibly inhibits microorganism growth on an overnight culture (Andrews, 2001). It is used to find the effective concentrations of antibiotics against different microorganisms.

The growth inhibition assay used in this work is designed to discover compounds with anti-bacterial activity and determine their respective MIC.

A known concentration of bacteria is inoculated with different concentrations of the compound to be tested. In wells where the compound is effective, bacteria will not grow and the growth medium will therefore stay clear, as opposed to wells where bacterial growth clouds the medium. The difference in optical density (OD), indicating the rate of bacterial growth, can be measured photometrically (Figure 8).

This assay was used to screen all available fractions for activities and subsequently determine the MIC value of active fractions in a second round using a dilution series.

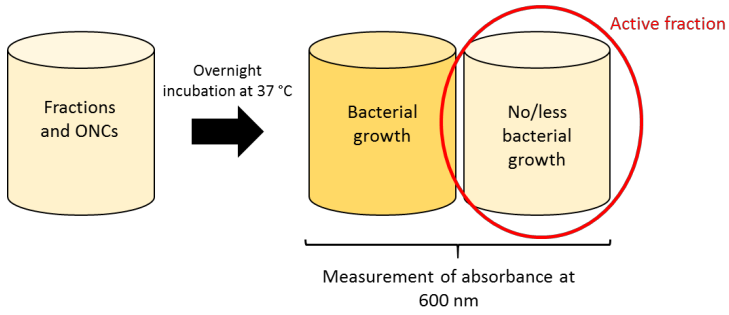


Figure 8 – Principle of growth inhibition assay. ONC: over night culture

Equipment:

Robot, Biomek® 3000	Beckman Coulter, USA
Victor 3 Multilabel Counter (Plate Reader)	Perkin Elmer, Waltham, MA, USA
Incubator 1000	Heidolph, Germany
Microtiterplates	Thermo Scientific, Denmark, 167008

Reagents:

Autoclaved MQ-H_2O (Milli-Q® Gradient 10)	Millipore, Billerica, MA, USA
Autoclaved 0.9% NaCl	Sigma Aldrich, Germany, S5886

Media:

Blood agar plates	Sump, University Hospital (UNN)
LB-agar plates	Sump, University Hospital (UNN)
Mueller-Hinton broth (MH)	DIFCO, USA, 275730
Brain-Heart-Infusion (BHI)	Sigma Aldrich, Germany, 53286

Bacteria:

Staphylococcus aureus (ATCC 25923)	LGC standards
Escherichia coli (ATCC 25922)	LGC standards
Enterococcus faecalis (ATCC 29212)	LGC standards
Pseudomonas aeruginosa (ATCC 27853)	LGC standards
Streptococcus agalactiae (ATCC 12386) (Control strain for: *Streptococcus* Group B)	LGC standards

Bacteria used in this assay (Table 5) were both, gram negative and gram positive types, representing different kinds of mostly facultative pathogens that often show antibiotic resistances (Beovic, 2006; Tenover, 2006).

Table 5 - Bacteria used in the growth inhibition assay (MH: Mueller-Hinton broth, BHI: Brain-Heart-Infusion)

Bacteria	Strain	Gram staining	Medium
S. aureus	ATCC 25923	positive	MH
E. coli	ATCC 25922	negative	MH
E. faecalis	ATCC 29212	positive	BHI
P. aeruginosa	ATCC 27853	negative	MH
S. agalactiae	ATCC 12386	positive	BHI

The growth inhibition assay was performed according to the Marbio protocol, which had been established according to Hasselmann (2003).

Bacteria were stored at -80 °C in growth medium with 10% glycerol, grown on blood agar overnight at 37 °C, then a scoop of bacteria was transferred from the plates to 8 ml of respective medium (Table 5) and incubated overnight on a shaker at 37 °C. Bacterial plates were kept at 4 °C for up to one month, restreaking once after two weeks.

For the growth inhibition assay, 2 ml of the bacterial solution was transferred to 25 ml of the suitable medium (Table 5) and incubated 1.5 hours or 2.5 hours. This corresponds to a turbidity of 0.5 McFarland standard (1.0×10^8 bacteria/ml). McFarland standards are used as a possibility to adjust and compare the density of bacterial suspensions that are used for identification and susceptibility testing.

For the first screening, a concentration of 50 µg/ml (DWF) was used for all samples. Samples were tested in duplicates. Bacteria were added to sample fractions in a 1:2000 end dilution and incubated overnight at 37 °C. On every microtiter plate a negative control consisting of growth medium and autoclaved MQ water, as well as a positive control composed of bacterial suspension and autoclaved MQ water, were included.

An extra plate was used to confirm normal bacterial susceptibility to gentamycin, and the number of colony forming units (CFU) was tested. For the gentamycin control, a dilution series of gentamycin in a concentration range from 16 µg/ml to 0.01 µg/ml with 11 steps was applied. Bacteria were added in the same way as to the sample plates. The CFU was measured by diluting the bacterial suspension at 0.5 McFarland in 0.9% NaCl to approximately $1x10^2$-$1x10^3$ bacteria/ml and plating out 100 µL on LB Agar plates. Gentamycin and CFU controls were incubated at 37 °C overnight together with sample plates.

After incubation of the sample plates, the absorbance of each well was measured at 600 nm wavelength in a plate reader. Extracts showing an OD-value of 0.05 or lower were considered active, while extracts exhibiting an OD-value between 0.05 and 0.09 were considered questionable and everything above that was designated inactive.

The samples rated as active or questionable underwent a second round of screening to find the MIC value. A dilution series, performed in duplicate, of the respective samples, containing 50 µg/ml, 25 µg/ml, 10 µg/ml and 1 µg/ml (DWF) was tested. Both values were used to calculate a mean. The protocol was the same as used for the initial screening.

Colonies on the CFU plates were counted and CFU/ml calculated. The values' range is indicated in Table 6. Gentamycin plates were inspected for bacterial growth (clouded wells) visually. When a well appeared clear this was noted as inhibition of bacterial growth. The CFU control was used to confirm a constant growth rate of the bacterial strains, while the Gentamycin control plate tested constant susceptibility towards anti-bacterial substances. It is important to ensure that these values stay at a constant level.

Obtained MIC values for bacterial cultures were monitored in between assays for their consistency and compared with literature values to make sure bacterial susceptibility did not change over time.

Table 6 -Values for gentamycin and CFU controls. Used in the growth inhibition assay for bacterial susceptibility towards gentamycin and bacterial density

Bacteria	MIC Gentamycin [µg/ml]	Bacterial density (colony forming units – CFU)
S. aureus	10.00	0.5-3x10^5 CFU/ml (2500-15000 CFU/well)
E. coli	0.25	0.5-3x10^5 CFU/ml (2500-15000 CFU/well)
E. faecalis	0.50	0.5-3x10^5 CFU/ml (2500-15000 CFU/well)
P. aeruginosa	0.25	3-7x10^4 CFU/ml (1500-3500 CFU/well)
S. agalactiae	4.00	0.5-3x10^5 CFU/ml (2500-15000 CFU/well)

2.4.2 Biofilm

A biofilm is an aggregation of microorganisms that form their own microenvironment by secreting extracellular polymeric matrix. Biofilm formation is a problem in many places. It is considered a key virulence factor by enabling bacteria to evade antibiotic effects for example in infections of implants, urinary tract infections and cystic fibrosis (Tenover, 2006). Biofilm formation can also cause problems in different industries including for example food industry and shipping (Mattilasandholm & Wirtanen, 1992). New compounds that inhibit biofilm formation are therefore interesting for a wide variety of applications.

The biofilm assay applied in this thesis uses *Staphylococcus epidermidis*, a facultative pathogen on human skin that shows a pronounced antibiotic resistance and is frequently involved in nosocomial and implant infections (Cafiso *et al.*, 2004), as an example for a biofilm forming bacterium. A *Staphylococcus haemolyticus* strain, which does not form biofilms in this growth medium, is used as a control group. Bacterial cultures are inoculated on polystyrene plates overnight, and biofilm formation is measured with the help of crystal violet solution. Bacteria are grown in medium with additional glucose to make them express the *ica*-operon, which is known to be related to biofilm formation (Cafiso *et al.*, 2004). The biofilm is fixated and determined photometrically (Figure 9).

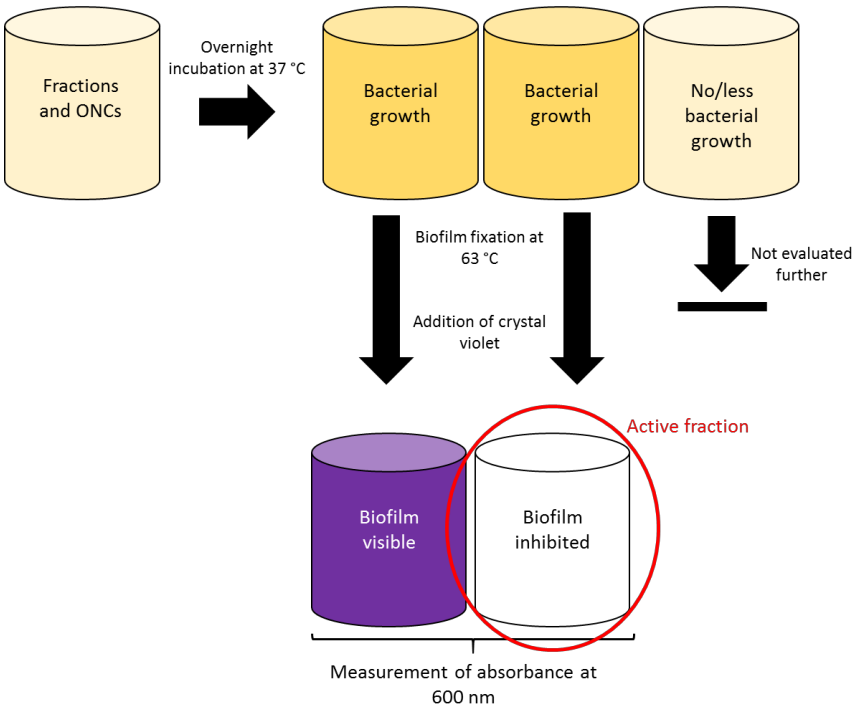


Figure 9 – Principle of biofilm assay

Equipment:

Robot, Biomek® 3000	Beckman Coulter, USA
Plate reader	Perkin Elmer, USA
Microtiterplates	Thermo Scientific, Denmark, 167008
Paper	

Reagents:

Autoclaved MQ-H_2O	Millipore, Billerica, USA
0.1% Crystal violet	Merck Millipore,115940
Glucose (Dextrose, D-(+) glucose-dextrose)	Sigma Aldrich, Germany D9434
70% Ethanol	Sigma Aldrich, Germany 24106

Media:

Tryptic soy broth (TSB)	Merck, Germany, 1.054590500)
Blood agar plates	University Hospital (UNN) at UIT

Bacteria:

Staphylococcus epidermidis (RP62A 42-77) ATCC 35984	University Hospital (UNN) at UIT
Staphylococcus haemolyticus (clinical isolate 8-7 A)	University Hospital (UNN) at UIT

Bacteria were stored at -80 °C in growth medium and 10% glycerol, grown on blood agar plates overnight at 37 °C, then a scoop of bacteria was transferred from the plates to 5 ml TSB and

incubated on a shaker overnight at 37 °C. Bacterial plates were kept at 4 °C for up to one month, restreaking them every second week.

50 µL of sample fractions from the sample stock plate were transferred to microtiter plates. Bacterial overnight cultures were diluted 1:100 in TSB with 1% glucose and added to the sample fractions in the microtiter plate. Sample fraction end concentrations in the microtiter plate was 50 µg/ml (DWF). On every plate, three controls were included: *S. epidermidis* with water served as a positive control, *S. haemolyticus* with water as a negative control and a medium blank. Sample fractions were tested in triplicates. Plates were incubated at 37 °C overnight.

The next day, the plates were checked visually for clear wells indicating dead cells. These wells were excluded from the analysis. Bacteria were removed from the plates by pouring them out onto paper. Wells were washed carefully with tap water 2-3 times and then incubated at 63 °C for 1 hour to fixate the biofilm. 70 µL 0.1% crystal violet solution was added to the wells, incubated for 10 minutes at room temperature, removed, and wells again washed with tap water. Plates were then left at 63 °C for approximately one hour until they were dry. 70 µL of 70% ethanol was added, plates incubated on a shaker for 5-10 min at room temperature and absorbance measured at 600 nm.

A sample was considered active when biofilm formation was inhibited to an OD-value of 0.25 or lower in at least two of the three triplicates. All three values were checked for comparability and if so, they were used to calculate a mean.

2.4.3 Cell Viability

Cancer is already a major cause of death and will probably become an even bigger issue in the future all over the world. It is a diverse group of diseases that is not decreasing, like many others do, because of the development of a certain region (Bray *et al.*, 2012). A satisfactory and selectively effective cure against cancer has not been found yet and looking out for potential compounds that could lead to a cure that is preferably able to selectively kill cancer cells is a major topic among scientists all over the world. It is understandable that an assay testing for activity against at least some kind of cancer is part of a pipeline searching for bioactive compounds.

Equipment:

Cell cultivation flasks	Nunclon, Denmark, 156367
96 well Microtiterplate	Thermo Scientific, MA, USA
DTX 880 Multimode Detector	Beckmann Coulter, USA

Multichannel pipettes	
Incubator 37 °C, 5 % CO_2	
Microtiterplate	Thermo Scientific, Denmark, 167008

Reagents:

AQOS (Cell Titer 96™ Aqueous One Solution Reagent)	Nalgene, USA, G358B
FBS	Biowest, S1810
Gentamycin	Biochrom AG, Germany, A2712

Media:

D-MEM (Dulbecco`s Modified Eagle Medium) (with 10% FBS and 0.1% Gentamycin)	Gibco®, UK, 32430
RPMI-1640 (with 10% FBS and 0.1% Gentamycin)	Merck, Germany, F61385
E-MEM (Eagle's Minimum Essential Medium) (with 10% FBS, 0.1% Gentamycin, 1% L-glutamin, 1% NEAA, 1% Natriumpyruvat, 2% $NaHCO_3$)	Biochrom AG, Germany, F4315

Cells:

A2058 ATCC CRL.11147 (human melanoma cells)	LGC standards
MRC5 ATCC CCL-171 (human lung fibroblasts)	LGC standards

This assay is a colorimetric method to determine the amount of living cells in proliferation or cytotoxicity assays. Metabolically active cells reduce a yellow substance (tetrazolium salt) into a dark blue product (formazan product), which is soluble and can be transported across the cell membrane. The number of living cells is directly proportional to the amount of photometrically measurable formazan product (Figure 10).

Cells were stored in liquid nitrogen. During cultivation, cells were split every 2-3 days depending on their growth.

