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„Production of arbutin and bergenin in callus cultures of *Bergenia pacumbis* (Buch.-Ham. ex D.Don) C.Y.Wu & J.T.Pan"

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Abbreviations

2,4-D	2,4,-Dichlorophenoxyacetic acid
BAP	6-Benzylaminopurine
DW	Dry weight
FW	Fresh weight
HPLC	High performance liquid chromatography
IAA	Indole-3-acetic acid
Kin	Kinetin
MS	Murashige & Skoog Medium
NAA	1-Naphthaleneacetic acid
PGR	Plant growth regulator
PVP	Polyvinylpyrrolidone
rpm	Revolutions per minute
SM	Secondary metabolite

1. Abstract

Secondary plant metabolites (SM) are of ever-increasing interest for treating various ailments, from cancerous diseases to hyperpigmentation of the skin. Grand-scale cultivation of plant material, however, can be environmentally problematic in the long term, especially if profit margin is of more interest than sustainability of natural resources. Plant tissue culture under controlled, aseptic conditions, especially plant cell suspension culture, might present a potential alternative here.

The biggest drawback of the method though is the low production rate of secondary metabolites compared to the parent plant, which is why the industrialization of cell suspension cultures is limited to just a few compounds available on the market. This could be attributed to the lack of differentiated structures. For example, it was shown that undifferentiated callus cells obtained from *Atropa belladonna* did not produce the tropane alkaloid hyoscyamine until root formation was induced (Bhandary et al., 1969). Another reason might be the lack of endophytes in aseptic cultures, which are known to influence secondary metabolite formation in plants in nature. Endophytes are endosymbiotic microorganisms that dwell inside a living plant. Contrasting to phytopathogenic microorganisms they do not cause any apparent symptoms of infection or disease. Endophytes appear ubiquitously and have been found in all species of plants investigated to date. The exact extent of the relationship between endophytes and their host plants are species specific and are not fully understood yet. We do know, however, that both partners partake in each other's metabolism. Endophytes have been shown to enhance certain plant secondary metabolites (SM). Thus, eliciting plant cell cultures with endophytes previously isolated from this plant species, or extracts thereof, could possibly induce the accumulation of typical SM.

The present study deals with the establishment and characterization of *Bergenia pacumbis* callus cultures and the identification of endophytes isolated from this plant. The findings gained from this work will serve as basics for future investigations into the impact of endophytes as biotic elicitors of the biosynthesis of plant secondary metabolites in cell cultures of *B. pacumbis,* with special focus on arbutin and bergenin.

The first step was to establish callus cultures using explants from leaves of *B. pacumbis* specimen. Two different basal nutrient media supplemented with various concentrations of auxins and cytokinins were screened for optimal callus growth. Callus cultures were propagated for several months to grow sufficient cell mass. The best results were achieved using MS medium with 10 μM of 6-benzylaminopurine (BAP) and 1-naphthaleneacetic acid (NAA) each.

Subsequently, samples of callus lines were lyophilised, extracted and analysed with High-Performance-Liquid-Chromatography (HPLC) to determine their arbutin and bergenin contents. The results were opposing expectations based on what was found in extracts of *Bergenia* leaves. Callus cells contained much more bergenin than arbutin.

Another part of the work was to amplify and sequence of the 16S rDNA extracted from endophyte strains previously isolated from different organs of *B. pacumbis* plants. Sequences were referenced to http://www.straininfo.net and http://ncbi.nlm.nih.gov databases and 47 isolates could be identified successfully. These results could aid future research on whether endophytic extracts could act as biotic elicitors to enhance the production rate of arbutin and bergenin in suspension cultures of *B. pacumbis*.

1.1 Zusammenfassung

Pflanzliche Sekundärmetabolite sind von zunehmendem Interesse für die Behandlung von verschiedenen Beschwerden, von Krebserkrankungen bis hin zu Hyperpigmentierung der Haut. Die Kultivierung von Pflanzen in großem Stil kann jedoch auf lange Sicht problematisch für die Umwelt sein, besonders wenn Gewinn über Nachhaltigkeit gestellt wird. Pflanzenzellkultur unter kontrollierten, sterilen Bedingungen, besonders Zellsuspensionskulturen, stellen hier eine mögliche Alternative dar.

Ihr größter Nachteil besteht jedoch darin, dass sie im Vergleich zur intakten Pflanze meistens geringere Mengen an Sekundärmetaboliten produzieren, weswegen die großangelegte Nutzung von Zellkulturen auf ein paar wenige Endprodukte am Markt beschränkt ist.

Ein Grund für diesen Umstand könnte das Fehlen von differenzierten Strukturen in Gewebekulturen sein. Es wurde beispielsweise gezeigt, dass Kalluszellen aus **Atropa** *belladonna* das Tropanalkaloid Hyoscyamin erst produzierten, nachdem das Wachstum von Wurzeln angeregt wurde (Bhandary et al., 1969). Eine weitere Ursache könnte die Abwesenheit von Endophyten in der *in vitro*-Kultur sein, welche erwiesenermaßen die Bildung von Sekundärmetaboliten in der Pflanze beeinflussen können.

Endophyten sind Mikroorganismen, die in einer intakten Pflanze in den Pflanzengeweben vorkommen. Anders als phytopathogene Mikroorganismen lösen sie keine Symptome von Infektionen oder Krankheiten aus. Endophyten kommen universell vor und wurden in allen bisher untersuchten Pflanzenspezies nachgewiesen. Die exakten Einzelheiten der Gemeinschaft von Endophyten und ihren Wirtspflanzen sind artspezifisch und noch nicht ganz verstanden. Es ist jedoch bekannt, dass beide Symbiosepartner aktiv den Metabolismus des jeweils anderen Partners verändern können. Es konnte gezeigt werden, dass Endophyten die

Produktion spezieller Pflanzensekundärmetabolite erhöhen können. Wenn man daher Pflanzenzellkulturen mit lebenden oder extrahierten Endophyten, die zuvor aus ihrer Wirtspflanzenart isoliert wurden, elizitiert, könnte man so möglicherweise die Produktion von typischen Sekundärmetaboliten erhöhen.

Die vorliegende Arbeit beschäftigt sich mit der Etablierung und Charakterisierung von Kalluskulturen von *Bergenia pacumbis* sowie der Identifizierung von aus dieser Pflanze isolierten Endophyten. Die gewonnenen Erkenntnisse sollen als Grundlage für künftige Untersuchungen zum Einfluss von Endophyten als biotische Elizitoren der Biosynthese von Sekundärmetaboliten, im Besonderen Arbutin und Bergenin, in Zellsuspensionskulturen von *Bergenia pacumbis* dienen.

Der erste Schritt war die Etablierung von Kalluslinien aus den Blättern von *B. pacumbis*. Zwei unterschiedliche Medien mit verschiedenen Konzentrationen an Zytokininen und Auxinen wurden hinsichtlich eines optimalen Wachstums des Kallus- getestet. Um ausreichend Biomasse zu produzieren, wurden die Zelllinien mehrere Monate lang hochvermehrt. Die besten Resultate bezüglich des Wachstums konnten mit MS Medium und Zusatz von je 10 µM Benzylaminopurin (BAP) und 1-Naphthylessigsäure (NAA) erzielt werden.

In weiterer Folge wurden Proben verschiedener Zelllinien lyophilisiert, extrahiert und mittels High Pressure Liquid Chromatography (HPLC) auf ihren Gehalt an Arbutin und Bergenin analysiert. Die Ergebnisse waren das exakte Gegenteil dessen, was in den Blättern von B. *pacumbis* gefunden wurde. Die Kalluszellen enthielten deutlich mehr Bergenin als Arbutin.

Ein weiterer Teil der Arbeit war das Sequenzieren von 16S rDNA aus Endophyten, die aus verschiedenen Organen von *B. pacumbis* isoliert worden waren. Die Sequenzen wurden mit den Datenbanken http://www.straininfo.net und http://ncbi.nlm.nih.gov referenziert.

Insgesamt konnten 47 Isolate erfolgreich identifiziert werden. Diese Ergebnisse könnten für künftige Untersuchungen hilfreich sein, um herauszufinden, inwieweit Extrakte aus Endophyten als biotische Elizitoren die Produktion von Arbutin und Bergenin in Suspensionskulturen von *B. pacumbis* erhöhen.

2. Aim of this project

The main goal of this project was to perform basic research on callus cultures of *Bergenia pacumbis* as well as the identification of endophytes previously isolated from the plant.

As part of this study, the following objectives had to be met:

- **Media screening for optimal callus growth**

Different plant growth regulators and media had to be tested to determine which combination was most suitable to induce and propagate callus of *B. pacumbis*.

- **Identification of endophytes found in *Bergenia pacumbis***

Previously isolated endophytes from *Bergenia* plants had to be identified using 16S rDNA sequencing.

- **Extraction of secondary metabolites from lyophilised callus cells**

For the determination of bergenin and arbutin contents in callus cultures a suitable method for extraction had to be found.

- **Establishing a suitable method for quantification via HPLC**

A working HPLC method had to be established using the equipment at hand.

- **Quantification of secondary metabolites in callus cells and plant tissues using HPLC**

Finally, arbutin and bergenin contents of leaves of adult plants, as well as callus cultures had to be determined.