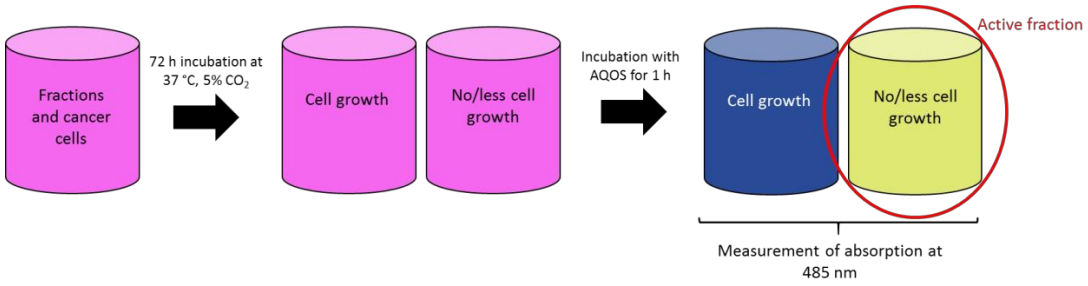


Figure 10 – Principle of the cell viability assay

For the assay, cells were counted with a Neubauer cell counting chamber and seeded out into a microtiter plate (2000 cells in 100 µL medium per well for A2058, 4000 cells for MRC5). Cells were incubated at 37 °C, 5% CO_2 for 24 hours, then examined under the microscope for normal growth and the old growth medium replaced by fresh RPMI-1640 containing 10% FBS and 0.1% gentamycin. Sample fractions were added to an end concentration of 50 µg/ml (DWF) in three parallels. A negative control, consisting of cells and medium (RPMI-1640 containing 10% FBS and gentamycin) without added fractions, was included on every plate. Triton X 100 at an end concentration of 0.5% was used as a positive control. Plates were incubated at 37 °C, 5% CO_2 for three days. 10 µL AQOS was added per well, plates were incubated at 37 °C, 5% CO_2 for one hour and absorption was measured at 485 nm using a DTX 880 Multimode Detector. Sample fractions were considered active when cell survival was rated below 50%.

After the initial screening of all fractions at 50 µg/ml (DWF), a second test for concentration dependence was performed with the active fractions at 50 µg/ml (DWF), 25 µg/ml (DWF) and 10 µg/ml (DWF) of sample concentration in duplicates. A mean value was calculated. The applied protocol was the same as in the initial screening.

The outer wells of the microtiter plate were not used for the analysis, as edge effects had been observed in this assay, cells and medium where added nevertheless.

2.4.4 Diabetes

The inhibition of protein tyrosine phosphatase 1B (PTP1B) causes increased sensitivity towards insulin (Elchebly *et al.*, 1999). This can be used in type 2 diabetes treatment. PTP1B dephosphorylates the insulin receptor and its substrates, and inhibits the effect of insulin.

T-cell PTP (TC-PTP) is closely related to PTP1B; they share 74% of homology in their catalytic domain, but have different cellular locations, functions and regulations (Bourdeau *et al.*, 2005). In contrast to PTP1B, TC-PTP is not supposed to be inhibited by this kind of treatment (Heinonen *et al.*, 2004; YouTen *et al.*, 1997). For this reason, the same assay was performed using TC-PTP instead of PTP1B to ensure that active fractions did not inhibit TC-PTP.

Equipment:	
Black microtiterplates and lids	Nunc, 437111
DTX 880 Multimode Detector	Beckmann Coulter, USA
Reagents:	
HEPES	Sigma Aldrich, Germany, H3375
NaCl	Sigma Aldrich, Germany, S9625
Dithiotreitol	Sigma Aldrich, Germany, D5545

Ethylendiamin tetraacetic acid	Sigma Aldrich, Germany, D5545
Bovine Serum Albumin	Sigma Aldrich, Germany, A2153
Protein tyrosine phosphatase 1B (PTP1B)	Merck Millipore, Germany 539735
T-cell PTP (TC-PTP)	Merck Millipore, Germany 14-646
Protein tyrosine phosphatase inhibitor IV	Merck Millipore, Germany 540211
6,8-difluoro-4-methylumbelliferylphosphat, DiFMUP	
Dimethyl sulfoxide (DMSO ≥99.5% (GC)	Sigma Aldrich, Germany D4540

In this assay the substrate 6,8-difluoro-4-methylumbelliferylphosphate (DiFMUP) is added to PTP1B, and the enzyme converts DiFMUP to its fluorescent form, DiFMU. The enzyme activity is proportional to the measured fluorescence (Figure 11).

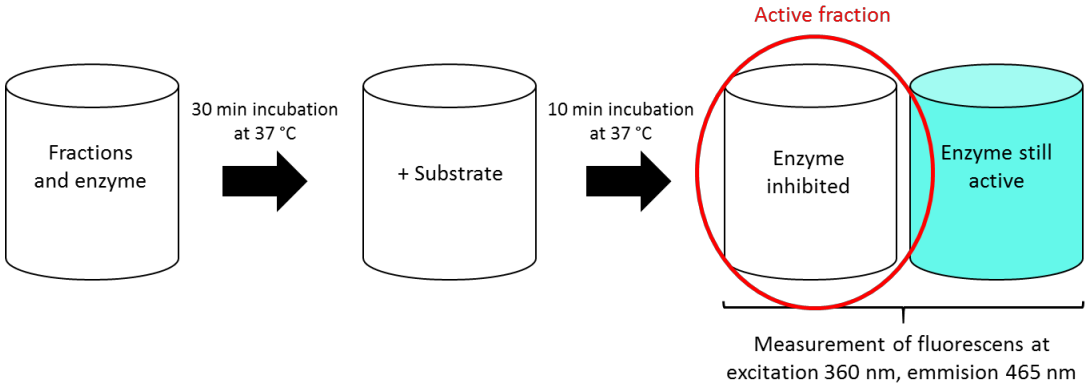


Figure 11 – Principle of diabetes (PTP1B enzyme) assay

The sample fractions were diluted to the desired concentration with assay buffer (25 mM HEPES, 50 mM NaCl, 2 mM Dithriothethreiol, 2.5 mM EDTA, 0.01 mg/ml BSA (pH 7.2)). 25 µL of each fraction were added to the wells of a black microtiter plate. 50 µL of the enzyme PTP1B at an end concentration of 15.6 ng/ml was added to each well and the plate was incubated for 30 minutes at 37 °C. 25 µL of DiFMUP with an end concentration of 2.5 µM was added to all the wells and the plate was incubated for another 10 minutes at 37 °C. Fluorescence was measured at an excitation wavelength of 360 nm and emission wavelength of 465 nm using a DTX 880 Multimode Detector. Enzyme activity was calculated and sample fractions were rated as active when they decreased the enzyme activity to 30% or lower.

Protein tyrosine phosphatase inhibitor at a concentration of 40 µM in assay buffer was used as a negative control. Plain assay buffer was used as a positive control. The assay was performed in triplicates and repeated with sample fractions at concentrations of 50 µg/ml (DWF) and 10 µg/ml (DWF) end concentration. A mean was calculated for comparable values in the

repetitions. Active sample fractions were also tested against TC-PTP using the same protocol except with TC-PTP instead of PTP1B.

2.5 Mass Spectrometry

Mass spectrometry (MS) is a method that separates ions in the gas phase according to their ratio of mass to charge (*m/z*). The mass spectrometer consists of an inlet that can include a separation system, ion source, mass analyzer for ions, and detector. The advantage of MS compared to other spectrometric techniques is that every peak in the resulting chromatogram is assigned a mass spectrum.

Here, reversed phase UHPLC was used as an inlet to separate the compounds before they enter the ion source. Electrospray ionization (ESI) was used as an ion source, transferring the compounds from the liquid phase into the gas phase and giving them charge. ESI is a relatively soft ionization method that does not cause much fragmentation of the sample compounds and is carried out at atmospheric pressure, making it suitable for biomolecules. Before ions are introduced into the actual mass analyzer, they are separated by drift time using gas and an electrical field. For mass analysis, a Time of Flight detector (ToF), separating ions according to their mass to charge ratio (*m/z*) by applying an electrical field, was used. MS is routinely used in research on natural compounds. The advantages of MS especially in natural product research are fast sample preparation and small sample sizes (Bouslimani *et al.*, 2014). It is mostly used for identifying compounds.

Equipment:

HPLC glass vials	Waters
VION® IMS QTof	Waters
PDA Detector	Waters
Column-/sample-/binary solvent manager	Waters
Column, Acquity UPLC, BEH C18, 1.7 µm	Waters

Programs:

UNIFI
MVA Tool EZ-Info
Progenesis

Reagents:

Methanol (Chromasolv® for HPLC, ≥ 99.9%, 34960)	Sigma Aldrich, Germany, 34960

For sample analysis via MS, dried samples were dissolved and diluted in 80% methanol and transferred to HPLC glass vials. Samples were analyzed using a VION IMS QTof. A 100 mm column was used for sample separation using a gradient of 10-100% acetonitrile (CH_3CN) in H_2O (+ 0.1% formic acid) over 15 min with a flow rate of 0.45 ml/min. The column was kept at 40 °C, injection volume was either 1 or 3 µL. Samples were analyzed by positive or negative electrospray ionization, *m/z* data from 50 *m/z* to 2000 *m/z* were acquired at a scan time of 0.2 s. Capillary and cone voltages were set to 0.8 kV and 50 L/h respectively while source and desolvation temperatures were set to 120 °C and 450 °C. Data was evaluated using UNIFI, MVA Tool EZ-Info and Progenesis.

2.6 Dereplication and Metabolomics

Metabolomics investigates small molecular compounds, for example the set of metabolites within an organism. Its techniques identify and quantify cellular metabolites and allow statistical analysis of samples as well as correlation with other samples to learn about bioactive compounds (Wu *et al.*, 2016). Metabolomics is a tool to compare complex samples without immediately extracting certain compounds (Yuliana *et al.*, 2011) and can be used to compare different species or tissues and effectively select biomolecules of interest.

Dereplication is the process of tentative identification of compounds to recognize and eliminate from consideration those compounds that are already studied. This can be done by MS or NMR and the aim of this tactic is to save money and time. Crucial for dereplication are the databases used to assign known structural formulas. Metabolomics and dereplication are very close and often intertwined (Hubert, 2015), as in the process of metabolomics dereplication is one of the critical steps.

Principle component analysis (PCA) is a statistical, qualitative analysis method for metabolomics data. It finds the principle components that is an underlying structure in a set of data, and describes samples using those. The actual number of components is high but PCA assumes that only the first are enough to describe samples, while the rest is often nothing but noise. PCA analyses samples according to those principal components and shows how samples group according to that. PCA was used here to investigate the differences in samples. EZ-Info was used to create a PCA. All components detected during MS were used for the analysis.

To investigate possibly interesting components closer, bioassays were used to determine active fractions, which were then subjected to MS analysis. Active and inactive fractions were compared in their chromatographic profile, distinct peaks selected and evaluated for their *m/z*

composition. The calculated masses of these compounds were used to perform a database search with ChemSpider. Hits were assigned to the respective compounds. The goal was to find a compound that showed activity in at least one of the assays and that did not produce any promising hits in the databases, in other words a novel compound.

3. Results

Extractions of different plant samples were fractionated and tested in different bioassays for their biological activity. Active fractions were analyzed via mass spectrometry to uncover the observed bioactive compounds, which might be future candidates for novel drugs.

3.1 Dissimilarity of Samples

The dissolved extracts were analyzed using MS to compare their composition of low molecular weight compounds, their metabolomic profile. To evaluate whether different species of *Cuscuta* also showed a variable composition in low molecular weight compounds, a principle component analysis (PCA) plot (Figure 12, Figure 13) was generated comparing the three different species. Only one kind of Extraction (B in the example shown here) was compared to be able to only assess one aspect of difference, in this case the species. All the detected compounds, characterized by retention time and mass, were used as markers. A loadings plot (Figure 12) was generated, to show how compounds contribute to the difference among the samples. It shows that a high amount of compounds contribute to the similarity of the different samples. The black triangles depicting these compounds group around the origin. Other compounds contribute to the difference of the samples. These black triangles are located far off from the origin.

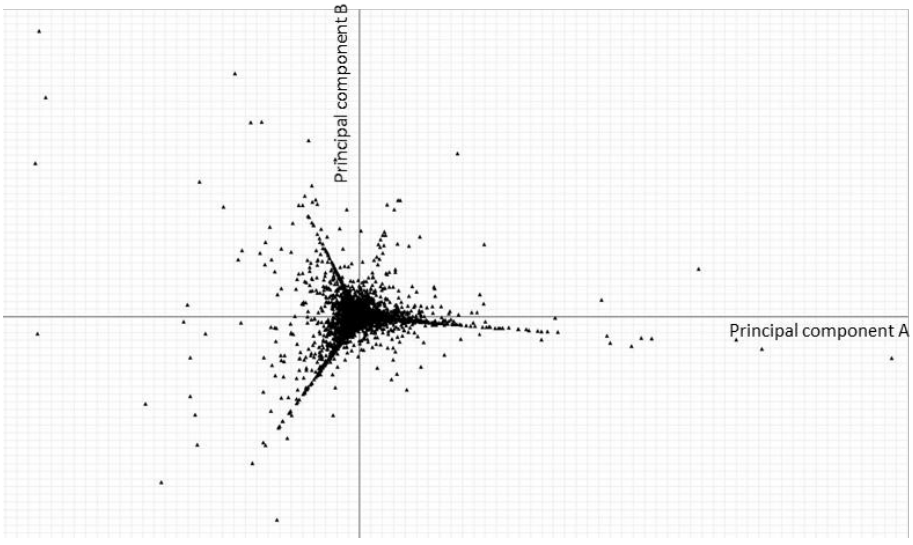


Figure 12 – Principle component analysis loadings plot of *Cuscuta* stem samples. The compounds that contribute to the different grouping of various *Cuscuta* samples are shown as triangles. The compounds centered around the origin of the axes contribute little to the variation of the samples, while compounds that are very distant from the origin contribute greatly.

A scores plot was generated to show how the samples group relatively to each other (Figure 13). Distinct injections of one sample group closely while different species are separated visibly. This indicates a difference in sample composition among the diverse *Cuscuta* species.

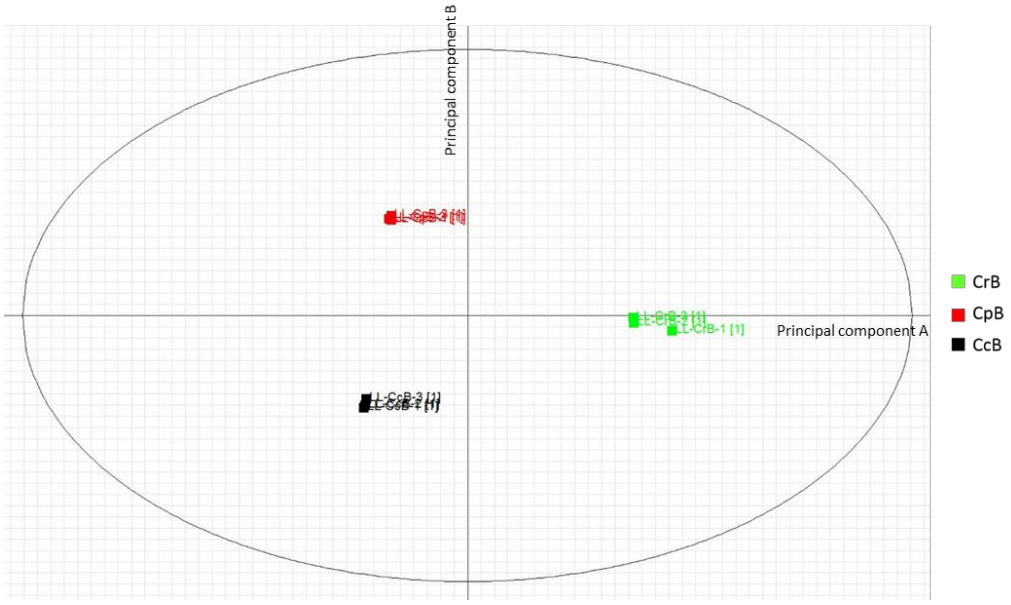


Figure 13 – Principle component scores plot of *Cuscuta* stem samples. The different grouping of the *Cuscuta* species *C. reflexa* (CrB), *C. platyloba* (CpB) and *C. campestris* (CcB) is shown, using one color for each of the species. Samples were injected in triplicates; single injections are shown in the plot as individual boxes.