3. Introduction

It is commonly known that plants can produce a wide array of natural compounds, also referred to as secondary metabolites. SM can vary drastically when it comes to their chemical structure and they have been made use of by mankind for millennia now. Their usage ranges from colorants to flavours and insecticides, but their utilization as medicinal products might be the most important (Taha et al., 2009). The production rate of SM however mostly depends on the physiological and developmental stage of the plant. The majority of pharmaceutically important SM are obtained via plant cultivation or collection of specimens growing in the wild. Attempts have been made to reproduce SM via chemical synthesis, however in most cases it came at a higher cost, which is why SM production still relies heavily on the extraction from plant material (Verma et al., 2012). Plant cell culture might serve as a promising compliment here, as it has several advantages over traditional field cultivation, especially over slowly growing plants, and is often times more financially feasible than chemical synthesis (Zhao and Verpoorte, 2007). Independence of geographical or seasonal situations, as well as a continuous, defined rate of production is possible as well (Smetanska, 2008). The commercial production of SM through plant *in vitro* culture, however, is limited to but a few compounds available on the global market. The most prominent reason for this might be our poor understanding of the biosynthetic pathways and technological processes required in production, extraction and purification of these compounds (Cai et al., 2012). For example, most common plant metabolites are stored intracellularly in plant cells (Cai et al., 2012). This makes recovery more challenging.

3.1. *Bergenia pacumbis (Buch.-Ham. ex D.Don) C.Y.Wu & J.T.Pan*

3.1.1. Botany

Bergenia pacumbis (buch.-ham.(Buch.-Ham. ex D.Don) C.Y.Wu & J.T.Pan (*Saxifragaceae*), formerly known as "*Bergenia ligulata* Wall." before it was taxonomically re-arranged, is a perennial, herbaceous and succulent plant with procumbent rhizomes that grows up to 50 cm tall. It is typically found in Central and East Asia, especially in the temperate Himalaya region at around 2000 – 2700 m altitude. Most notable are its large ovate leaves with bristly margins, often forming rosettes. During the flowering period (March-May) they are 5-15 cm long, however, they can reach 30 cm and more in length, turning to a bright red in autumn. The margin is fringed, with short, bristle like hair. Contrasting to *Bergenia ciliata*, there are small ligules found at the short leaf stalk. The root stock is stout, barrel shaped and around 2 cm in diameter. It appears reddish, with a white inner portion. Flowers are purple, white or pink with 5 petals, a hairless calyx, blunt lobes and a long style, growing up to 3,2 cm in diameter and organized in a cymose panicle with a flexible flowering stem. Fruits are bluish-white capsules that split along the inner edges of the carpels (Bhutya, 2011; Prashanth, 2016; Yeo, 1966).

Figure 1: Specimen of *B. pacumbis* situated in the medicinal plant garden of the Department of Pharmacognosy, University of Vienna.

3.1.2. Secondary metabolites

Bergenia pacumbis is rich in phenolic compounds (Yongsi et al., 2011). The most notable constituent is bergenin (figure 2, page 16), the C-glucoside of 4-O-methyl gallic acid (Jain and Gupta, 1962), which is predominantly found in the rhizomes (Bajracharya, 2015). Other phenolics include afzelchine (Tucci et al., 1969), leucocyanidin, gallic acid, methyl gallate, catechin (Dix and Srivastava, 1989) and paashaanolactone (Chandrareddy et al., 1998). The plant also contains β-sitosterol (Bhat et al., 1974; Shah et al., 1972) and starch (Srivastava and Rawat, 2008). The leaves are rich in arbutin, a glycosylated hydroquinone (Pop et al., 2009).

Bergenin containing herbs have a long tradition of usage in traditional Asian medicine. Plant preparations are utilized to treat lithiasis, dysuria and polyuria, and are used as an astringent and antipyretic (Khare, 2008). Antimicrobial activity was proved against *Candida albicans, C. tropicalis* and *C. guilliermondii*. Less activity was observed with *Aspergillus flavus*, *A. nidulans* and *A. niger* (Patel et al., 2012; Ping, 2012; Silva et al., 2009).

A significant antioxidant activity was found using β-carotene, DPPH and a heterogeneous Fenton assay (De Abreu et al., 2008; Rana et al., 2005; Srinivasan et al., 2007).

Methanolic and hydromethanolic extracts of *B. ligulata* containing bergenin showed an antiviral effect against the influenza virus with an IC50 value of 10 µg/ml *in vitro*. Cytotoxic activity did not occur (Rajbhandari et al., 2011; Rajbhandari and Wegner, 2003). Activity against herpes simplex viruses (HSV-1 and -2) has been proven as well (Khan et al., 2005).

Anti-inflammatory effects, especially with the inhibition of COX-2 was shown *in vitro* as well (Nunomura et al., 2009). Furthermore, a reduced production of Th1 cytokines like IL-2 could be shown in adjuvant-induced arthritic balb/c mice (Nazir et al., 2007).

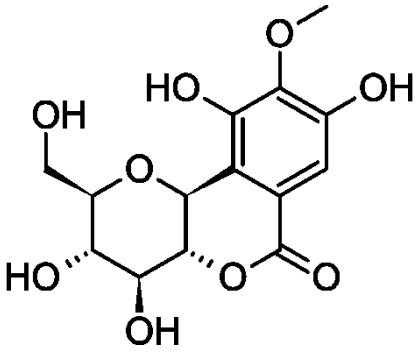

Figure 2: Chemical structure of bergenin.

Arbutin, hydroquinone-O-β-D-glucopyranoside (figure 3, page 18), is one of the main constituents found in *Arctostaphylos uva-ursi* (bearberry), which has long since been used to treat urinary tract infections (Jahodár et al., 1985; Vu and Vasi, n.d.). It is found in bergenia species as well (Pop et al., 2009). Although there is currently no strong evidence backing up its efficacy, bearberry is an integral part of traditional European medicine (Frohne, 2009). Arbutin acts as a prodrug. It is absorbed via the glucose carrier in the small intestine (Alvarado, 1965) and metabolized to sulfate and glucuronide conjugates, which are largely excreted via urine.

Siegers et al. showed that *Escherichia coli* ATCC6538 liberates hydroquinone from these conjugates (Siegers et al., 2003). It is speculated that, in patients suffering from urinary tract infections, bacterial enzymes liberate hydroquinone *in situ* (Schindler et al., 2002). Another widely documented use of arbutin is as a skin whitening agent, especially in Asian cosmetic products (Hori et al., 2004; Maeda and Fukuda, 1996). Tyrosinase is an enzyme responsible for the enzymatic oxidation of L-tyrosine to melanin, which in turn provides skin pigmentation (Thongchai et al., 2007). Over-activity of tyrosinase can result in unsightly skin depigmentation. Arbutin acts as a chelator, binding the tyrosinase's vital copper ion and thus inhibiting the first step of the biosynthetic pathway of melanin production. (Sugimoto et al., 2004; Zaidi et al., 2014). Tyrosinase may also play a role in the formation of neuromelanin in the substantia nigra of the human brain. This could be essential to the neurotoxicity of dopamine and the neurodegeneration associated with Parkinson's disease (Xu et al., 1997). Dadgar et al. demonstrated that intraperitoneal application of arbutin eased the typical, Parkinson's disease like symptoms caused by injections of MPTP (1-Methyl-4-phenyl-1,2,3,6-tetrahydropyridin) into mice (Dadgar et al., 2018).

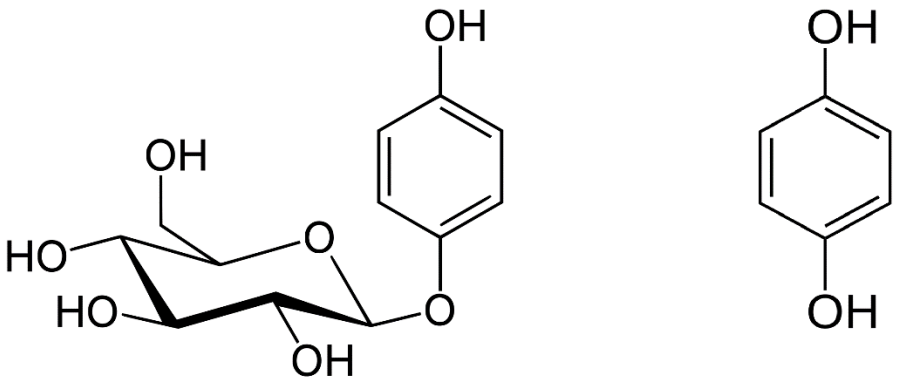

Figure 3: Chemical structures of arbutin (left) and hydroquinone (right)

3.2. Plant tissue culture

Plant tissue culture is the cultivation of single cells, tissues, organs or whole plants under aseptic conditions *in vitro.* With total control over environmental factors such as pH of the nutrient medium, temperature and humidity as well as nutrition one can easily optimize culture conditions for varying plant species. Plant tissue culture is largely used to multiply plants, for example the breeding of orchids (Murashige, 1974). Starting from a small section of plant tissue, named explant, a plant can be multiplied into hundreds and thousands of new individuals of identical genotype (Zimmerman et al., 1986). Other applications include disease elimination, plant improvement and the large-scale production of secondary plant metabolites (Hussain et al., 2012). One of the biggest advantages of plant tissue culture is the total independence of seasons and daylight, meaning year-round production is possible (Akin-Idowu, 1986). These techniques are also used to conserve and propagate endangered species like e.g. *Artemisia laciniata* (Kodym et al., 2018).

Plant tissue culture is considered to be the most effective technique for plant improvement, especially in terms of increased yields and enhanced disease and stress resistance via somaclonal and gametoclonal variations (Brown and Thorpe, 1995). This can be used to

produce commercially more successful variants as it is much faster than traditional techniques like cutting, grafting or propagation through seeds. Another advantage is the possibility of producing virus-free plants (García-Gonzáles et al., 2010). Sagare et al. (2000) used somatic embryogenesis to propagate *Corydalis yanhusuo*, an important species in traditional Chinese medicine that is very susceptible to downy mildew, without any fungal infections (Sagare et al., 2000). El-Dougdoug and El-Shamy (2011) worked on the elimination of banana bunchy top virus (BBTV) and brome mosaic virus (BMV) through meristem cultureto produce virus-free banana specimens for large-scale cultivation (El-Dougdoug, Kh. A., 2011).

Another use of plant tissue culture is the production of secondary metabolites for both analytical and commercial purposes. Primary metabolites include the universal base components of life, namely carbohydrates, lipids, amino acids and the like, which are necessary for development, growth and reproduction. Secondary metabolites (SM) serve a more differentiated purpose, especially within the adaption to the environment. Colorants, fragrances and flavourings serve as attractants for pollinators and seed dispersers. Alkaloids and tannins can act as a defence against natural enemies. Some of these compounds have been found beneficial for human and animal health (Kroymann, 2011).

For millennia, mankind has used natural products derived from plants and animals for medicinal preparations. Although in recent years drug discovery via *in silico* methods has been growing rather quickly, natural compounds are still valid for potential future drugs. According to the FDA, 34% of approved medicines between 1981 and 2010 were based on or derived from natural products, which included statins, anticancer drugs and immunosuppressants (Harvey et al., 2015).

Examples of important SM that are obtained from plants include the analgesic drug morphine from *Papaver somniferum*, the anti-malaria compound artemisinin from *Artemisia annua*, insecticides derived from *Azadirachta indica* (Neem), the anticancer drugs vinblastine and vincristine, which are used to treat childhood leukaemia and Hodgkin's disease, from *Catharantus roseus*, and the antiemetic emetin derived from *Cephaelis ipecacuanha* (Wilson and Roberts, 2012; Yoshimatsu and Shimomura, 1993).

For the industrialized production of SM large amounts of plant material are required if the content of the compound is low. This necessitates large field cultures; however, this is not always feasible. Plant tissue culture can serve as an alternate source for certain compounds. Examples include the immune system stimulating Echinacea polysaccharides from *Echinacea angustifolia* and *E. purpurea,* podophyllotoxin derived from *Podophyllum spp*. used in cancer treatment, *Panax ginseng* used for dietary supplements and of course the prime example paclitaxel from *Taxus sp*., which is a highly valuable drug for the treatment of ovarian and lung cancers (Wilson and Roberts, 2012).

3.2.1. Callus culture

Higher plants are usually not made up of one single type of cells, but rather a plethora of highly differentiated cell types with varying form, function and organization. Chloroplasts work vastly different than essential oil producing glandular trichomes, for example. In the event of pathogen infection or physical damage a plant will form so called callus tissue to close the wound outwardly and allow for proper wound healing. These callus cells are undifferentiated and unorganized parenchyma cells. If taken into culture, a single one of these callus cells can be regenerated into a fully functional, whole plant, due to totipotency (George et al., 2008).

For the induction callus formation, a previously sterilized explant from a plant is inoculated on nutrient medium supplemented with varying auxins and/or cytokinins. Alternatively, taking explants from already aseptic plant cultures is possible as well. Since callus is a type of wound tissue, it will most often form at the cut region of the explant (Smith, 2012). These callus cells can be separated from the explant and cultivated on suitable medium. Regular sub-culturing ensures a fresh source of nutrients, allowing for propagation over longer periods of time. Single dish cultivation on solid medium is only suitable for analytical purposes, however. For larger scale production of secondary metabolites, plant cell suspension culture is the preferred method. Since this type of culture relies on single cells suspended in liquid medium, it is important that the initial callus conglomerates be soft and friable, lest they form aggregates with uneven growth that are more difficult to work with. There exist various techniques to achieve a finer suspension, like filtering, pipetting, decantation, or by adding pectinase at very low levels. Pectinase breaks up aggregates by dissolving the intercellular pectins (Mustafa et al., 2011).

3.3. Endophytes

The expression “endophyte” stems from the Greek words *endon* = within and *phyton* = plant. The first indication for the existence of endophytes was provided by De Bary in 1866 when he discovered the presence of microbial cells when he was microscopically analysing plant tissues (Bary, 1866). While the term has had varying definitions over the decades, one of the commonly used definitions includes bacteria, fungi, archaea and unicellular eukaryotes such as algae and amoebae dwelling inside plants without causing damage to its host plant (Hardoim et al., 2015). In 1991, Orlando Petrini defined endophytes as “all organisms

inhabiting plant organs that at some time in their life cycle can colonize internal plant tissues without causing apparent harm to their host" (Petrini, 1991). It has been indicated that endophytes occur in virtually every plant studied to date (Strobel and Daisy, 2003). Endophytes have been proven to inhabit roots, stems, petioles, leaves, inflorescences, fruits, seeds and even dead and hollow hyaline cells (Hata and Sone, 2008; Specian et al., 2012; Stępniewska and Kuźniar, 2013).

Historically it was long believed, that endophytes were just pathogens with low virulence, thriving inside the plant without causing any apparent symptoms. More recently, however, endophytes were found to have beneficial effects on their host plant, such as plant growth promotion and increased resistance against biotic and abiotic stress, pathogens and parasites (Kogel et al., 2006). While the host provides assimilates as well as shelter to the endophytes, endophytes themselves can actively partake in the plant's metabolism. Diazotrophs (nitrogen fixating bacteria) for example can provide up to 90% of the plant's nitrogen requirements via atmospheric N_2 fixation within nodules of certain legumes (Richardson et al., 2009). In most cases, endophytes originate from the environment by horizontal transmission, but they can also be passed down via previously infected seeds in a pathway called vertical transmission (Truyens et al., 2015). While endophytes can enter a plant through natural openings as well as open wounds, some of them possess the ability to actively penetrate plant tissue by producing hydrolytic enzymes like cellulases and pectinases. Since this ability is shared by most pathogens as well, the host plant must possess some sort of regulation to recognise endophytes as non-hostile because of the lack of a hypersensitive response. To which extent is however still in need of further research. Evolutionary, endophytes may have been some sort of intermediate between plant pathogens and saprophytic bacteria and fungi, forming a symbiotic relationship with their hosts over time (Hallmann et al., 1997; Selosse et al., 2018).

Endophytic metabolites have become of great interest for human health recently (Alvin et al., 2014). Some chemical compounds originally attributed to the plant alone, have been proven to be produced by endophytes residing in that plant and they proceeded the production after being isolated and in axenic culture. The most prominent example for such a compound is paclitaxel, an antineoplastic found in the bark of *Taxus brevifolia* in 1971 (Wani et al., 1971). This compound is used successfully for the treatment of different cancers. Because of the enormous negative environmental impact of harvesting thousands of yew trees scientists were looking for an alternative source (Zhao et al., 2011). Fortunately, Stierle and co-authors discovered the endophytic fungus *Taxomyces andreanae* that produced this compound in axenic cultures (endophytes cultivated outside of their host plant tissue) alongside its host (Stierle et al., 1993). In 2008, Kusari et al. isolated the endophytic fungus strain INFU/Hp/KF/34B from *Hypericum perforatum* shoots that was capable to produce hypericin, as well as its potential precursor emodin *in vitro* (Kusari et al., 2008). These discoveries lead to an increased interest in fungal endophytes and their metabolites among scientists. The new research focus indeed led to novel discoveries of antibiotics, antivirals, antineoplastics, insecticides, antidiabetics and immunosuppressants (Strobel and Daisy, 2003).