Compounds that contribute most to the differences among samples can be selected and analyzed via database search to identify them. With this technique, a comparison of compound content is possible. This approach is, however, very time-consuming. To be able to select interesting compounds more effectively it was decided to filter the data further before analyzing MS data. This was done by fractionating the samples, performing bioassays to find active fractions, compare their composition to inactive fractions and hypothesize about possibly active compounds.

When fractionating the samples, the weight of the dried extracts was noted before (total extract), and after (fractions) flash fractionation, and compared to ensure that neither a big loss nor a gain of weight had occurred during fractionation (Figure 14). Flash fractionation was used as a preparative method, thus, the results obtained here are primarily meant to ensure a stable quality of fractionation. This is mandatory to allow the comparison of the results obtained later during bioassays and mass spectrometry.

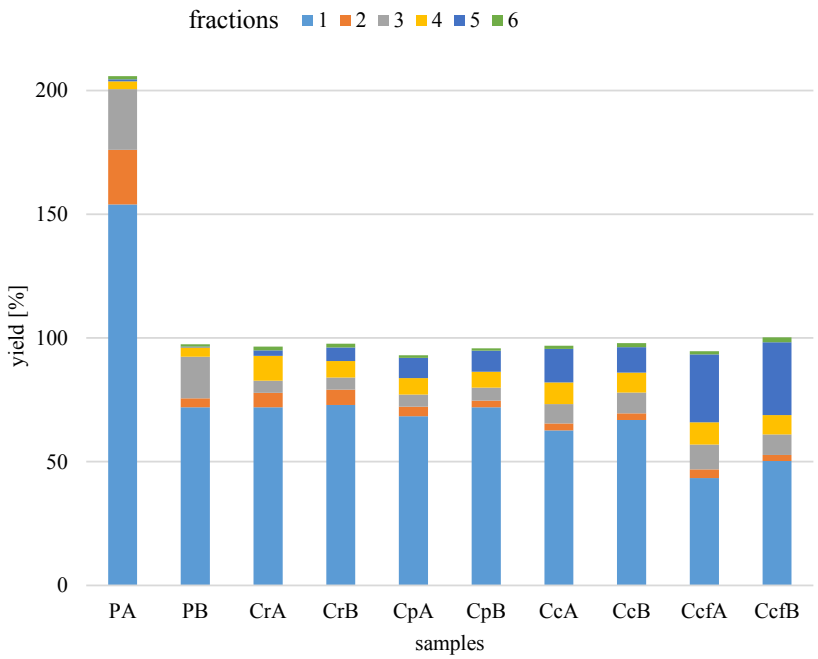


Figure 14 – Compositional difference of samples as a function of flash fractionation. Sample weight before fractionation was set as 100%. Sample fractions were weighed after fractionation and added up to compare them to the initial weight of the respective sample. Each bar shows the contribution of the six different fractions to the total weight of the respective sample. P: *P. zonale*, Cr: *C. reflexa*, Cp: *C. platyloba*, Cc: *C. campestris*, Ccf: *C. campestris* flowers, A: extraction at 4 °C, B: extraction at 27 °C

Figure 14 shows the sample yield after flash fractionation. Yields are close to 100% in all samples except for PA, the *Pelargonium zonale* extraction performed at 4 °C. PA shows a yield far exceeding 100%, which is most likely due to a mistake during the weighing process and a subsequently wrong calculation of yield. Fraction 6 has the lowest dry weight, while in all samples but the flower extract (Ccf), fraction 1 contains more than 60% of the total sample weight. The flower extract contains more weight in its fractions 5 than the other samples, which shows a distinctive difference between stem and flower extract. *Cuscuta* samples generally contain more material in fraction 5 than *Pelargonium* samples, which in return contain more weight in fraction 3. The other fractions (2, 4 and 6) contain a similar portion of weight in the different samples.

3.2 Identification of Active Fractions

To evaluate samples for bioactivity and thus select fractions for further investigation, assays against different bacteria, cancer cells and PTP1B, an enzyme involved in diabetes type 2, were performed.

3.2.1 Bacterial Growth Inhibition

To search for activities in *P. zonale* samples in *C. campestris* flower samples and in *Cuscuta* stem samples, an initial screening with sample fractions and total extracts at a concentration of 50 µg/ml (DWF) was conducted. The cut-off value for the measured OD_{600} was set to 0.05. All samples below that value were tested further at different concentrations to evaluate their MIC and concentration dependence (Figure 15, Figure 16) for bacterial growth inhibition.

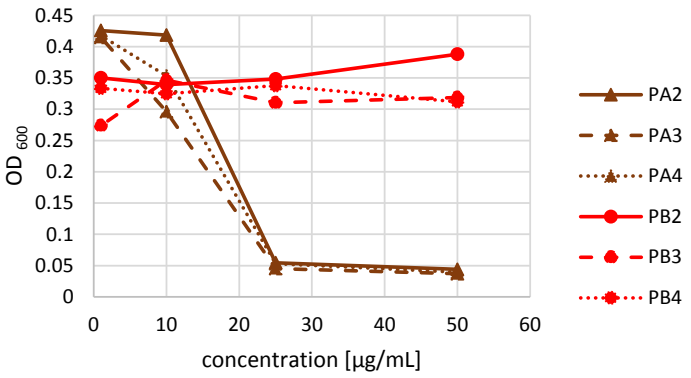
None of the samples was active against *E. faecalis*, *E. coli* and *P. aeruginosa* (data included in the appendix, Table 11, Table 12, Table 13). For *S. aureus,* fractions 2, 3 and 4 of both *P. zonale* extractions (PA and PB) showed activity (Table 7) and were analyzed again to identify the concentration dependence of this activity (Figure 15, Figure 16). Here the PB fractions had lost their activity while the PA fractions showed strong inhibitory activity at 50 µg/ml and 25 µg/ml (DWF).

In the screening for antibacterial activity against *S. agalactiae*, fractions 6 of the *P. zonale* samples as well as some of the *Cuscuta* samples showed activity. The *C. platyloba* (CpA and CpB) and *C. campestris* stem samples (CcA and CcB) all showed activity in fractions 6 while reduced bacterial growth could also be seen in fractions 6 of the *C. campestris* flowers (Table 7), but to a lesser extent that did not reach the cut-off value. CrA showed a higher activity in fraction 5 than in fraction 6, while CrB showed lower activity in fraction 6 than the rest of the stem samples. CrA5 was rated as active, CrB6 was rated as questionable and CrA6 was not taken into further consideration as the value did not reach the cut-off. The fractions rated as active and fraction CrB6, which had been rated as questionable, were tested further using different concentrations to evaluate their MIC value (Figure 16). They all repeatedly showed high activity at 50 µg/ml (DWF). PA6, PB6, CrA5, CpA6 and CcB6 showed the same high activity at 25 µg/ml (DWF), while CcA6 already showed slightly lower activity and CrB6 as well as CpB6 already were much less active. At 10 µg/ml (DWF) sample concentration, only PB6 inhibited growth visibly. To sum up, *Cuscuta* stem samples showed inhibitory potential against *S. agalactiae* in fractions 6 (fraction 5 in CrA) while *Pelargonium* samples exhibited various activities against two different kind of bacteria, *S. agalactiae* and *S. aureus*.

Table 7 – Summary of inhibition measurements on bacterial growth. Activity against two bacteria is expressed as optical density measured at 600 nm. Fractions were considered active when the OD_{600} value was below 0.05 (marked in red), questionable with an OD_{600} value in between 0.05 and 0.09 (marked in yellow) and inactive when the OD_{600} value was higher than 0.09. P: *P. zonale*, Cr: *C. reflexa*, Cp: *C. platyloba*, Cc: *C. campestris*, Ccf: *C. campestris* flowers, A: extraction at 4 °C, B: extraction at 27 °C, 1-6: fraction number, T: total extract

Sample type	*S. aureus*	*S. agalactiae*	Sample type	*S. aureus*	*S. agalactiae*
PA1	0.23	0.34	PB1	0.35	0.30
PA2	0.04	0.27	PB2	0.05	0.24
PA3	0.04	0.20	PB3	0.04	0.21
PA4	0.04	0.26	PB4	0.04	0.27
PA5	0.19	0.19	PB5	0.20	0.23
PA6	0.24	0.04	PB6	0.21	0.04
PAT	0.27	0.33	PBT	0.11	0.34
CrA1	0.36	0.28	CrB1	0.31	0.29
CrA2	0.40	0.35	CrB2	0.36	0.33
CrA3	0.41	0.33	CrB3	0.39	0.31
CrA4	0.33	0.33	CrB4	0.28	0.29
CrA5	0.23	0.04	CrB5	0.24	0.27
CrA6	0.30	0.14	CrB6	0.24	0.06
CrAT	0.26	0.31	CrBT	0.26	0.27
CpA1	0.39	0.44	CpB1	0.38	0.38
CpA2	0.35	0.37	CpB2	0.41	0.33
CpA3	0.37	0.39	CpB3	0.42	0.38
CpA4	0.37	0.37	CpB4	0.35	0.35
CpA5	0.26	0.36	CpB5	0.25	0.33
CpA6	0.25	0.04	CpB6	0.25	0.04
CpAT	0.33	0.34	CpBT	0.32	0.32
CcA1	0.35	0.34	CcB1	0.35	0.31
CcA2	0.40	0.33	CcB2	0.39	0.30
CcA3	0.40	0.39	CcB3	0.42	0.35
CcA4	0.34	0.31	CcB4	0.28	0.30
CcA5	0.23	0.32	CcB5	0.26	0.33
CcA6	0.26	0.04	CcB6	0.24	0.04
CcAT	0.23	0.29	CcBT	0.25	0.27
CcfA1	0.33	0.26	CcfB1	0.32	0.36
CcfA2	0.38	0.32	CcfB2	0.35	0.42
CcfA3	0.29	0.29	CcfB3	0.36	0.41
CcfA4	0.22	0.27	CcfB4	0.41	0.38
CcfA5	0.20	0.28	CcfB5	0.18	0.38
CcfA6	0.23	0.28	CcfB6	0.25	0.29
CcfAT	0.23	0.23	CcfBT	0.22	0.34

a

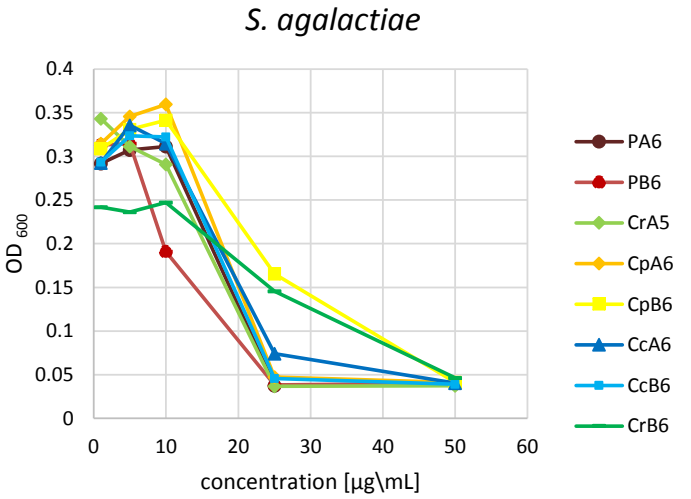


b

Sample type	MIC [µg/mL]
PA2	25
PA3	25
PA4	25

Figure 15 – Concentration dependence curves of fractions against *S.aureus*. a: The inhibitory effect of fractions that had been rated as active in the initial screening against *S. aureus* was tested using concentrations of 50 µg/ml, 25 µg/ml, 10 µg/ml and 1 µg/ml (DWF). Activity is expressed as optical density measured at 600 nm. b: The MIC value of the samples that had retained their activity were evaluated. The last concentration at which the OD_{600} value was below the cut-off value of 0.05 was designated as MIC value. P: *P. zonale*, A: extraction at 4 °C, B: extraction at 27 °C, 1-6: fraction number; mean values of two measurements are depicted, error bars have not been included

a



b

Sample type	MIC [µg/mL]
PA6	25
PB6	25
CrA5	25
CpA6	25
CpB6	50
CcA6	50
CcB6	25
CrB6	50

Figure 16 – Concentration dependence curves of fractions against *S. agalactiae*. a: The inhibitory effect of fractions that had been rated as active in the initial screening against *S. agalactiae* was tested using concentrations of 50 µg/ml, 25 µg/ml, 10 µg/ml and 1 µg/ml (DWF). Activity is expressed as optical density measured at 600 nm. b: The MIC value of the samples were evaluated. The last concentration at which the OD_{600} value was below the cut-off value of 0.05 was designated as MIC value. Cr: *C. reflexa*, Cp: *C. platyloba*, Cc: *C. campestris*, A: extraction at 4 °C, B: extraction at 27 °C, 1-6: fraction number; mean values of two measurements are depicted, error bars have not been included

To investigate active fractions further, the respective fractions were analyzed via MS. Chromatograms of active and inactive fractions were compared to find possibly responsible compounds for the observed bioactivities.

As during the screening for antibacterial activity, fractions 6 of different *Cuscuta* samples had been active, those fractions' chromatograms were evaluated comparing MS data. At first this yielded no distinctly different peak between active fractions 6 and inactive fractions 5. In the UV data, however, a difference became visible (Figure 17). A peak was found at a retention time of 10.60 min in the active fraction 6 but not in the inactive fraction 5.