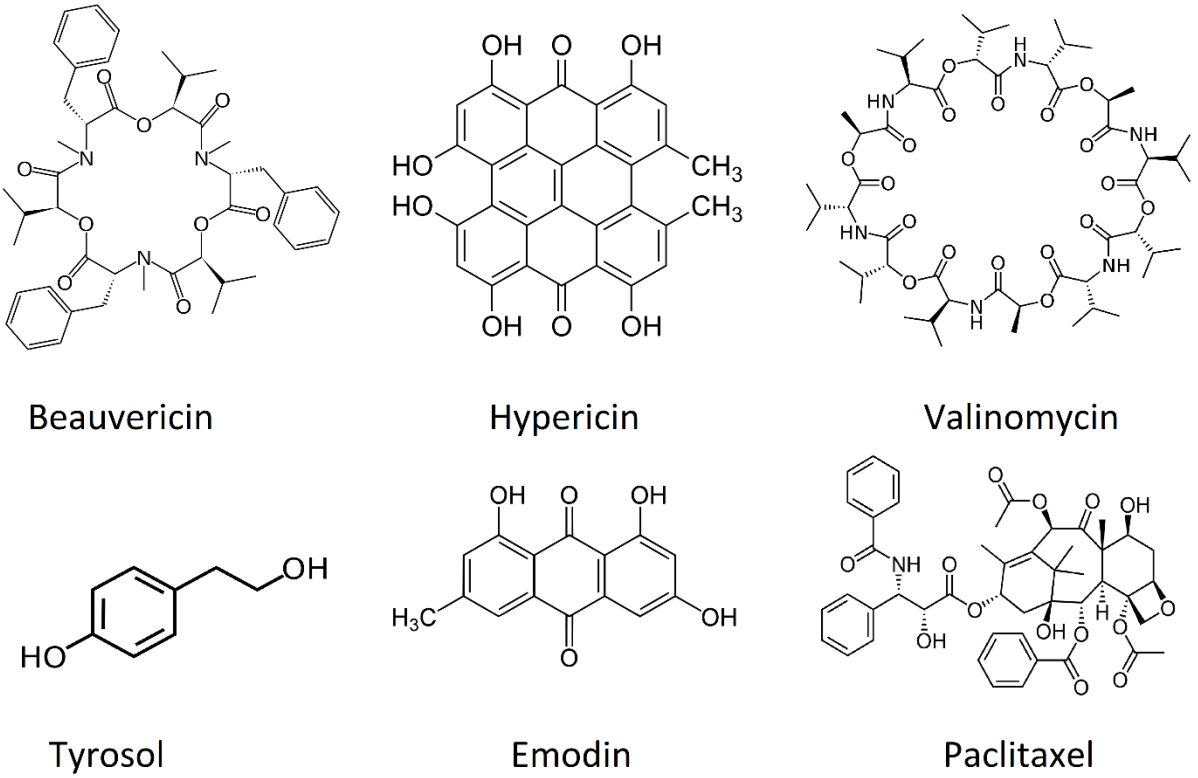

Figure 4: Selected bioactive compounds with medicinal importance isolated from endophytes (modified from Gouda et al., 2016).

Cultivating endophytes axenically is, however, not the only way to produce secondary metabolites. Endophytes or the organic solvent extracts thereof can serve as elicitors. Elicitors are extrinsic or foreign effector molecules, that may originate e.g from plant pathogens and lead upon recognition by the plant to different defence mechanisms resulting in increased synthesis of certain metabolites to decrease damage or heighten environmental stress resistance. Plant tissue or suspension cultures are known to generally contain low concentrations of these compounds (Smetanska, 2008). When used in *in vitro* plant culture, these elicitors can induce the production of secondary metabolites that were either absent before or synthesized at a very low level.

Artemisinin, extracted from *Artemisia annua* L., is one of the main compounds of artemisinin-based combination therapies (ACTs) recommended by the World Health Organization for the treatment and prevention of malaria. Attempts trying to produce artemisinin via tissue or cell cultures have been undertaken, however due to extremely high costs, production still relies on the whole plant. Li et al. showed in 2012 that treating hairy root cultures of *A. annua* with live endophytic actinomycetes strains increased the production of artemisinin in some cases. Similarly, inoculation of living endophytic fungi (*Rhizoctonia* sp.) to suspension cultures of *Atractylodes lancea* promoted the production of atractylodin (Tao et al., 2011). In 2014, Awad et al. presented a study about how the treatment of *Taverniera cuneifolia* (Roth) root cultures with living bacterial and fungal cultures increased the production of glycyrrhizic acid up to six-fold. Supplementing cell suspension cultures of *Dioscorea zingiberensis* with water-extracted mycelial polysaccharide (WPS) extracted from the endophytic fungus *Fusarium oxysporium* Dzf17 increased the yield of diosgenin almost 4-fold (Li et al., 2011).

3.3.1. Bacterial endophyte identification

16S rDNA sequencing plays a pivotal role when it comes to the fast identification of bacteria (Woo et al., 2008). A more accurate method but more demanding and thus often not feasible is genome sequencing of bacteria (Loman et al., 2012). Medical practitioners can use this barcoding technique using 16S rDNA to quickly determine the causative bacteria of an infectious disease to treat it with appropriate antibiotics (Woo et al., 2008). Since endophytes reside inside host plants, extensive surface sterilization is used to rid plant material of contaminating microorganisms that are not endophytes. Surface sterilisation does not reach the inside of intact tissues, meaning that endophytes are still be alive after sterilisation. In

2016, Liaqat and Eltem noticed bacterial growth among their peach and pear rootstock cultures. Since MS plates inoculated with the final wash solution of the surface sterilization procedure didn't show any signs of contamination, they proposed that these bacteria must stem from inside the rootstock cultures themselves, indicating the presence of endophytes. Those bacteria were then isolated from the plant tissue culture plates and streaked onto nutrient agar plates and then purified. Bacterial colonies were separated based on their morphological characteristics and maintained in pure culture. The isolates were processed for Gram-staining reaction, as well as partial 16S rRNA gene sequencing (Liaqat and Eltem, 2016).

3.3.2. Genetic barcoding

Morphological characteristics such as cell size, shape, flagellas and the like have been found to not be suitable for properly studying bacterial phylogeny and diversity. Instead, DNA analysis of evolutionary conserved gene regions with highly variable sections in between offer a more streamlined identification. In bacteria, the ribosomal operon is responsible for transcribing the rRNA genes as 30S rRNA precursor molecules, which are then cleaved by RNase III into 16S, 23S and 5S rRNA molecules. Because the 16S rRNA is seen as the most conserved one, scientists have successfully relied on 16S sequences for the identification and classification of bacteria for almost three decades now (Weisburg et al., 1991). The 16S rRNA gene in particular contains short, highly conserved regions with low variability, which can be used as primers to amplify highly variable sections in between two primers which then can be used for species discrimination (Bodilis et al., 2012). Bacterial isolates of unknown strands are identified based on 16S sequence homology analysis with already existing sequences saved in

a database. Comparative tools such as BLAST (https://www.ncbi.nlm.nih.gov/) or Seq-match (http://rdp.cme.msu.edu/) are used to look for the closest related matches in their databases. Exact identification, however, remains a challenge to a certain degree, since comparative genome analyses have shown the shortcomings of this method including false positive and negative results. The bacterial ribosomal DNA is a multicopy gene with intraspecific variations, whereby a random copy of this gene is amplified for barcoding. Unfortunately, copy numbers per genome vary from one up to fifteen or more copies. While the number of copies seems to be taxon-specific to a certain degree, variations among strains belonging to the same species have been recorded as well (Větrovský and Baldrian, 2013).

4. Material and Methods

4.1. Plant material

In June 1993 a stock plant of *Bergenia pacumbis* was collected in Phulchoki, Nepal, at an altitude of 2,715 m and was brought to Austria by Dr. Namita Maskay (Tribhuvan University, Kathmandu, Nepal). Ever since then it is been cultivated in the greenhouse of the Department of Pharmacognosy, University of Vienna. After *in vitro* propagation cloned specimens were transferred to the medicinal plant garden of the same institute. These individuals served as donors for the establishment of callus cultures.

Callus cell lines were initiated using lamina, petiole and midrib explants of *B. pacumbis* leaves. They were harvested over the course of October, November and December 2017 and surface sterilised (refer to chapter 4.2.). For analysis of arbutin and bergenin contents additional leaves were collected in August and December 2017 as well as in May 2018. Rhizome and root samples were also collected in May 2018.

4.2. Surface sterilization

Collected leaves were washed thoroughly under running tap water for about 10 minutes before being divided into petiole, midrib and lamina. For easier handling they were then cut into smaller pieces of about 10 cm length. The sterilizing agent used subsequently was an aqueous solution of sodium hypochlorite supplemented with two drops of TWEEN-20 per litre as a wetting agent. For this purpose, 12 parts of a concentrated solution of NaOCl manufactured by NEUBER were mixed with 88 parts of tap water, resulting in 1.5% of active chlorine. Explants were steeped in the sterilant for 20 minutes and then rinsed with

autoclaved, distilled water. To remove any traces of chlorine the plant material was further soaked in said water three times for 10 minutes each. Washing solutions were discarded after each step. To avoid premature browning on the explants they were stored in a sterile 0.1% solution of PVP10 during the process of culture initiation.

4.3. Culture media

MS medium (Murashige and Skoog, 1962) served as the basal medium used for callus cultivation. For the preparation of media stock solutions were used, which were 100-fold concentrated for macro elements, 1000-fold for the micro elements and 200-fold for the vitamins. Plant growth regulators were prepared as 1 mM stock solutions (Table 1 on page 29). All stock solutions were stored in the fridge at 4 °C, except for the KNO_3 solution which was kept at room temperature to avoid precipitation. For preparation of media an Erlenmayer flask was filled halfway with distilled water. 30 g/L sucrose and 100 mg/L myo-inositol were added, followed by the appropriate amounts of all stock solutions and growth regulators. An additional 3 g/L Gelrite® (Roth, 71010-52-1) was added to achieve a semi solid consistency. During the first stage of callus induction, 0.1% of PVP was added to prevent browning (Malla, 1999). The pH was adjusted to 5.7 ± 0.1 with 0.1 M NaOH. For the induction of callus growth glass test tubes were filled with 13 ml medium each before being closed with Magenta A-caps. For the propagation of callus cell lines later, HIPP®-jars (height , Ø 55 mm) were filled with 40 ml each, which were then capped with Magenta B-caps. All media in glass containers were autoclaved at 121 °C for 20 minutes. For cultivation in petri-dishes (92 x 16 mm) the medium was autoclaved in Schott-flasks, cooled to about 50 °C and subsequently approximately 30 ml was poured into each dish.

4.4. Culture conditions

Cultures in general were kept in the dark at 25±1 °C and were subcultured approximately every six weeks.

About halfway through the study, half of the callus cultures were placed in an incubator at 28 °C to test for change in growth.

4.5. Optimization of callus culture

For screening of the optimal supplement of plant growth regulators for callus cultivation five different MS media were prepared, using different auxins and cytokinins at varying concentrations (table 1). Cell lines showing a lack of growth or excessive browning were discarded.

Table 1: Composition of nutrient media for callus cultivation.

Medium	Basal Medium	BAP [µM]	NAA [µM]	2,4-D [µM]	IAA [µM]	KIN [µM]
BP 1	MS	5	2			
BP 2	MS	10	10			
BP 3	MS			5		
BP 4	MS			10		
BP 5	½ MS				7.5	7.5

4.6. Identification of endophytes from *Bergenia pacumbis*

4.6.1. Origin of isolates

Previously, endophytes of *B. pacumbis* were isolated by Dr. Martina Oberhofer (Oberhofer, personal communication) from several clonal individuals situated in the medicinal plant garden of the Pharmacognosy Department of the University of Vienna. It was possible to isolate 156 different strains of endophytes, which were then stored in a 50% glycerol stock at -80 °C until further use.

4.6.2. Preparation of bacterial samples

After being removed from the -80 °C freezer the isolates were slowly thawed on ice for later use. For liquid culture inoculation, 4 ml of Tryptic soy broth (TSB) medium (Sigma-Aldrich, Darmstadt) was filled into 15 ml Falcon tubes. Bacterial samples were then inoculated using a sterile loop. The Falcon tubes were then incubated at 28 °C at 200 rpm in a rotary shaking incubator (Dr. Oberhofer, personal communication) until noticeable growth of bacteria was visible through cloudiness of the media, respectively a pellet formed on the bottom of the tube. For DNA extraction, each tube was centrifuged at 200 rpm for 10 minutes, the supernatant was discarded, and the bacterial pellet was transferred into a microcentrifuge tube.

4.6.3. Genomic DNA extraction

From these bacterial pellets, 100 µl were transferred into a new microcentrifuge tube and then re-suspended with 275 µl of the Digestion Solution Master Mix (see table 3, page 34). The tubes were left to incubate for 60 minutes at 55 °C on a heating block to achieve sufficient cell lysis. Subsequently, 200 µl Wizard ® SV Lysis Buffer was added and each tube vortexed to

interrupt the lysis process. This procedure is a variation of the original protocol provided by the Wizard ® SV Genomic DNA Purification System. Instead of 16-18 h incubation time on the heating block, tubes were only incubated for one hour.

Table 2: Composition of the digestion solution master mix.

Materials	Concentration or Amount (µl)
Nuclei Lysis Solution	200 µl
EDTA	50 µl
Proteinase	20 µl
RNAse	5 µl
Total	275 µl

4.6.4. Purification of genomic DNA

The tubes contents were transferred into Wizard ® SV minicolumns, which were assembled with collection tubes. The assemblies were centrifuged at 13.000 rpm for 3 minutes, the minicolumns were removed and the liquid in the collection tubes was discarded. Each assembly then received 650 µl of Column Wash Solution and was again centrifuged at 13.000 rpm for 1 minute. The filtrate was discarded, and the washing step was repeated once again. The assemblies were then centrifuged empty for 2 minutes at 13.000 rpm to dry the matrix. Each Wizard ® SV minicolumn was then transferred into a new 1.5 ml tube. To eluate the genomic DNA, 100 µl nuclease free water was added and the assemblies were centrifuged for 60 seconds at 13.000 rpm.

4.6.5. Gel electrophoresis

Gel electrophoresis was used to estimate the quality and quantity of the PCR products. Agarose gel (0,8%; Merck, 101630616) with an addition of 5 µl/100ml GelRed™ (Biotium, Fermont, 41002) was prepared using TBE buffer 10x. To estimate the quality, quantity and length of the amplicons, a 1 kb ladder (Biolabs Massachusetts, N3232L) was used for referencing. The well on the first lane of the gel received 2 µl of this 1 kb ladder. Into each subsequent well, a mixture of 2 µl of genomic DNA samples and 1 µl of blue loading dye was pipetted. Smaller gels were left to run in the electrophoresis at 100 V for 30-45 minutes, while bigger gels with 2 rows of samples were run at 120 V. Next, the gels were placed inside the Gel Doc ™ imaging system (Bio-Rad, Hercules), where they were exposed to UV light. Gel red stains the DNA in presence of UV-light and made the bands visible to the human eye. Results were photographed with the GelDoc device and interpreted. If the PCR was successful, the 16S rDNA gene products showed intense bands at the same height as the 1500 kb reference band from the ladder. For estimating the appropriate amount of genomic DNA needed for the PCR amplification another gel was run. If the bands were weak the amount of sample was doubled from 1 µl to 2 µl to allow for sufficient amplification. When 2 µl were used, the water content of the PCR master mix (table 3) was adjusted accordingly.

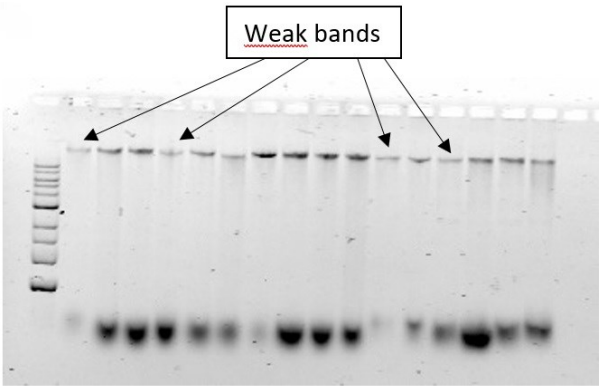

Figure 5: Result of gel electrophoresis of genomic DNA.

4.6.6. Gel PCR master mix and cycling conditions

The 16S rDNA serves as a marker for studying bacterial phylogeny and taxonomy. Amplification was achieved via polymerase chain reaction: PCR tubes were placed into a -20°C cooling block for the entire preparation process. Into each tube, 39 µl of PCR master mix (table 3) was added, as well as 1 µl of previously purified DNA. When the DNA samples showed a weak band after gel electrophoresis the amount of sample was doubled to 2 µl. A new master mix was prepared, containing only 29 µl of nuclease free water, amounting to a total of 38 µl of master mix. The primers used for the master mix were 27F (5'AGAGTTTGATCMTGGCTCAG-3') as the forward primer and 1492R (5'-TACCTTGTTACGACTT-3`) as the reverse primer. The PCR tubes were closed and placed into the Eppendorf Mastercycler nexus X2 (Gerke et al., 2013), were they underwent the temperature program seen in table 4.

Table 3: Master Mix for one PCR reaction.

Materials	Concentration or Amount (µl)
Standard Taq reaction buffer 10X with $MgCl_2$	4
Deoxynucleotide solution mix (10mM)	0,5
27F forward primer (10µM)	1
1492R reverse primer (10µM)	1
Taq polymerase	0,5
DMSO (dimethylsulfoxide) 100%	2
Nuclease free water	30

Table 4: PCR cycling program performed by the Eppendorf Mastercycler nexus X2: First, denaturation of double DNA strands at 95 °C for 2 min, followed by 30 cycles of denaturation at 90 °C for 30 seconds, annealing at 60 °C for 30 seconds and extension at 72 °C for 3 minutes. The last extension was performed at 72 °C for 5 minutes with a final hold at 4 °C unlimited. Samples were then stored at 4 °C in the fridge.

Step	Temperature	Duration (min)
Initial Denaturation	95 °C	2
30 Cycles	95 °C	0.5
	60 °C	0.5
	72 °C	3
Final extension	72 °C	5
Hold	4 °C	

4.6.7. Purification of amplicons for sequencing

After successful PCR amplification the last step was to purify the product. For this the protocol of the DNA Clean and Concentrator ™ 5 (Zymo Research) kit was used as follows: In a 1.5 ml microcentrifuge tube the leftover 37 µl of PCR product was mixed with 185 µl of Binding Buffer (1:6) by briefly vortexing. The mixture was transferred into a Zymo-Spin™ Column which was assembled with a Collection Tube. The assembly was centrifuged for 30 seconds at 14000 rpm, after which the flow-through was discarded. Next, 200 µl of Wash Buffer to the assembly which was again centrifuged for 30 seconds at 14000 rpm. This washing step was repeated once, the filtrates were discarded both times. To dry the membrane the assembly was centrifuged once more, this time without any additives. Next, the Zymo-Spin™ Column was

transferred into a new 1.5 ml microcentrifuge tube. 32 µl of Elution Buffer was added directly onto the column matrix and was left to incubate for 2 minutes at room temperature. This new assembly was again centrifuged for 30 seconds at 14000 rpm to elute the purified DNA. For sequencing, the forward and reverse reactions were pipetted in separate tubes (Mix2Seq, Eurofins, Ebersberg, Germany). 15.5 µl of purified DNA was mixed with 2 µl of primers, either forward or reverse. Pre-labled tubes were then sent to Eurofins Genomics and sequenced overnight. Results were received via e-mail. Obtained sequences were edited, forward and reverse DNA strands were assembled, and the assemblies were then submitted to NCBI Genbank BLAST search and compared to the database.