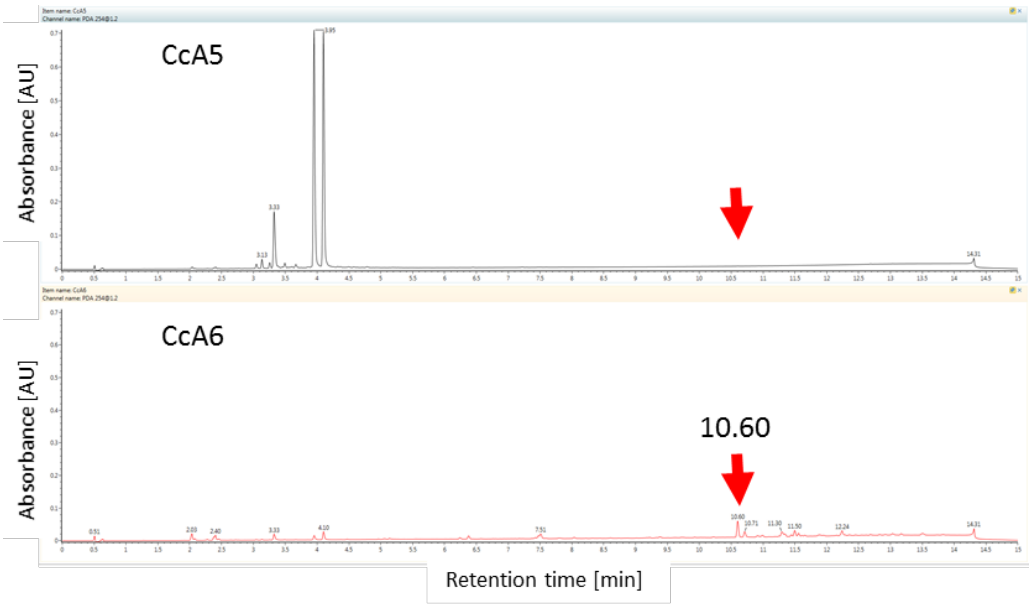


Figure 17 – UV chromatograms of inactive fraction CcA5 and active fraction CcA6. Arrows indicate the peak of interest at a retention time of 10.60 min.

As the UV data is collected before ionization of the sample, it is not linked to a spectrum to identify the corresponding *m/z* within that peak. Thus the corresponding peak in the base peak intensity (BPI) chromatogram had to be identified first before the spectrum could be evaluated. This, however, indicated a mix of many different compounds, where none seemed dominant. The issue was therefore not followed up further.

3.2.2 Biofilm Inhibition

To search for specific inhibitory activities against biofilm formation, an initial screening using all the sample fractions and total extracts at a concentration of 50 µg/ml (DWF) was applied and delivered a variety of samples that were rated as active (Table 8). PA3 and PB3 were rated as active but excluded because the wells had been clear and thus without bacterial growth.

PB2, PB4, PB5, PB6 as well as the total extract PBT were rated as active. CrA5 and CrA6 both showed activity while in all the other *Cuscuta* samples only fraction 6 showed activity within the cut-off value.

Table 8 – Summary of inhibition measurements on biofilm formation. Measured OD_{600} values of *Staphylococcus epidermidis* incubated with 50 µg/ml (DWF) of respective sample fractions. Activity is expressed as optical density measured at 600 nm. Fractions were considered active when the OD_{600} value was below 0.25 (marked in red). P: *P. zonale*, Cr: *C. reflexa*, Cp: *C. platyloba*, Cc: *C. campestris*, Ccf: *C. campestris* flowers, A: extraction at 4 °C, B: extraction at 27 °C, 1-6: fraction number, T: total extract

Sample type	Activity	Sample type	Activity	Sample type	Activity
PA1	0.97	CpA1	1.16	CcfA1	0.93
PA2	0.27	CpA2	1.07	CcfA2	0.90
PA3	0.08	CpA3	1.04	CcfA3	0.86
PA4	0.47	CpA4	1.14	CcfA4	1.07
PA5	0.34	CpA5	0.87	CcfA5	1.09
PA6	0.42	CpA6	0.14	CcfA6	0.16
PAT	0.29	CpAT	0.63	CcfAT	1.01
PB1	0.78	CpB1	1.05	CcfB1	0.99
PB2	0.18	CpB2	1.07	CcfB2	0.82
PB3	0.06	CpB3	1.08	CcfB3	0.91
PB4	0.16	CpB4	1.03	CcfB4	0.97
PB5	0.14	CpB5	1.21	CcfB5	1.07
PB6	0.23	CpB6	0.18	CcfB6	0.24
PBT	0.11	CpBT	1.10	CcfBT	1.02
CrA1	0.95	CcA1	1.09		
CrA2	0.93	CcA2	1.08		
CrA3	0.88	CcA3	1.07		
CrA4	0.95	CcA4	1.08		
CrA5	0.17	CcA5	1.24		
CrA6	0.14	CcA6	0.15		
CrAT	1.01	CcAT	1.09		
CrB1	1.06	CcB1	1.07		
CrB2	1.03	CcB2	1.10		
CrB3	1.07	CcB3	1.13		
CrB4	0.85	CcB4	0.97		
CrB5	0.89	CcB5	1.24		
CrB6	0.17	CcB6	0.15		
CrBT	1.01	CcBT	1.17		

3.2.3 Anti-cancer and Cytotoxicity Studies

To detect activities against cancer cells, all samples were screened against A2058 cells, a human melanoma cell line that is known to be relatively sensitive, using 50 µg/ml (DWF) to identify interesting fractions. Due to issues with reproducibility and solubility, the DMSO concentration (DMSO is needed here to dissolve the hydrophobic substances in the samples that are not

soluble in water) was raised to an end concentration of 1% DMSO in the test wells. The screening and second test were done at the same time to eliminate possible changes of dissolved sample composition. As a result, and due to the time limit, some of the fractions found to be active in the depicted screening have not been tested for concentration dependence. The other fractions that had been active in the initial screening against A2058 cells (Table 9) were tested again at different concentrations (Figure 18, Figure 19).

Data of the first screening and retest has been included in the appendix (Table 14, Figure 29, Figure 30).

Table 9 – Summary of inhibition measurements on A2058 human melanoma cells. Calculated cell survival [%] after incubation with sample fractions at 50 µg/ml (DWF) is shown. Samples showing 50% or lower cell survival were considered active (marked in red). Results shown are derived from the assay done with 1% DMSO end concentration in the test wells. P: *P. zonale*, Cr: *C. reflexa*, Cp: *C. platyloba*, Cc: *C. campestris*, Ccf: *C. campestris* flowers, A: extraction at 4 °C, B: extraction at 27 °C, 1-6: fraction number, T: total extract

Sample type	Activity	Sample type	Activity	Sample type	Activity
PA1	84,31	CpA1	71,17	CcfA1	76,75
PA2	11,00	CpA2	72,62	CcfA2	82,64
PA3	9,07	CpA3	88,35	CcfA3	80,89
PA4	10,02	CpA4	53,93	CcfA4	-0,66
PA5	92,50	CpA5	-5,00	CcfA5	-5,88
PA6	80,75	CpA6	60,93	CcfA6	85,55
PAT	10,64	CpAT	78,40	CcfAT	-5,19
PB1	88,23	CpB1	58,59	CcFB1	76,38
PB2	12,21	CpB2	68,09	CcFB2	91,61
PB3	15,19	CpB3	107,15	CcFB3	82,63
PB4	11,10	CpB4	34,87	CcFB4	84,05
PB5	85,53	CpB5	-5,50	CcFB5	-5,98
PB6	74,88	CpB6	62,47	CcFB6	-2,61
PBT	71,93	CpBT	66,22	CcFBT	-4,53
CrA1	97,13	CcA1	79,39		
CrA2	114,18	CcA2	81,54		
CrA3	104,40	CcA3	80,73		
CrA4	89,83	CcA4	61,91		
CrA5	-5,33	CcA5	-5,96		
CrA6	80,65	CcA6	60,40		
CrAT	101,12	CcAT	70,43		
CrB1	73,46	CcB1	79,67		
CrB2	108,20	CcB2	88,78		
CrB3	92,02	CcB3	72,77		
CrB4	96,13	CcB4	56,79		
CrB5	-7,31	CcB5	-5,40		
CrB6	93,81	CcB6	39,22		
CrBT	85,37	CcBT	63,73		

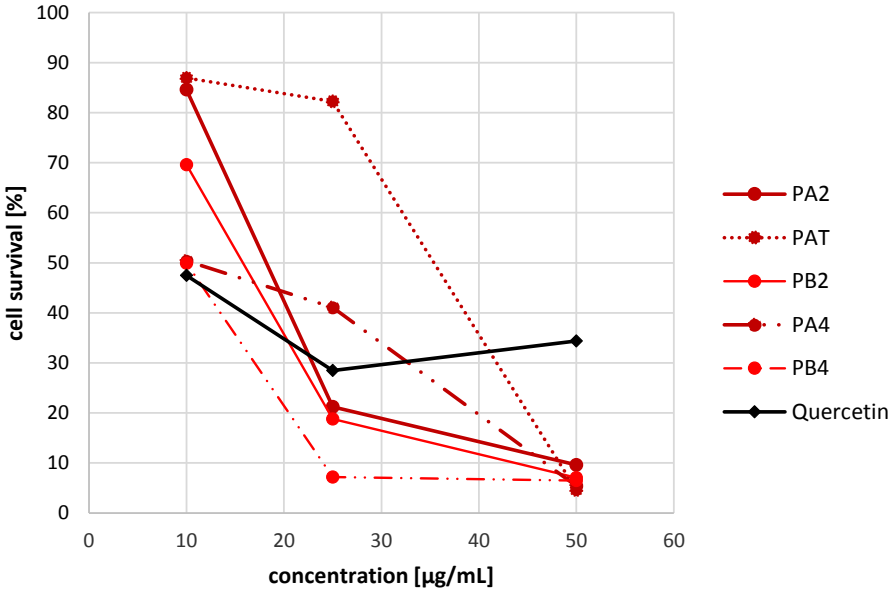


Figure 18 – Concentration dependence of *Pelargonium* samples against A2058 human melanoma cells. Samples were tested at concentrations of 50 µg/ml, 25 µg/ml and 10 µg/ml (DWF). The black curve shows a quercetin standard solution. P: *P. zonale*, A: extraction at 4 °C, B: extraction at 27 °C, 1-6: fraction number, T: total extract; mean values of two measurements are depicted, error bars have not been included

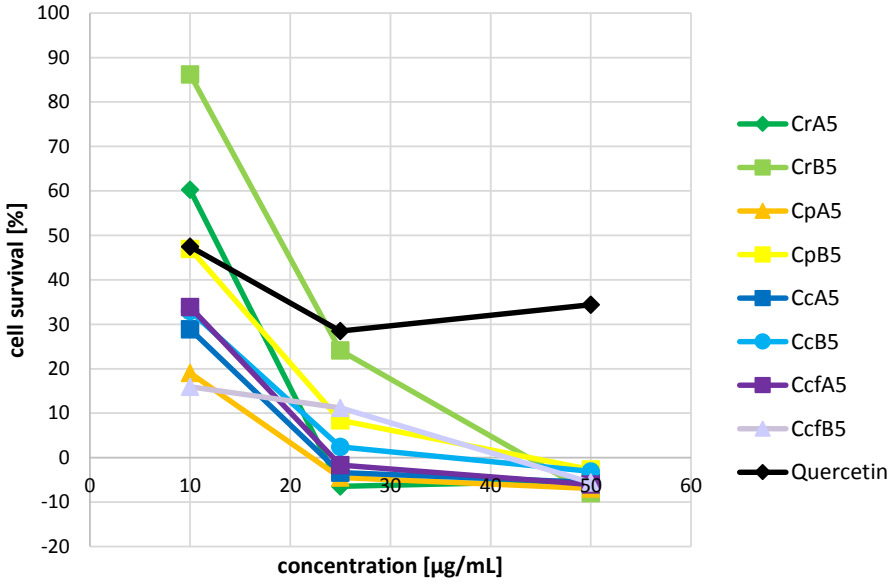


Figure 19 – Concentration dependence of *Cuscuta* samples against A2058 human melanoma cells. Samples were tested at concentrations of 50 µg/ml, 25 µg/ml and 10 µg/ml (DWF). The black curve shows a quercetin standard solution. Cr: *C. reflexa*, Cp: *C. platyloba*, Cc: *C. campestris*, Ccf: *C. campestris* flowers, A: extraction at 4 °C, B: extraction at 27 °C, 1-6: fraction number, mean values of two measurements are depicted, error bars have not been included

In the concentration dependence testing, of the cell viability assay, of *Pelargonium* samples (Figure 18) the total extract PAT only showed activity at a concentration of 50 µg/ml (DWF).

All other samples also showed an inhibition of cell growth with cell survival rates lower than 50% at a concentration of 25 µg/ml (DWF). PB4 even stayed active at a cell survival rate of around 50% at a concentration of 10 µg/ml (DWF).

Figure 19 shows the testing of concentration dependence for *Cuscuta* samples. All tested samples show cell survival rates below 50% at concentrations of 50 µg/ml and 25 µg/ml (DWF) and only fractions CrA5 and CrB5 show a cell survival rate higher than 50% at 10 µg/ml (DWF), while the rest is still rated as active at this concentration.

In the concentration dependence assay against MRC5 lung fibroblasts, all Pelargonium samples showed activity around 50% cell survival at a concentration of 50 µg/ml (DWF) but had already lost this activity at an concentration of 25 µg/ml (DWF) (Figure 20). In *Cuscuta* samples (Figure 21) CcA5, CcB5, CcfA5 and CcfB5 showed activity at all three concentrations (50 µg/ml, 25 µg/ml and 10 µg/ml (DWF)), CrA5 showed activity at 50 µg/ml and 25 µg/ml (DWF), and CcfA4, CpA5 and CpB5 showed activity only at 50 µg/ml (DWF). Cell survival values of CpB4 and CrB5 were above 50% at all tested concentrations.

In addition to the sample fractions, a quercetin standard was tested against both cell lines. It showed activities that were highest at a concentration of 25 µg/ml (DWF) and set in the range of sample effects. Many of the active sample fractions showed higher activities than the quercetin standard did.

To evaluate whether the observed effect on A2058 human melanoma cells is specific for cancer cells or a general cytotoxic effect, the same fractions that had been used in the retest, were also tested against MRC5 human lung fibroblasts as a non-cancer cell line. Table 10 shows which samples had been rated as active against A2058 and MRC5 cells and whether this renders them relevant for further investigation. Most of the fractions that were active against the cancer cells were also active against MRC5 cells. Samples PA2, PB2, PA4 and CcfA4, all at 25 µg/ml (DWF) as well as samples PB4 and CpA5 at 25 µg/ml and 10 µg/ml (DWF) were active against A2058 but not against MRC5 cells. The same is true for sample CrB5 at concentrations of 50 µg/ml and 25 µg/ml as well as CpB5 at 10 µg/ml (DWF). The biggest differences in activity against A2058 and MRC5 were observed in sample CrB5.