4.7. Phylogenetic analysis

The database http://www.straininfo.net was used to search the actual type of strains, while the database http://ncbi.nlm.nih.gov was used to find the most similar match in the sequence data base. Both were used as reference strains. For the phylogenetic analysis of the 16S rDNA the software Molecular Evolutionary Genetics Analysis Version 10.0.5 (MEGA X) was used. Fasta files from reference and experimental sequences were imported into MEGA X. The sequences were then aligned by using the ClustalW logarithm with default settings (Gap Opening Penalty: 15.0; Gap Extension Penalty: 6.66) in repeated steps. If necessary, reverse compliment was applied to change the strand of the amplicon and the alignment algorithm ClusterW was applied once more. If the alignment was satisfying, fray ends of sequences were cut off. The alignment block was used for calculating the best fitting base substitution model for maximum likelihood analysis. This was the one with the lowest -LN value of -9011,433156. Finally, a maximum likelihood tree was estimated.

4.8. Harvest of callus cells and fresh plant material

Callus cells were removed from the culture media and thoroughly cleaned of any residual medium. Next, they were weighed to determine their fresh weight. To gain enough material for the subsequent extraction at least 1 gram of fresh callus cells had to be gathered. The samples were then lyophilized for 24 hours to ensure complete dryness. Until further use samples were stored in paper bags in a desiccator over silica gel.

Fresh plant material was harvested by cutting parts of the rhizome, roots and the lamina of a number of Bergenia specimens situated in the Medicinal Plant Garden at the Department of Pharmacognosy, University of Vienna. To prevent degradation of secondary metabolites freshly cut material was immediately stored in liquid nitrogen at the harvest site. After cutting the samples into smaller pieces they were lyophilized in the same fashion as the callus cells. Until extraction the plant tissue was stored in Falcon tubes at -80 °C.

4.9. Extraction of secondary metabolites

For the extraction as described in the European pharmacopoeia for bearberry leaves (EDQM, 2017), previously lyophilised leaf lamina material was pulverised (250) using a grinder. 400 mg of the sample were extracted in a round-bottom flask with 10 ml of distilled water in a water bath (100 °C) with reflux cooling for 30 minutes. The extract was filtered using a wad of cotton wool. The grounds, with the addition of the piece of cotton, was again extracted with 10 ml of distilled water for 30 minutes. After filtration through a paper filter both filtrates were united and diluted to 25 ml with distilled water. During another filtration with a paper filter, the first

5 ml of filtrate were discarded. A subsequent millilitre was collected in an Eppendorf tube, which was centrifuged at 13.000 rpm for 10 minutes before being analysed by HPLC.

For the extraction method following Boros et al. (2014), the lyophilized callus samples were pulverized by hand using a mortar and pestle. For each sample, 30 mg were weighed into 2 ml Eppendorf tubes. After the addition of 0.5 ml of solvent mixture [methanol:water = 1:1 (v/v)] the extraction was performed using ultrasonication for 2x15 minutes. Extracts were purified by centrifugation at 14000 rpm for 10 minutes. The supernatant was collected using a pipette and stored in 1.5 ml Eppendorf tubes in the dark at 4 °C until the analysis was carried out.

For the extraction of leaf lamina, rhizome and root tissue the previously lyophilized samples were pulverized by intensely vortexing the material in 30 ml Falcon tubes stocked with 10 glass beads. This allowed for finely grinding small amounts quickly. Extraction was performed by weighing in 30 mg of sample material and adding 1.5 ml of solvent mixture [methanol:water = 1:1 (v/v)]. The rest of the procedure was the same as above.

4.10. High Performance Liquid Chromatography (HPLC)

High Performance Liquid Chromatography was used to identify and quantify arbutin and bergenin in extracts of callus cells and plant tissues.

4.10.1. Instruments

The different components of the HPLC equipment used for analysis are shown in table 5.

Table 5: Components of the HPLC unit (Shimadzu Corp.)

Instrument	Model
Degasser	DGU-20A5R
Pump	LC-20AT
Auto sampler	SIL-20AC HT
Detector	Photodiode array detector (SPD-M20A)
Column oven	CTO-20AC
Controller	CBM-20A

4.10.2. Columns

Columns used to establish the HPLC protocol and for analysis are shown in Table 6.

Table 6: Columns used for HPLC analysis

Column	First column used	Second column used
Name	Acclaim 120 C18	Luna ® 5 µm C18 (2) 100 Å
Length	150 mm	250 mm
Diameter	2.1 mm	4.6 mm
Particle size	3 µm	5 µm

4.10.3. Solvents

The various solvents used for the HPLC are shown in table 7.

Table 7: Solvents used for HPLC analysis.

Solvents	Manufacturer
Distilled water	Produced in house
Acetonitrile	HiPerSolv CHROMANORM® Super gradient for HPLC, vwr chemicals
Methanol	Vwr chemicals
Formic acid	Vwr chemicals
Trifluoroacetic acid	Vwr chemicals

4.10.4. HPLC method establishment

Three methods for the measurement of arbutin and bergenin were tested. The first method followed the monography of bearberry leaves (*Uvae ursi folium*) from the European pharmacopoeia (EDQM, 2017) for testing arbutin contents via HPLC and external standard. It was performed with the Acclaim 120 C18 column and an aqueous solution of phosphoric acid (pH = 3.0; solvent A) and acetonitrile (solvent B) as mobile phase. The detection wavelength was set to λ = 280 nm because both compounds of interest have good absorption at this value. The runtime was elongated to 28 minutes because for bergenin a longer retention time than that of arbutin was expected. The flow rate was 0.5 ml/min and the gradient as shown in table 8.

Table 8: Gradient profile for method one

Time (min)	A (%V/V)	B (%V/V)

0.00	90	10
15.00	90	10
15.10	5	95
28.00	5	95

The second method was a variation of the standard method used in the Pharmaceutical Biotechnology Group at the Department of Pharmacognosy, University of Vienna, as shown in table 9.

This method was used in conjunction with the Acclaim column as well. Solvents used with this method were distilled water with an addition of 0.1% formic acid (solvent A) and acetonitrile (solvent B) at a flow rate of 0.5 ml/min and a column oven temperature of 25 °C. The detection wavelength was set to λ = 280 nm as described above.

Table 95: Gradient profile of method 2

Time (min)	A (%V/V)	B (%V/V)
0.0	90	10
8.0	90	10
8.1	5	95
20.0	5	95
20.1	90	10
35.0	Stop	Stop

The third method used in this study was a variation of the HPLC method established by Boros et al. (2014), customized to the equipment at hand. This method was carried out with the Luna

® 5 µm C18 (2) column. Solvents were distilled water (solvent A) and analytical grade methanol (solvent B). Instead of formic acid this method used an addition of 0.1 v/v % trifluoroacetic acid to both methanol and water to achieve a narrow peak shape (see table 10 for the solvent gradient). Flow rate was adjusted to 2 ml/min while the column oven temperature stayed at 25 °C. The detection wavelength was also set to λ = 280 nm.

Table 60: Gradient profile of method three

Time (min)	Solvent A (%V/V)	Solvent B (%V/V)
0.0	100	0
30.0	70	30
30.1	5	95
38.0	5	95
38.1	100	0
46.0	100	0
46.1	Stop	Stop

4.10.5. Identification of peaks

The first step was to prepare sample solutions of the reference substances arbutin and bergenin at a concentration of 1000 µg/ml each, using analytical grade methanol as a solvent.

To ensure complete dissolution ultrasonication was applied for 10 minutes. Both solutions were injected into the HPLC to determine the retention time of both substances. The peaks of arbutin and bergenin were identified by increasing the concentration of both arbutin and bergenin in each standard solution, thus resulting in enlarged peak areas. During method optimization a combined standard solution of 500 µg/ml of both arbutin and bergenin was used for a more time efficient method screening.

4.10.6. Calibration curve

For the quantification of arbutin and bergenin in extracts an external standard was used. For the calibration curve a series of dilutions was prepared. For both arbutin and bergenin the series consisted of 1000, 500, 100, 50, 10, and 5 µg/ml each, for bergenin there was an additional dilution at 1 µg/ml. Of each individual dilution 5 µl were injected into the HPLC three times. Calculations and the construction of graphs were performed using Microsoft Excel (Microsoft Office 365 ProPlus, Version 1908, build 11929.20708).

4.10.7. Measurements

Prior to measuring each extract sample was centrifuged for 5 minutes at 13000 rpm to clear the extract of any precipitation. 120 µl of the supernatant were pipetted into HPLC sample vials. For each individual run 5 µl of sample was injected into the HPLC.

4.10.8. Quantification

For the identification and quantification of arbutin and bergenin in plant extracts as well as standard solutions the program LabSolutions, Version 5.82 was used. Integration of each peak was performed valley to valley.

The limit of detection (LOD) and limit of quantification (LOQ) were both determined mathematically via the use of Microsoft Excel as well. Following the multiplier suggested by the ICH standard (International Conference on Harmonization of Technical Requirements for Registration of Pharmaceuticals for Human Use) the standard deviation (sd) and the slope (S) of the calibration curve had to be determined first (Singh, 2015). Next, both values were calculated from the following equations:

$$LOD = 3 \times (sd/S)$$

$$LOQ = 10 \times (sd/S)$$

5. Results

5.1. Development of the callus culture

To achieve optimal callus growth, 5 different media were prepared to screen various types and concentrations of cytokinins and auxins (table 1, page 30).

Explants on media BP 3 (figure 6) and BP 4 (figure 7, page 46) lacked any signs of growth. Even after twelve weeks of cultivation, there was no noticeable formation of callus. Since the explants just turned brown, they were discarded. Thus, both media supplemented with 2,4-dichlorophenoxyacetic acid alone were deemed to be unsuitable for the induction of callus formation.

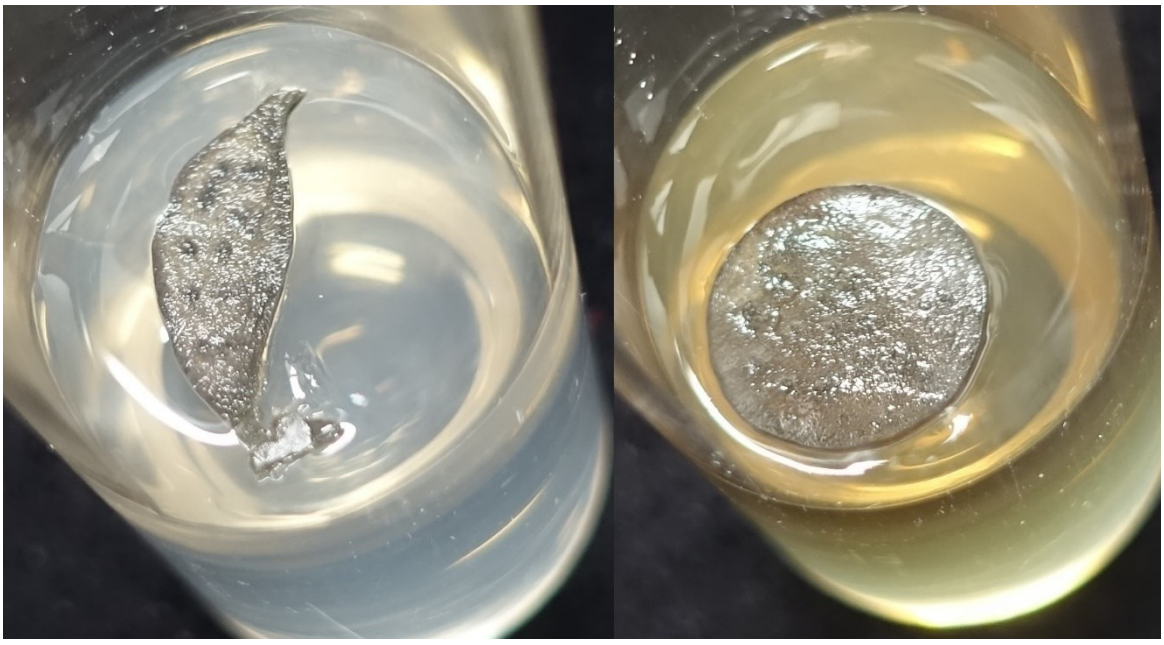

Figure 1: Petiole and midrib explants on medium BP 3 after twelve weeks.

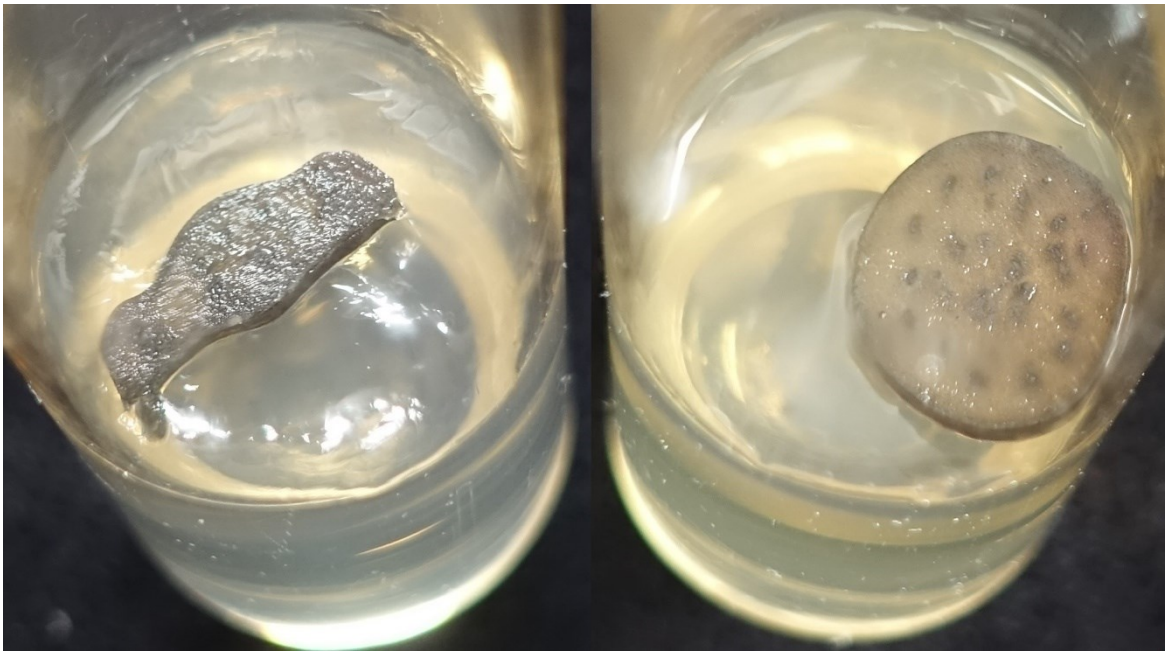

Figure 2: Petiole and midrib explants on medium BP 4 after twelve weeks.

Media BP 1 and BP 2, however, clearly led to the desired callus formation. Explants on medium BP 1 (figure 8, page 51) produced callus which was slightly darker than that formed on medium BP 2 (figure 9, page 51), and it generally was of harder, almost crystal-like consistency, which is not desirable when establishing a suspension culture later on. Some of the explants turned dark brown, even browning the medium underneath, but callus was still able to grow. Overall growth was comparably slow, and the callus aggregates were miniscule compared to those on medium BP 2.

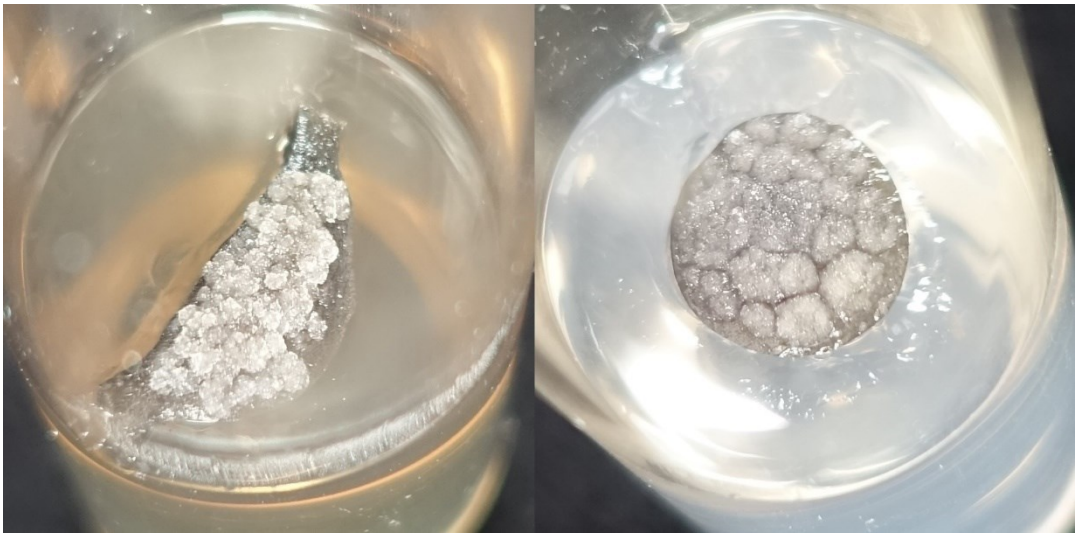

Figure 3: Midrib and petiole explants on medium 1, seven weeks after initiation.

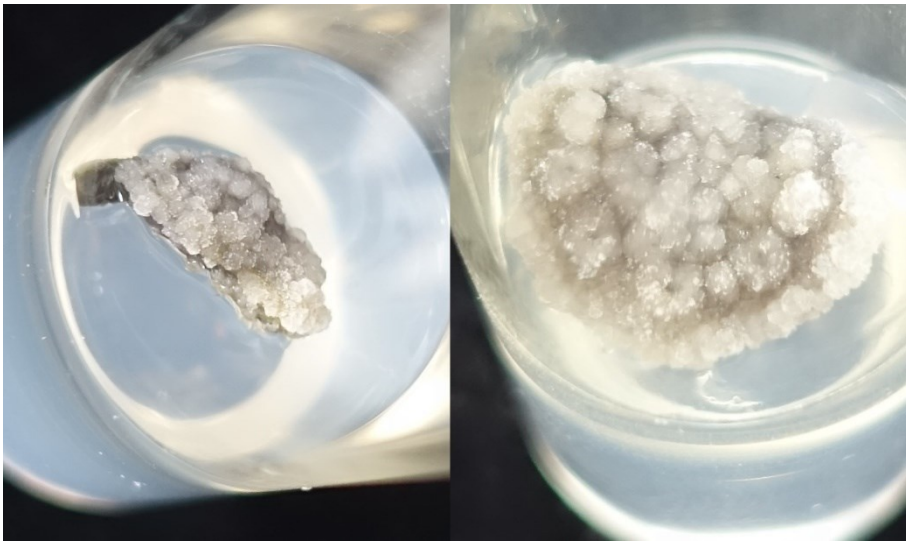

Figure 4: Explants of midrib and petiole on medium 2, after 13 weeks of propagation.