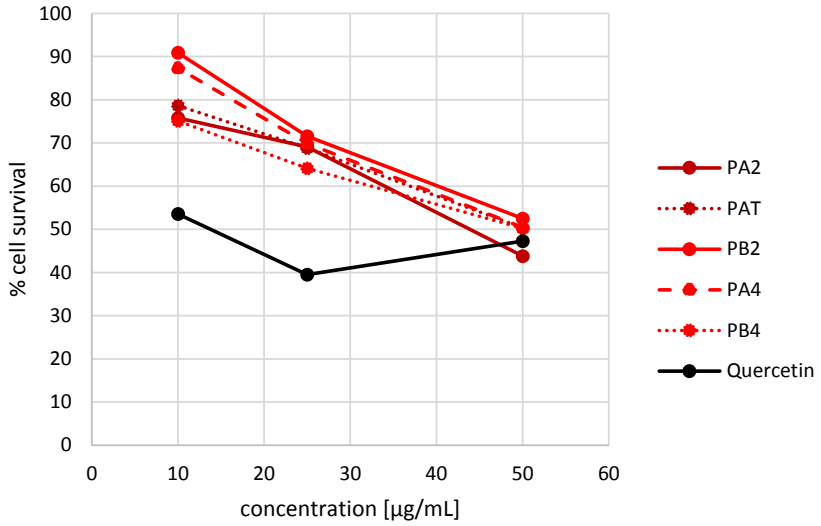


Figure 20 – Concentration dependence of *Pelargonium* samples against MRC5 human lung fibroblasts. Tested concentrations were 50 µg/ml, 25 µg/ml and 10 µg/ml (DWF). The black curve shows a quercetin standard solution. P: *P. zonale*, A: extraction at 4 °C, B: extraction at 27 °C, 1-6: fraction number, T: total extract, mean values of two measurements are depicted, error bars have not been included

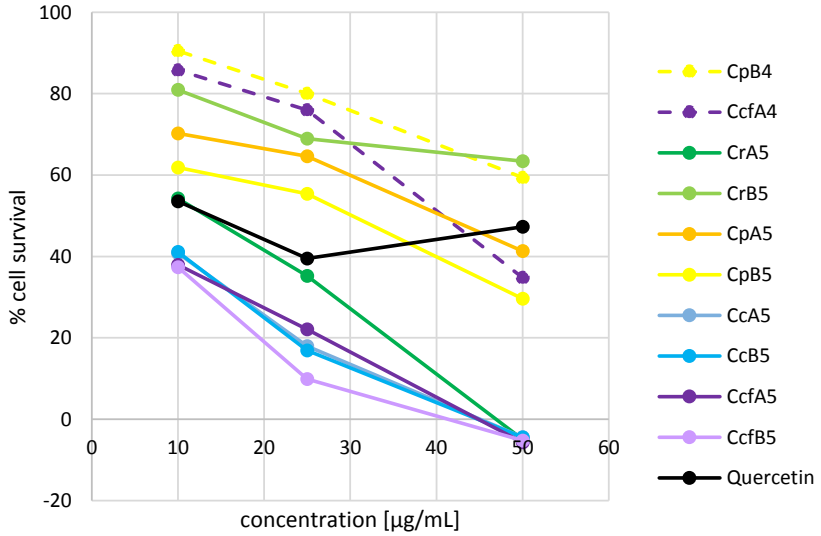


Figure 21 – Concentration dependence of activity of *Cuscuta* samples against MRC5 human lung fibroblasts. Tested concentrations were 50 µg/ml, 25 µg/ml and 10 µg/ml (DWF). The black curve shows a quercetin standard solution. Cr: *C. reflexa*, Cp: *C. platyloba*, Cc: *C. campestris*, Ccf: *C. campestris* flowers, A: extraction at 4 °C, B: extraction at 27 °C, 1-6: fraction number; mean values of two measurements are depicted, error bars have not been included

Table 10– Comparisons of activity against A2058 and MRC5 cells. Activities of samples rated as active in the cell viability screening in the retests against A2058 and MRC5 cells at different. +: active (<50% cell survival), +/-: questionable (50-60% cell survival), -: inactive (>60% cell survival). P: *P. zonale*, Cr: *C. reflexa*, Cp: *C. platyloba*, Cc: *C. campestris*, Ccf: *C. campestris* flowers, A: extraction at 4 °C, B: extraction at 27 °C, 1-6: fraction number, T: total extract

	A2058			MRC5		
	µg/mL			µg/mL		
Sample	50	25	10	50	25	10
PA2	+	+	-	+	-	-
PA6	-	-	-	-	-	-
PAT	+	-	-	+/-	-	-
PA4	+	+	+/-	+/-	-	-
PA5	-	-	-	-	-	-
PB2	+	+	-	+/-	-	-
PB6	-	-	-	-	-	-
PBT	-	-	-	-	-	-
PB4	+	+	+	+/-	-	-
PB5	-	-	-	-	-	-
CrA4	-	-	-	-	-	-
CrA5	+	+	-	+	+	+/-
CrB4	-	-	-	-	-	-
CrB5	+	+	-	-	-	-
CpA4	+/-	-	-	-	-	-
CpA5	+	+	+	+	-	-
CpB4	+/-	-	-	+/-	-	-
CpB5	+	+	+	+	+/-	-
CcA4	-	-	-	-	-	-
CcA5	+	+	+	+	+	+
CcB4	-	-	-	-	-	-
CcB5	+	+	+	+	+	+
CcfA2	-	-	-	-	-	-
CcfA3	-	-	-	-	-	-
CcfA4	+	+	-	+	-	-
CcfA5	+	+	+	+	+	+
CcfB2	-	-	-	-	-	-
CcfB3	-	-	-	-	-	-
CcfB4	-	-	-	-	-	-
CcfB5	+	+	+	+	+	+

To investigate active fractions further, the respective fractions were analyzed via MS. Chromatograms of active and inactive fractions were compared to find possibly responsible compounds for the observed bioactivities

Fractions 5 of all *Cuscuta* extracts had been rated as active in the cell viability screening. Fractions 4 had also shown some inhibition of cell growth, but were mostly not rated as active

because cell survival rates remained above the cut-off value of 50%. To find the compound responsible for the cytotoxic effect, chromatograms of fractions 5 and 4 were compared to inactive fractions. These chromatograms were searched for a peak in fraction 5 and, to a lesser extent, in fraction 4, but not in any of the inactive fractions. Figure 22 shows chromatograms of samples CpA2-CpA6 and the corresponding extracted mass chromatograms for an *m/z* value of 303.05101. The peak at a retention time of 3.6 minutes appeared in fractions 4 and 5, not in the inactive fractions 2 and 3 and only to a very small extent in inactive fraction 6. Peaks with the same mass appeared at a retention time of 2.06 minutes in fractions 3, 4 and to a lesser extent in fractions 5 and 6. This hinted to a different compound with the same *m/z* ratio but a different structure.

After identifying a candidate compound with an *m/z* value of 303.05101 for the cytotoxic activity, observed in the cell viability assay, ChemSpider, a chemical structure database, was used to search for its identity. Hits included many polyphenolic compounds with several equal hit scores. One of them, quercetin, ($C_{15}H_{10}O_7$) was selected (Figure 23) and verified by comparing with a commercial standard.

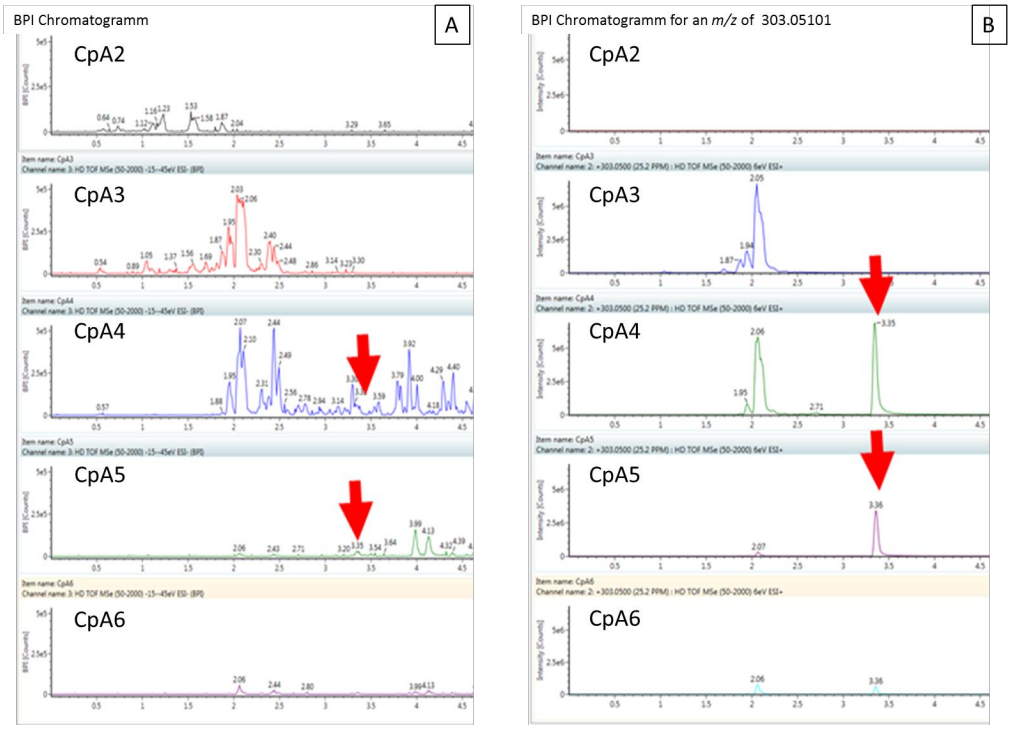


Figure 22 – Chromatograms and extracted mass chromatograms for fractions CpA2-CpA6. A: Comparison of chromatograms. Arrows indicate the presence of the relevant peak at a retention time of 3.36 min which is present in active fractions CpA5 and CpA4 (whose activity was slightly above the cut-off value) of the cell viability assay. B: Comparison of extracted mass chromatograms, searching for an *m/z* value of 303.0500. Arrows indicate the presence of the relevant peak at a retention time of 3.36 min present in active fractions CpA5 and CpA4 (whose activity was slightly above the cut-off value) of the cell viability assay. Cp: *C. platyloba*, A: extraction at 4 °C, 1-6: fraction number

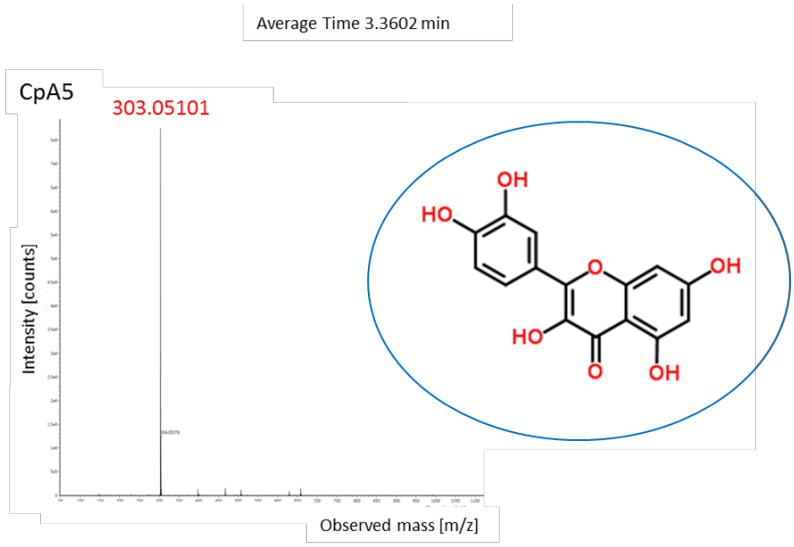


Figure 23 – The candidate compound for the observed anti-cancer activity. Spectrum of the peak at a retention time of about 3.6 minutes in CpA5 illustrating that its dominating component is a compound with an *m/z* value of 303.05101. To the right the structure of quercetin, the result of the database search for this *m/z* with an molecular formula of $C_{15}H_{10}O_7$.

3.2.4 Anti-PTP1B Activity

This assay was performed to find inhibitory activity against PTP1B, an enzyme related to diabetes type 2. In an initial screening, all samples were tested at a concentration of 50 µg/ml (DWF) against PTP1B, analogous to the other assays. The *Pelargonium* samples showed inhibition in fractions 2 throughout 6 and in the total extract while the *Cuscuta* samples were active in fractions 6 of all the different stem samples. *C. campestris* flowers did not show any activity against PTP1B (Figure 24). To evaluate activity at lower concentrations, the screening was repeated at 10 µg/ml (DWF) sample concentrations. At this concentration only the *Pelargonium* sample fractions 3 retained their enzyme inhibition (Figure 24).

To ensure a selective inhibition of PTP1B, a second screen against TC-PTP was done (Figure 24), using all samples that had been active against PTP1B at 50 µg/ml (DWF) or lower. All tested *Cuscuta* fractions as well as most of the *Pelargonium* samples showed activity against TC-PTP. The only samples inactive against TC-PTP were PA5, PA6, PB2 and PB6. As those samples had not shown activity against PTP1B at lower concentrations of 10 µg/ml (DWF) they were not evaluated further.

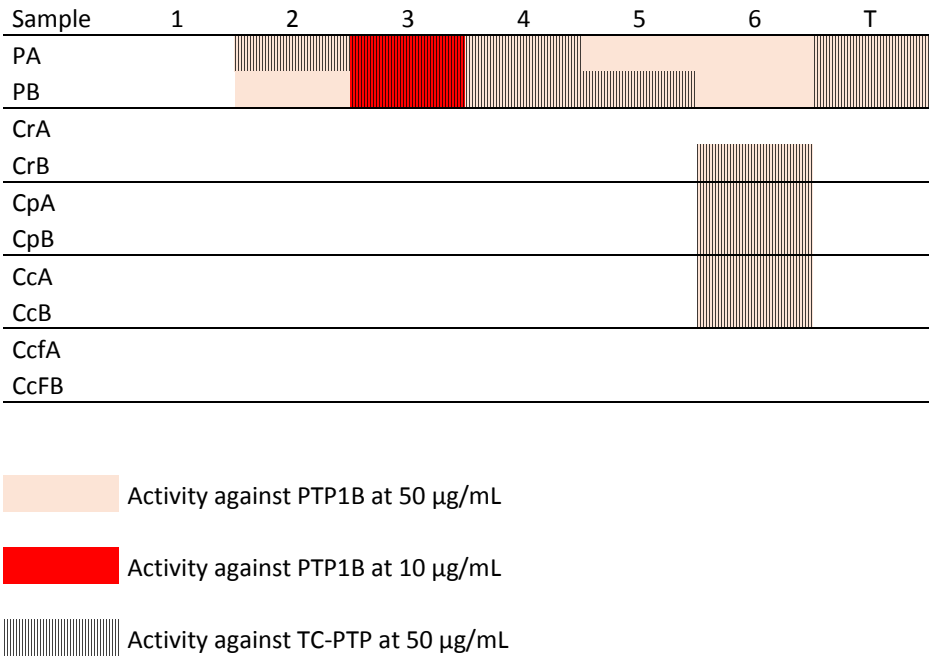


Figure 24 – PTP1B enzyme inhibiting activities of sample fractions. A sample fraction was rated as active when it was able to inhibit enzyme activity to a value of 30% or lower of the original activity. The assay was done against PTP1B at two different sample concentrations: 50 µg/ml and 10 µg/ml (DWF). Active fractions were then tested against TC-PTP. Interesting fractions show activity against PTP1B but no activity against TC-PTP. P: *P. zonale*, Cr: *C. reflexa*, Cp: *C. platyloba*, Cc: *C. campestris*, Ccf: *C. campestris* flowers, A: extraction at 4 °C, B: extraction at 27 °C, 1-6: fraction number, T: total extract

4. Discussion

The aim of this thesis was to compare different *Cuscuta* species and different tissues of one *Cuscuta* species among each other as well as to their host plant *Pelargonium zonale* in terms of activities that were tested in different bioassays. Candidate compounds responsible for those activities should be identified, while the influence of different extraction temperatures on the results of the bioactivity assays was also of interest. The discussion is composed of three blocks: The first one addresses the influence of the methods on the experimental results (4.1), the second part compares the obtained results among different *Cuscuta* species, between different *Cuscuta* tissues and between parasite and host respectively (4.2) and the third part discusses the results in context with previous findings (4.3).

4.1 Methods' Influence on Results

It is hardly possible to choose any method that does not influence the results. Thus, it is important to be aware of the influences it has on the results one obtains and, to adjust the methods to the goals of the study at hand. Here the intended use of the compounds as drugs defines to a certain degree, which properties they must show to be of interest. An example for this is a limited degree of hydrophobicity, as high hydrophobicity would interfere with purification methods. Methods in high throughput eliminate substances that might have a relevant activity but do not fit other requirements. When interested in those, they have to be evaluated in a different approach.

The first method whose influence on the obtained results is evaluated here is extraction. It is usually applied when investigating natural compounds as the first step. It cuts down the number of analyzable compounds notably and is thus crucial and limiting at the same time for the respective study. The method as well as the solvent used will in most of the cases favor compounds with certain properties, while discriminating against others. In this study the loss of activity of samples PB in the concentration dependence study of the growth inhibition assay (Figure 15) is a tentative example for the influence of the extraction method on the results. Here, samples extracted at a lower temperature (PA), had kept their initial activity during later repetitions of an assay, while samples extracted at a higher temperature (PB) had lost theirs. On the other hand, PB showed activity in many fractions during the biofilm assay, while in PA only some of those fractions were active (Figure 27). *C. reflexa* samples showed activity in fraction 6 only in extraction B during the diabetes assay (3.2.4). While the extractions at Marbio, where the focus is on marine organisms, are usually conducted at 4 °C, extractions with plant samples

often use higher temperatures (Behbahani, 2014; Islam, 2015). However, it appears from the results of this study that this comes at the expense of compound stability. Extraction at a lower temperature conserves the activity of the compounds for a longer time (4.3). Another issue related to the properties of the extraction solvent became evident when pigments were dissolved in 100% methanol, while other compounds were only soluble after the addition of water (2.2). These difficulties make it highly plausible that also in subsequent steps, when dissolving fractions in DMSO for storage (2.3) and afterwards dissolving it again in a DMSO/water mixture (2.4), some insoluble substances are lost (Di & Kerns, 2006). The issue of solubility will be addressed in more detail below. One method to be able to extract a higher amount of compounds from plants, which is often applied is, to use two separate extractions. One of them uses a polar solvent (like methanol), the other a non-polar solvent like chloroform or petroleum ether (Mazumder *et al.*, 2003; Rahmatullah *et al.*, 2010). The use of methanol as a solvent however, should include many potentially interesting and applicable compounds. It is preferred as the exclusive solvent in multiple studies on *Cuscuta* compounds (Behbahani, 2014; Lee *et al.*, 2011). Moreover, as the capability of downstream separation methods and analysis tools (flash fractionation and MS) was restricted and did not allow the analysis of highly hydrophobic compounds, in this work only a methanol extraction was performed.

Another technical challenge became clear when performing the cell viability assay, where results were at first not reproducible. Low solubility of an active compound was identified as the reason for those unstable activities. The concentration dependent assay following the first screening that was performed here is a means to detect and estimate such effects. To avoid them, fractions were added directly to the assay media instead of performing series of aqueous dilutions first (Di & Kerns, 2006). All screens (A2058 screening, A2058 concentration dependence and MRC5 concentration dependence, 3.2.3) were performed simultaneously and immediately after dissolving the dried samples in a mixture of DMSO and water with an increased portion of DMSO. DMSO content should not, however, be increased further than to an end concentration of 1% in the cell media, as this could harm the cells (Lilly, 2007). The first screening itself, however, as fractions are still composed of many different compounds, has to be done at a rather high concentration (DWF) to ensure having enough of every single substance in the sample to still perceive its bioactivity. Solubility issues like the one observed here can notably influence tests for biological activity. Low solubility of a compound in a certain solvent can decrease its concentration drastically and therefore lead to false or contradictory results (Di & Kerns, 2006). This was especially relevant here, as tests were done

in a different solvent than MS analysis, which was used to identify compounds. Moreover, freeze-thaw cycles and low storage temperatures that were applied to prevent oxidation processes and other reactions that might alter the original compounds, precipitate poorly soluble compounds (Di & Kerns, 2006). This again leads to concentration variations in dilutions.

Another factor that needs to be kept in mind are the limits of applied methods. When evaluating the MS data for a candidate for the observed anti-bacterial activities in fractions 6, the peak of interest could only be found in the UV data (Figure 17). Mass spectrometry can provide powerful results, calculating the mass and, with the help of databases, suggesting a probable identity and structure of a compound. Nevertheless, it is not possible to account for all compounds in a sample when using MS. Some compounds might for example be ionized very poorly and thus be invisible. The presence of very abundant compounds like phenols in plant samples can further mask other compounds making them difficult to distinguish.

4.2 Comparisons Among Samples

Stem extracts of three different species of the parasitic plant *Cuscuta*, one flower extract and the host plant *P. zonale* were analyzed in this thesis. Differences as well as similarities among the samples were expected and are evaluated in this part of the discussion. In regard of different species, the *Cuscuta* plant family has been found to vary greatly in, for example, photosynthesis and xanthophyll composition (Kruk & Szymanska, 2008). As those are important and thus rather conserved mechanisms, while secondary metabolites vary highly between different taxa, it is likely that *Cuscuta* species that differ within primary functions also vary in secondary products. To restrict these differences, all parasitic species were grown on the same host, at the same time of the year and under the same temperature and light conditions. Starting the comparison with the sample fractions after flash fractionation (Figure 14), they differ only marginally among the different species in term of weight ratios. As chlorophylls elute late in reversed phase chromatography and because of the green color in fractions 6, it can be assumed that they were contained there (Saitoh *et al.*, 1995). Consistently, *C. platyloba*, which is the least green one of the three species, contains less weight in fraction 6. Throughout the bioassays, the three different *Cuscuta* species showed no major differences (Figure 26, Figure 27, Figure 28). Slight deviations of activity in fraction numbers can be attributed to variations during flash fractionation. In the cell viability assay, *C. reflexa* samples show only activity in fraction 5 and high cell survival in fractions 4, while the other species reach values very close to the cut-off of 50% cell survival in fractions 4 as well. Another difference worth investigating is the differing activity of the species towards the non-cancerous cell line MRC5. *C. campestris* shows

high activity against those cells throughout, while both other species exhibit lower activity against MRC5 cells compared to A2058 cancer cells. Again, a potential difference in composition of the active compounds could be observed, this time leaving *C. campestris* as the outsider. All this points to a different composition of compounds in *Cuscuta* species and encourages further comparative investigation.

As flowers have differential transcriptomic activity compared to stems, it can be assumed that their tissue also contains a different balance of compounds, maybe even new compounds that are exclusively found there. The weight ratio of the fractions after flash fractionation shows a higher variability between flowers and stem of the same species than between stems of different species (Figure 14). In flower samples, the relative amount of fraction 1 is lower than in stem samples, while fraction 5 is enhanced. As, after personal communication (Jeanette Hammer Andersen), the fractions that are most probable to yield bioactive compounds in this set of methods are fractions 5, this suggests higher or more diverse bioactivity in flower extracts compared to stem extracts. This was only partly confirmed, however, in the results obtained from bioactivity assays in this thesis. Flower extracts showed less activity than stem extracts in the anti-bacterial assay for growth inhibition where fractions 6 of all stem samples where active, while flower samples where completely inactive (Figure 26). In contrast to this, flower extracts showed activity in biofilm assay. Whether this activity is specific for biofilm formation or an anti-bacterial effect directed against *Staphylococcus epidermidis* should be evaluated in future research. In the cell viability assay, activity of flower extracts was spread over fractions 4-6 and also included the total extract, while in stem samples it only included fractions 5 and occasionally a fraction 4 (Figure 28). This implies a higher abundance of a cytotoxic compound in the flowers distributed across fractions 4, 5 and 6. However, flower extracts screened for activity against the non-cancerous cell line MRC5 showed just as much activity against those as against A2058 cancer cells. The same was true for *C. campestris* stem samples and can thus not be related to a difference between stem and flowers. As flowers might contain a higher abundance of compounds and the other species showed activity that is more selective against cancer cells, flowers of other *Cuscuta* species should be investigated for their anti-cancer activity. However, a problem with that is that flowering in the different *Cuscuta* species is induced under different temperature/light regimes, thus violating the comparability of the growth conditions.

Though they are two different plants, a parasitic plant is intimately connected to its host. Molecules are exchanged between the two conjoined organisms (for which the exact mechanism is not known yet) and the host should thus be taken into account when considering the compounds present in the parasite *Cuscuta*. The host plant used to grow all three different species of *Cuscuta* plants, is *Pelargonium zonale* of the *Geranium* family. Its common name is "horse-shoe pelargonium" or "Wildemalva" in Afrikaans. It is native to southern Africa, though the various colorful flowers of its cultivated forms can be seen on balconies all over the world.

Figure 25 – *Pelargonium zonale* (Phytotron UiT Tromsø)

Pelargonium samples show a different ratio of weights between the six flash fractions compared to *Cuscuta* samples (Figure 14). This variation is not surprising as these plants belong to very different taxonomic groups and contain very different sets of secondary metabolites and other compounds (Croteau *et al.*, 2000). Generally, *Pelargonium* samples showed higher activity than *Cuscuta* throughout all assays, which is consistent with the fact that *Pelargonium* species have a long tradition as medicinal plants and have been shown to have anti-oxidant, anti-microbial and anti-cancer activity (Saraswathi *et al.*, 2011) as well as being effective against respiratory tract infections like bronchitis (Agbabiaka *et al.*, 2008). Those results, however, were generally not pursued further as this thesis was focused on *Cuscuta*. Moreover it was not observable in this work, if and which compounds were taken up by *Cuscuta* from *P. zonale*. Compounds that appear in both plants and were altered in *Cuscuta* were observed in Behbahani (2014), where lutein, lupeol and eugenol were found in both, host and parasite, while the epoxide forms of all three substances were only found in *Cuscuta.* Closer comparison to find compounds like these and comparisons between the same species of *Cuscuta* plants grown on different host plants are necessary to answer this question.

4.3 Identification of Fractions with Potential Biotechnological Value

Different *Cuscuta* extracts were fractionated and evaluated for various biological activities. From the two different kinds of extractions that were performed on *P. zonale* samples, the one at 4 °C showed a more stable activity in the bacterial assay after lengthy storage time than the extraction at 27 °C. Some of the *Cuscuta* fractions showed promising anti-cancer activity

combined with low or no cytotoxic activity against a non-cancerous cell line. Additionally, MS analysis yielded a phenolic compound, quercetin, as a candidate for the observed anti-cancer activity. For the interpretation of the obtained results, the activities found in bioassays are a first hint to a promising compound present in the respective fraction. As fractions are still a complex mixture of compounds, however, synergistic effects can strongly influence the activity (experience of the Marbio research group). Therefore, it is not possible to simply link a certain activity to a compound with the available data after bioassays and MS. To ascertain which compound causes activity and to what extent it is active, it is necessary to isolate and purify that compound and test it again in pure from. Additionally, cut-off values applied here were not based on biological considerations. They were used to sort the data in active and inactive fractions, to narrow down the candidates to the most promising ones. Thus, in further analysis of this data the exact values should also be considered to interpret the data, as ratings for active and inactive might be too strict when trying to interpret the biological background.

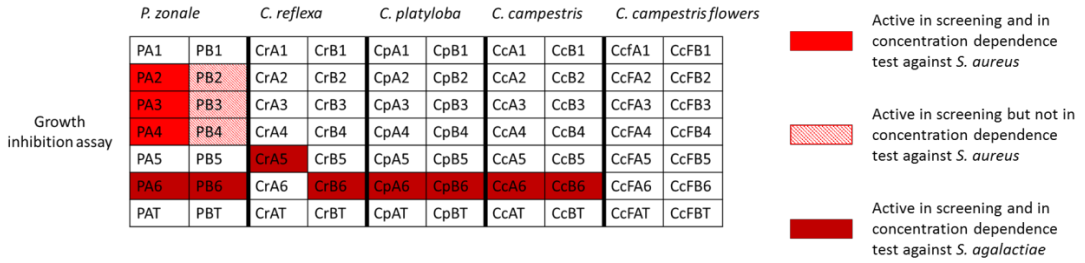


Figure 26 – Summarized results of the growth inhibition assay. Colored scheme showing in red the activities that were found (using a cut-off value of OD_{600} 0.05) in the growth inhibition assay screening at 50 µg/ml (DWF) against either *S. aureus* or *S. agalactiae* and the ones that were confirmed or not confirmed during the concentration dependence test. P: *P. zonale*, Cr: *C. reflexa*, Cp: *C. platyloba*, Cc: *C. campestris*, Ccf: *C. campestris* flowers, A: extraction at 4 °C, B: extraction at 27 °C, 1-6: fraction number, T: total extract

During evaluation, it stood out that the results of the growth inhibition assay were not entirely consistent in screening and retest (Figure 26). It is worth noting that samples affected by the observed loss of activity in the concentration dependence test belonged all to extraction B that had been performed at high temperature. Additionally, the respective sample plate had been stored for a longer period before performing this concentration dependence test. The factor that only the concentration dependence test against *S. aureus* had been affected by loss of activity can probably be attributed to the time-difference in between the two assays, as the concentration dependence test against *S. agalactiae* had been performed earlier and did not show any reduced activity. The observed loss of activity might thus be attributed to oxidation or other degradation processes that occur more rapidly at higher temperatures. While there was no difference visible

during the first screening, these effects might have advanced during storage of the sample plate, and thus led to the observed loss of activity after some storage time.

The activities observed in the growth inhibition assay were limited to *S. aureus* and *S. agalactiae.* (Table 7). Activities of *C. reflexa* extract against *S. aureus* has been discovered before when testing with the cup plate method (D. K. Pal *et al.*, 2006), but contrary to the discoveries here, D. K. Pal *et al.* (2006) also detected anti-bacterial activity of *C. reflexa* extract against *E. coli* and *P. aeruginosa*. The cup-plate method, however, measures zone of inhibition against bacteria on a plate with the respective sample extract administered in the middle (Autore *et al.*, 1984). As performing an assay in solution like it was done here might pose a severe difference, results cannot be compared directly. Additionally, the cut-off value used here may mask lower activities, which would have to be investigated closer for their significance. It is, however an impulse for future investigations, especially considering the issue of compound solubility discussed earlier, to employ and compare different types of assays when testing for the same phenomenon. *C. reflexa* extracts have also been found to be active against other strains of bacteria. Islam (2015) concluded however that petroleum ether extracts were more effective than methanol extracts. Together with the activity in the most lipophilic fraction 6 and mass spectrometry data that did not yield a responsible compound for anti-bacterial activity due to the complex mixture of compounds in fraction 6 (3.2.1), this indicates that anti-bacterial substances present in *Cuscuta* are probably hydrophobic and therefore require a less polar solvent in order to be evaluated better. While there are a few studies investigating the anti-bacterial activity of *C. reflexa*, this is not the case for the other species examined here. As in this study all three species tested show the same activity, a comparison can be used in future studies to identify the responsible compounds and possible differences in amount and composition of substances. In contrary to the activity of *Cuscuta* against bacterial strains, where studies of the actually active anti-bacterially compounds are still rather scarce, a compound found in *Pelargonium* species, geranium oil, which is a mixture of many compounds dominated by citronellol and geraniol, has been identified to be active against various *S. aureus* strains (Bigos *et al.*, 2012). Though isolated from *Pelargonium graveolens* in the mentioned study, it is plausible that a similar mixture of compounds causes the inhibiting effect of *P. zonale* extracts against *S. aureus*.

The second bacterial assay applied was designed to search for biofilm inhibition. However, it does not measure the difference between killed bacteria and actual inhibition of biofilm formation. Thus, bacterial growth was checked visually at the corresponding point of the assay.

If bacteria are killed, no measurement of specific biofilm inhibiting properties is possible. Instead, the fractions in question show anti-bacterial activity, which is not of interest in this assay. Control, however, is only visual and thus not very reliable. To estimate the real influence of anti-bacterial activity on the results of this assay, the active fractions of growth inhibition assay and biofilm assay were compared (Figure 27). The results obtained from the biofilm assay agreed with the ones obtained during the growth inhibition assay. No concentration dependence assay was done as this similarity was assumed to verify growth inhibition effects rather than specific inhibition of biofilm formation. In the current literature there is no biofilm inhibiting activity attributed to *Cuscuta* so far. Yet, fractions 6 of the flower samples showed activity only during the biofilm assay, but not in the growth inhibition assay. There is no study indicating an anti-bacterial activity of *Cuscuta* species against *Staphylococcus epidermidis*, it might thus be possible to detect some specific activity against biofilm formation in the flower extract. However, it is also highly probable that the anti-bacterial compounds present in *C. campestris* stem are also present in *C. campestris* flowers and for some reason only show their activity in the biofilm assay, not against the bacteria of the growth inhibition assay. The reason for that might be a lower concentration or a different kind of compound mixture in the flowers.

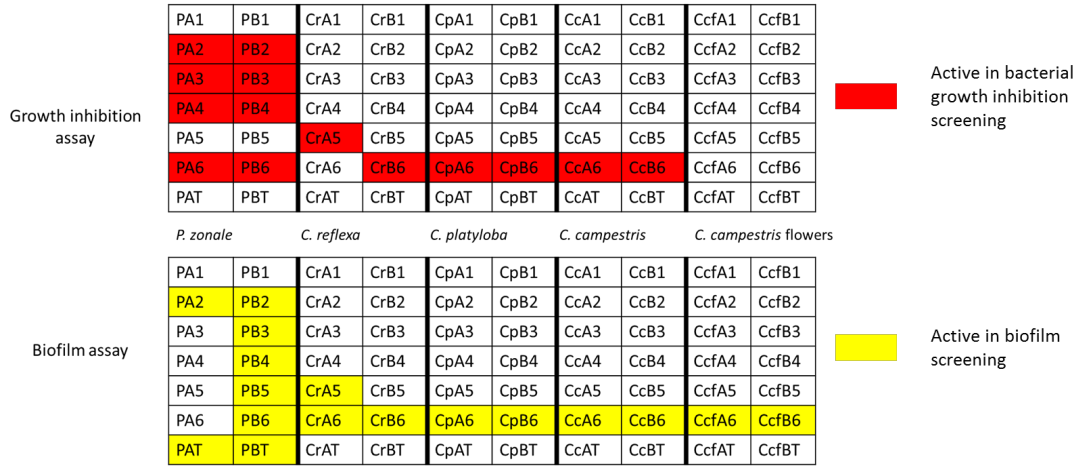


Figure 27 – Summarized results of the growth inhibition and the biofilm assay. Colored scheme showing in red the activities that were found (using a cut-off value of OD_{600} 0.05) in the growth inhibition assay screening at 50 µg/ml (DWF) (upper part) as well as in yellow the activities that were found (using a cut-off value of OD_{600} 0.25) in the biofilm screening at 50 µg/ml (DWF) (lower part). P: *P. zonale*, Cr: *C. reflexa*, Cp: *C. platyloba*, Cc: *C. campestris*, Ccf: *C. campestris* flowers, A: extraction at 4 °C, B: extraction at 27 °C, 1-6: fraction number, T: total extract

While activities in the prokaryotic assays where observed in fractions 6 of the *Cuscuta* samples, activities in the eukaryotic cell viability assay were concentrated mainly around fractions 5 (Figure 28). When considering the cell survival values in Table 9 it becomes obvious that while some of the fractions 4 are also rated as active, many of the inactive fractions 4 of the *Cuscuta*

samples are also close to the cut-off value of 50% cell survival. Those deviations indicate a slightly different distribution of active compounds by flash fractionation. An exception to that phenomenon is *C. reflexa* (samples CrA and CrB) where only fraction 5 shows noteworthy activity. This implies that in *C. reflexa* there is a difference in compounds compared to the other species, which should be investigated further in the future. The flower extract shows a more widespread activity, including fractions 4, 5 and 6, which could be attributed to variations in flash chromatography, but also total extracts, which points to a higher overall concentration of anti-cancer compounds. This should be investigated further, as there seem to be no studies addressing this issue.

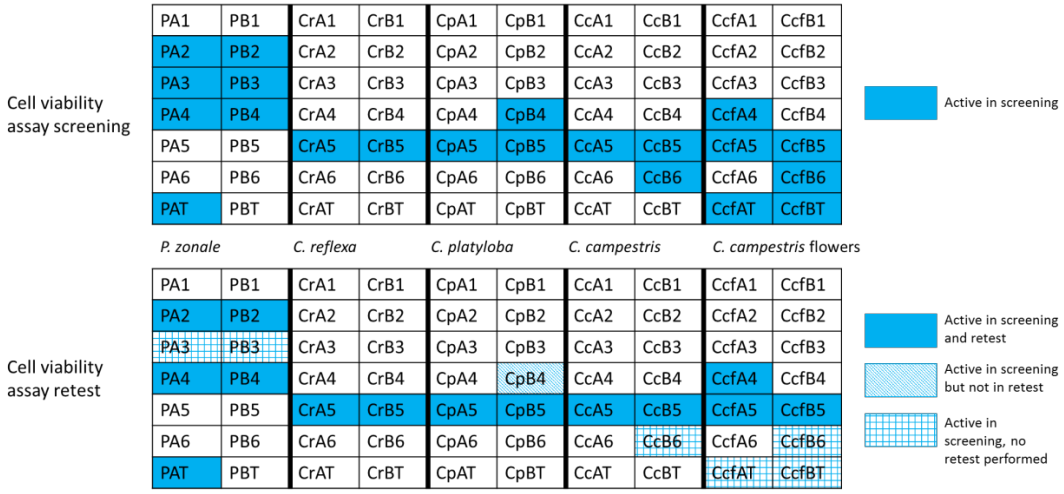


Figure 28 – Summarized results of the cell viability assay. Colored scheme showing in blue the activities that were found (using a cut-off value of 50% cell survival) in the cell viability assay screening (upper part) at 50 µg/ml (DWF) and the ones that were confirmed or not confirmed during the retest (lower part). It also indicates the active fractions that were not included in the retest. P: *P. zonale*, Cr: *C. reflexa*, Cp: *C. platyloba*, Cc: *C. campestris*, Ccf: *C. campestris* flowers, A: extraction at 4 °C, B: extraction at 27 °C, 1-6: fraction number, T: total extract

By mass spectrometry and a database comparison, quercetin was identified as an anti-cancer compound present in *Cuscuta* samples. This result confirms that workflow and methods are valid for achieving the goal of identifying bioactive compounds. Quercetins' presence in *Cuscuta* and its anti-cancer properties, however, had already been described (Lee *et al.*, 2011; Loffler *et al.*, 1995), so this compound is not itself of particular interest. When comparing the activity of the active *Cuscuta* fractions to pure quercetin (Figure 19, Figure 21), some of the fractions exhibit a higher activity than quercetin alone. This might indicate a mixture of components acting separately and giving an added effect. Alternatively, it could also be caused by synergistic effects within the fractions caused by other active compounds in the *Cuscuta* sample fractions that could enhance the bioactivity that is exhibited by pure quercetin. Literature research shows that there have been a number of studies conducted on anti-cancer

activity of different *Cuscuta* species. While Suresh *et al.* (2011) found the watery extract of *C. reflexa* to possess anti-cancer and anti-inflammatory activity and described some molecular mechanisms responsible for the observed activity, no responsible compound for those effects was identified in this study. Additionally, other *Cuscuta* species like *Cuscuta chinensis* and *Cuscuta epithymum* were found to be active against different cell lines using chloroform and hydroalcoholic extracts (Jafarian *et al.*, 2014) and activities of *Cuscuta* species against melanoma and lymphoma cell lines were discovered (Ghazanfari *et al.*, 2013). Moreover, Riaz *et al.* (2016) identified some compounds and evaluated their activity against colorectal cancer cell lines and *Cuscuta kotschyana* extract was found to show activity in a cell viability test against human breast cancer cell line MCF7 in a study conducted by Sepehr *et al.* (2011). This study attributed the anti-cancer activity mainly to quercetin and kaempferol ($C_{15}H_{10}O_6$) and thus confirms the result of this thesis. However, comparing the studies mentioned in this paragraph it is obvious that quercetin is not the only compound contributing to anti-cancer activity, as its effect would for example be lost in a watery extract as investigated by Suresh *et al.* (2011) due to its poor solubility. While in Cuscuta sample fractions anti-cancer activity was limited to fractions 4 and 5, this was different in *P. zonale*. Activities covered factions 2-4 and the total extract, which points to a high concentration of anti-cancer compounds as well as different compounds than those showing activity in *Cuscuta* samples. If those compounds are by any means related to the ones acting in *Cuscuta* and if a connection can be found that links them, for example transport from the host to the parasite and subsequent modification, should be evaluated. Probably there is also more than only one responsible compound as activity is spread over three fractions. Also other studies discovered an anti-cancer activity related to *P. zonale* extract (Hassanein *et al.*, 2011; Lewtak *et al.*, 2014). In the concentration dependent re-test (Figure 18) however, PA2 shows high activity at 50 µg/ml (DWF) and no activity at lower concentrations, while other active fractions show activity curves that resemble concentration dependence. The sharp decline in activity in PA2 indicates unspecific inhibition due to high concentrations of a compound, and can thus be identified as a false positive. Aggregation already happens at concentrations >10 µM of compound concentration. It can also result from super-saturation of compounds in a concentrated DMSO solution (Di & Kerns, 2006), which was used for sample storage (2.3). As the aim of this study were the bioactive compounds of *Cuscuta*, the substances responsible for anti-cancer activity in *P. zonale* were not evaluated further. This might however be an option in future studies.

When evaluating the activity of fractions against PTP1B, none of the fractions showed any relevant activity without being active against TC-PTP at the same time, which could cause severe problems in a medical application and was therefore used as exclusion criterion. Yet, this assay only tests against one specific enzyme, while anti-diabetic activity can have various other targets. It is known from other studies that *C. reflexa* exhibits anti-diabetes activity (Rahmatullah *et al.*, 2010; Rath *et al.*, 2016). Both, Rahmatullah *et al.* (2010) as well as Rath *et al.* (2016) detected a lowering effect of *C. reflexa* methanol extract on blood glucose levels in diabetic rats without any side effects. However, the compounds and the exact mechanism behind this effect are still unknown and should be investigated further. This could be done by further fractionation, isolation and characterization of compounds. Including and comparing different species like in this thesis could help finding the responsible compounds either by discovery of the same effect in other *Cuscuta* species or the possibility to compare to inactive species of the same plant family.

4.4 Conclusion and Outlook

In this thesis, extracts of the parasitic plant *Cuscuta* from different species and tissues, and the host *P. zonale* were tested for activities in different bioassays. Additionally, active samples were analyzed via MS to identify the active compounds. A compound with the molecular formula $C_{15}H_{10}O_7$ named quercetin was found to be partly responsible for anti-cancer activity in all *Cuscuta* samples. As pure quercetin showed less activity than the tested plant fractions, it was concluded that there are other compounds in the plant samples that exhibit an even greater anti-cancer activity or a synergistic effect together with quercetin. The method of bioactivity tests followed by MS analysis to discover novel compounds with potentially interesting activity was confirmed to be effective, even if quercetin itself was already known. Some sample fractions showed anti-cancer activity in a cell viability assay against A2058 human melanoma cells but no cytotoxic effect against MRC5 human lung fibroblasts. To further investigate this possibly specific anti-cancer effect, MS data has to be evaluated in more detail to identify a candidate substance. To be able to do this, a second fractionation of the active flash-fractions should be applied to separate the abundant polyphenols from other, less abundant, active compounds in future research. Preceded by an isolation and purification step, structure elucidation via NMR and a bioactivity profiling of the respective compound can then be done. Additionally, an investigation of synergy effects within the *Cuscuta* plant extracts could provide valuable knowledge on how to increase quercetin's effects. Further fractionation steps could be

applied to find the "minimal mixture" still showing the synergy followed by analysis of the contained compounds.

No striking differences were observed among different *Cuscuta* species or tissues in terms of bioactivity at first, but the PCA analysis showed a variation in compound composition among species. Also, in the cell viability assay, differences in activity that are worthy of further investigation could be singled out. For future studies, it is possible to use metabolomics approaches, identifying and quantifying metabolites to investigate variations among different species. Furthermore, the setup of the cell viability assay could be adapted to more in-depth comparisons of different species, also including flower extracts of the other species. It would be interesting to explore, moreover, the relationship of compounds between parasites and host more thoroughly, especially if uptake and metabolization of bioactive compounds or precursors by *Cuscuta* could be identified. Learning more about compounds which are transferred between parasite and host could help understanding the solute transfer between parasitic and host plants, a still very poorly understood process. Knowledge about transferred compounds' metabolization could yield insights into survival strategies of the parasitic plant and give access to a new, possibly unique set of compounds. Such compounds may show new properties that could be used biotechnologically. Behbahani (2014) for example showed that compounds epoxidized by *Cuscuta* exhibited a higher cytotoxicity than the unprocessed metabolites. Besides the influence of the *Cuscuta* plant itself on its secondary metabolites, also the microbiome of the plant, the microbial community living in symbiosis with the plant, should be taken into consideration. Especially as it is known that they are involved in the production of a number of bioactive secondary metabolites (Koeberl *et al.*, 2013). A comparison of these microbiomes especially between host and parasite should be evaluated in further research. Additionally, when investigating thoroughly the compounds contained in Cuscuta, a different method of extraction or at least solvent in combination with a different set of downstream methods to test the extracted compounds should be considered in order to add a different set of compounds to the known components of *Cuscuta*.

Here, the applied bioassays were focused on selected medicinal applications and promising results were found. This is, however, a competitive field of research and compounds have to fulfill a certain standard to be considered further. For example, compounds should be novel and applicable under certain medicinal circumstances. This selective pressure is reflected in the strict discarding during the usual workflow of the Marbio pipeline. The results obtained in this thesis might be interesting for further research in the field of *Cuscuta* but do not fit the high

throughput requirements of the Marbio pipeline for further investigation. Nevertheless, there is a plethora of other biotechnological applications, including nutraceuticals, cosmetics and herbicides. *Cuscuta* has already been used as a supplement in drinks and food in traditional medicine, while quercetin, here found to have anti-cancer activity, is already sold as a dietary supplement and several studies are promoting *Cuscuta* extracts as an effective agent to support hair growth (Pandit *et al.*, 2008; Patel *et al.*, 2014). Also worth an investigation is the question whether *Cuscuta*, as an obligate parasite dependent on being able to infest a host shortly after germination, can produce substances that enhance plant growth and could thus be used in agriculture. Thus, much remains unknown and open for further research. Parasitic plants in particular and nature in general still harbor many secrets that are far from being unlocked. As the possibilities grow, new opportunities arise and research might bring up new ways to describe and use the same natural resources that our ancestors already made part of their daily life and used for their own benefit.

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Appendix

Table 11 – Inhibition measurements on bacterial growth. Activity against *E. faecalis* is expressed as optical density measured at 600 nm. Fractions would have been considered active when the OD_{600} value was below 0.05, questionable with an OD_{600} value in between 0.05 and 0.09, and were rated as inactive when the OD_{600} value was higher than 0.09, which was the case in all the examples shown here. P: *P. zonale*, Cr: *C. reflexa*, Cp: *C. platyloba*, Cc: *C. campestris*, Ccf: *C. campestris* flowers, A: extraction at 4 °C, B: extraction at 27 °C, 1-6: fraction number, T: total extract

Sample type	*E. faecalis*	Sample type	*E. faecalis*	Sample type	*E. faecalis*
PA1	0.25	CpA1	0.28	CcfB1	0.28
PA2	0.18	CpA2	0.28	CcfB2	0.27
PA3	0.21	CpA3	0.27	CcfB3	0.27
PA4	0.18	CpA4	0.28	CcfB4	0.27
PA5	0.18	CpA5	0.27	CcfB5	0.24
PA6	0.21	CpA6	0.16	CcfB6	0.26
PAT	0.18	CpAT	0.28	CcfBT	0.29
PB1	0.30	CpB1	0.27	CcfA1	0.25
PB2	0.19	CpB2	0.28	CcfA2	0.28
PB3	0.21	CpB3	0.28	CcfA3	0.26
PB4	0.19	CpB4	0.28	CcfA4	0.21
PB5	0.16	CpB5	0.26	CcfA5	0.21
PB6	0.10	CpB6	0.17	CcfA6	0.24
PBT	0.21	CpBT	0.26	CcfAT	0.25
CrA1	0.31	CcA1	0.26		
CrA2	0.29	CcA2	0.27		
CrA3	0.27	CcA3	0.28		
CrA4	0.29	CcA4	0.28		
CrA5	0.10	CcA5	0.24		
CrA6	0.21	CcA6	0.15		
CrAT	0.30	CcAT	0.26		
CrB1	0.29	CcB1	0.25		
CrB2	0.29	CcB2	0.29		
CrB3	0.28	CcB3	0.28		
CrB4	0.28	CcB4	0.27		
CrB5	0.24	CcB5	0.23		
CrB6	0.17	CcB6	0.14		
CrBT	0.27	CcBT	0.26		

Table 12– Inhibition measurements on bacterial growth. Activity against *E. coli* is expressed as optical density measured at 600 nm. Fractions would have been considered active when the OD_{600} value was below 0.05, questionable with an OD_{600} value in between 0.05 and 0.09, and were rated as inactive when the OD_{600} value was higher than 0.09, which was the case in all the examples shown here. P: *P. zonale*, Cr: *C. reflexa*, Cp: *C. platyloba*, Cc: *C. campestris*, Ccf: *C. campestris* flowers, A: extraction at 4 °C, B: extraction at 27 °C, 1-6: fraction number, T: total extract

Sample type	*E. coli*	Sample type	*E. coli*	Sample type	*E. coli*
PA1	0.43	CpA1	0.47	CcfB1	0.55
PA2	0.19	CpA2	0.52	CcfB2	0.56
PA3	0.18	CpA3	0.46	CcfB3	0.52
PA4	0.19	CpA4	0.52	CcfB4	0.47
PA5	0.35	CpA5	0.41	CcfB5	0.40
PA6	0.42	CpA6	0.48	CcfB6	0.41
PAT	0.19	CpAT	0.47	CcfBT	0.41
PB1	0.51	CpB1	0.53	CcfA1	0.42
PB2	0.20	CpB2	0.53	CcfA2	0.41
PB3	0.18	CpB3	0.52	CcfA3	0.46
PB4	0.19	CpB4	0.52	CcfA4	0.37
PB5	0.25	CpB5	0.40	CcfA5	0.38
PB6	0.40	CpB6	0.44	CcfA6	0.37
PBT	0.20	CpBT	0.42	CcfAT	0.35
CrA1	0.51	CcA1	0.42		
CrA2	0.48	CcA2	0.54		
CrA3	0.51	CcA3	0.48		
CrA4	0.47	CcA4	0.45		
CrA5	0.36	CcA5	0.38		
CrA6	0.54	CcA6	0.49		
CrAT	0.43	CcAT	0.41		
CrB1	0.43	CcB1	0.40		
CrB2	0.47	CcB2	0.50		
CrB3	0.49	CcB3	0.49		
CrB4	0.45	CcB4	0.47		
CrB5	0.37	CcB5	0.38		
CrB6	0.37	CcB6	0.44		
CrBT	0.39	CcBT	0.40		

Table 13 – Inhibition measurements on bacterial growth. Activity against *P. aeruginosa* is expressed as optical density measured at 600 nm. Fractions would have been considered active when the OD_{600} value was below 0.05, questionable with an OD_{600} value in between 0.05 and 0.09, and were rated as inactive when the OD_{600} value was higher than 0.09, which was the case in all the examples shown here. P: *P. zonale*, Cr: *C. reflexa*, Cp: *C. platyloba*, Cc: *C. campestris*, Ccf: *C. campestris* flowers, A: extraction at 4 °C, B: extraction at 27 °C, 1-6: fraction number, T: total extract

Sample type	*P. aeruginosa*	Sample type	*P. aeruginosa*	Sample type	*P. aeruginosa*
PA1	0.40	CpA1	0.49	CcfA1	0.51
PA2	0.36	CpA2	0.48	CcfA2	0.47
PA3	0.31	CpA3	0.61	CcfA3	0.47
PA4	0.33	CpA4	0.64	CcfA4	0.47
PA5	0.40	CpA5	0.62	CcfA5	0.51
PA6	0.42	CpA6	0.52	CcfA6	0.48
PAT	0.32	CpAT	0.54	CcfAT	0.50
PB1	0.57	CpB1	0.44	CcfB1	0.63
PB2	0.39	CpB2	0.48	CcfB2	0.55
PB3	0.34	CpB3	0.51	CcfB3	0.56
PB4	0.32	CpB4	0.61	CcfB4	0.53
PB5	0.32	CpB5	0.59	CcfB5	0.56
PB6	0.41	CpB6	0.52	CcfB6	0.55
PBT	0.39	CpBT	0.53	CcfBT	0.64
CrA1	0.55	CcA1	0.50		
CrA2	0.55	CcA2	0.48		
CrA3	0.60	CcA3	0.47		
CrA4	0.64	CcA4	0.59		
CrA5	0.64	CcA5	0.49		
CrA6	0.56	CcA6	0.50		
CrAT	0.42	CcAT	0.52		
CrB1	0.56	CcB1	0.51		
CrB2	0.52	CcB2	0.48		
CrB3	0.55	CcB3	0.49		
CrB4	0.59	CcB4	0.56		
CrB5	0.56	CcB5	0.47		
CrB6	0.47	CcB6	0.52		
CrBT	0.49	CcBT	0.49		

Table 14 – Activities of samples rated as active in the first cell viability screening against A2058 cells. DMSO concentrations were later raised to ensure better solubility. Numbers represent % of cell survival: red – active (<50% cell survival), yellow –questionable (50-60% cell survival), white – inactive (>60% cell survival) The first set of concentrations (a) depicts the first concentration dependence assay, the second set (b) a repetition. P: P. zonale, Cr: C. reflexa, Cp: C. platyloba, Cc: C. campestris, Ccf: C. campestris flowers, A: extraction at 4 °C, B: extraction at 27 °C, 1-6: fraction number, T: total extract

	a			b		
	µg/mL					
	50	**25**	**10**	**50**	**25**	**10**
PA2	20	15	57	8	62	98
PA5	92	93	97	10	51	91
PA6	80	84	96	99	97	96
PAT	16	77	79	28	96	87
PB5	8	8	76	84	85	87
PB6	73	89	89	97	96	123
PBT	54	98	94	106	99	96
CrA4	108	107	89	68	68	73
CrA5		-		-1	11	31
CrB4	102	98	104	45	84	83
CrB5	29	111	100	13	77	75
CpA4	86	86	83	92	86	70
CpA5		-		-6	72	37
CpB4	95	94	94	90	92	92
CpB5		-		-6	67	75
CcA2		-		95	97	88
CcA3		-		98	100	93
CcA4	83	97	90	86	87	89
CcA5		-		92	98	83
CcB4	77	86	81	47	41	90
CcB5	8	42	92	-5	51	91
CcfA2	91	102	91	95	94	82
CcfA3	94	97	92	97	102	89
CcfA4	82	89	88	53	90	96
CcfB2	101	100	114	98	98	92
CcfB3	96	103	106	97	97	92
CcfB4	96	107	104	85	95	92

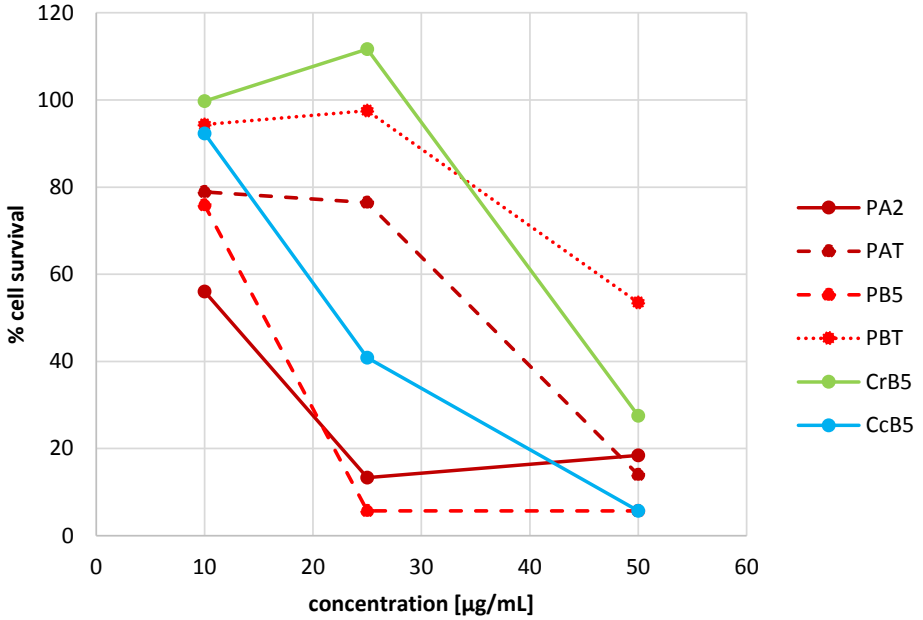

Figure 29 –Concentration dependence assay of samples against A2058 cells. The samples depicted were rated reproducibly as active in the first cell viability screening against A2058 cells at different sample concentrations (DWF) and at lower DMSO concentrations that were later raised to ensure better solubility. Tested were concentrations of 50 µg/mL, 25 µg/mL and 10 µg/mL, P: P. zonale, Cr: C. reflexa, Cp: C. platyloba, Cc: C. campestris, Ccf: C. campestris flowers, A: extraction at 4 °C, B: extraction at 27 °C, 1-6: fraction number, T: total extract, mean values of two measurements are depicted, error bars have not been included

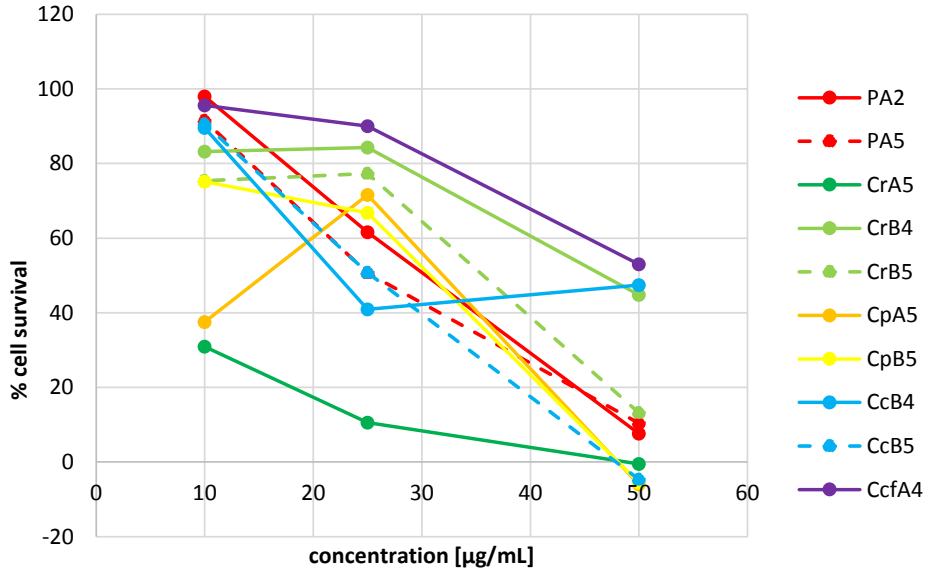

Figure 30 – Second Concentration dependence assay of samples against A2058 cells. The samples depicted were rated reproducibly as active in the first cell viability screening against A2058 cells at different concentrations of samples (DWF) and at lower DMSO concentrations that were later raised to ensure better solubility.. Tested were concentrations of 50 µg/mL, 25 µg/mL and 10 µg/mL, P: P. zonale, Cr: C. reflexa, Cp: C. platyloba, Cc: C. campestris, Ccf: C. campestris flowers, A: extraction at 4 °C, B: extraction at 27 °C, 1-6: fraction number, T: total extract, mean values of two measurements are depicted, error bars have not been included