Explants on medium BP 2 produced callus which was comparably quick in growth, but the most important feature was the lighter colour compared to all the other media. Overall growth was most abundant on medium BP 2. The consistency of the aggregates was softer and more friable, in stark contrast to callus formed on the other media.

No PVP had been added to the first batch of medium BP 5 which resulted in explants turning almost black in colour (figure 10), presumably dying. This demonstrated the importance of an absorbent added to the media when working with plants that are high in polyphenols, such as *Bergenia sp.* (Malla, 1999). All explants of this first batch were discarded after 4 weeks and cultivated no further.

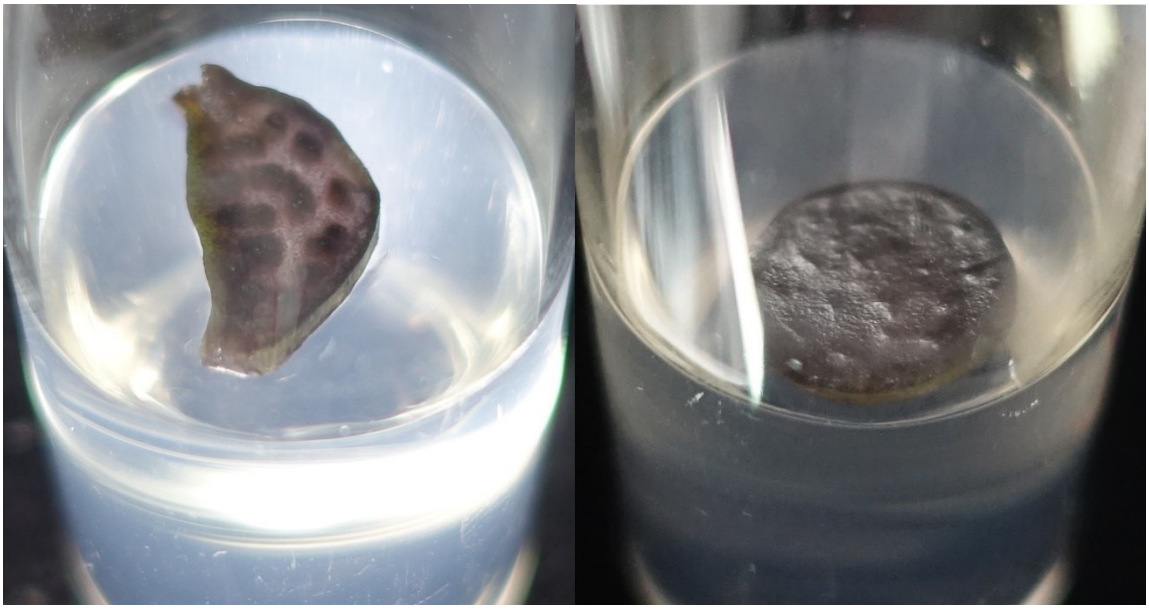

Figure 5: Midrib and petiole explants on medium 5 lacking the addition of PVP.

All subsequent cultures on medium BP 5 supplemented with 0.1 % PVP showed noticeable callus growth (figure 11, page 53). Compared to callus obtained on media BP 1 and BP 2 the consistency was harder and the colour was of an overall darker shade, which are both undesirable attributes. During the propagation of callus parts of the cultures turned brown, which did not happen as often on media BP 1 or BP 2. These brown sections were removed during subculturing and discarded. Growth was rather slow as well, compared to medium BP 2.

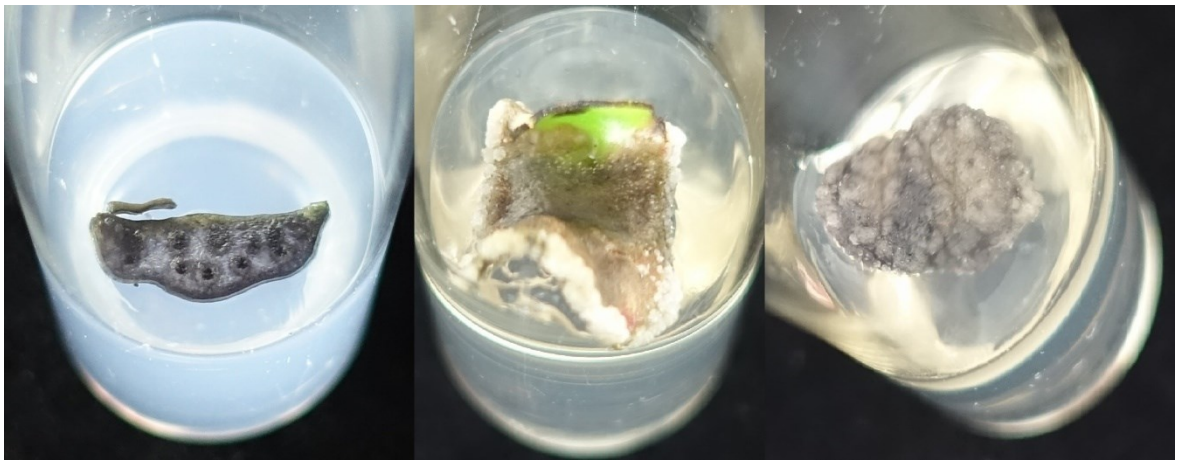

Figure 6: Midrib, lamina and petiole explants on medium 5 after 10 weeks of propagation.

After about three months of cultivation, callus aggregates with sufficient growth were separated from the explants to be further propagated (figure 12).

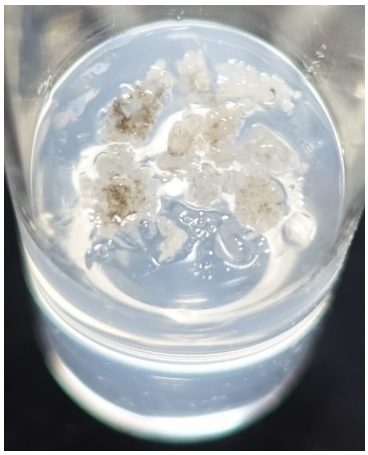

Figure 7: Callus aggregates separated from petiole explant on medium BP 2.

In order to investigate a possible influence of the temperature some of the callus cultures on the different media were incubated at 28 °C, increasing the temperature by 3 degrees compared to the usual 25 °C (figure 13, page 54). After only two weeks the callus colour turned significantly darker (figure 14, page 54), especially with the explants on medium BP 5. Since

overall growth did not improve, but rather stagnate, this experiment was aborted and not pursued further.

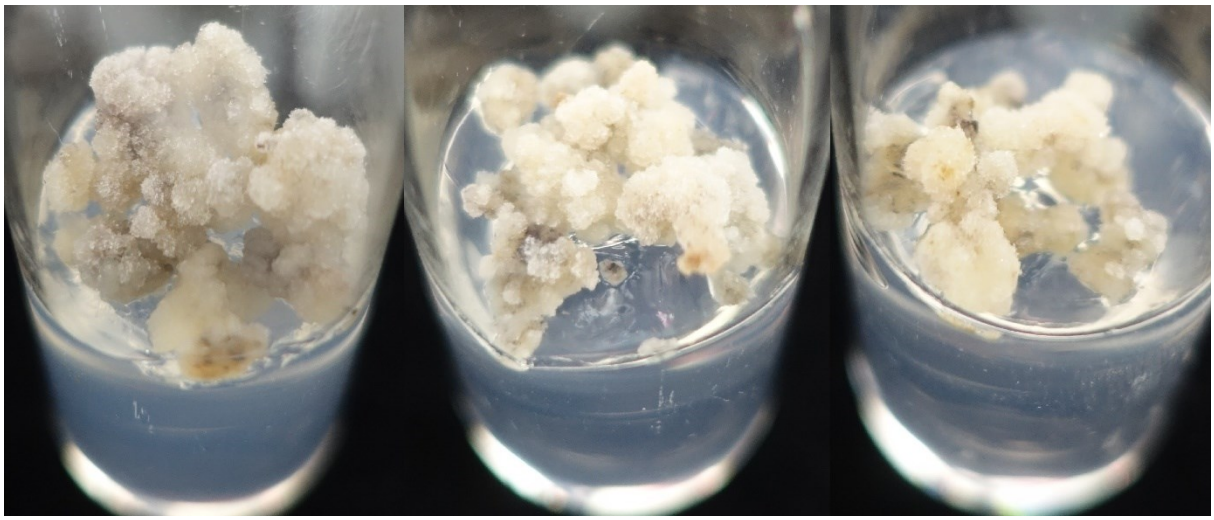

Figure 13: Callus lines on media BP 2 (left), BP 5 (middle) and BP 5 (right) cultivated at 25 °C.

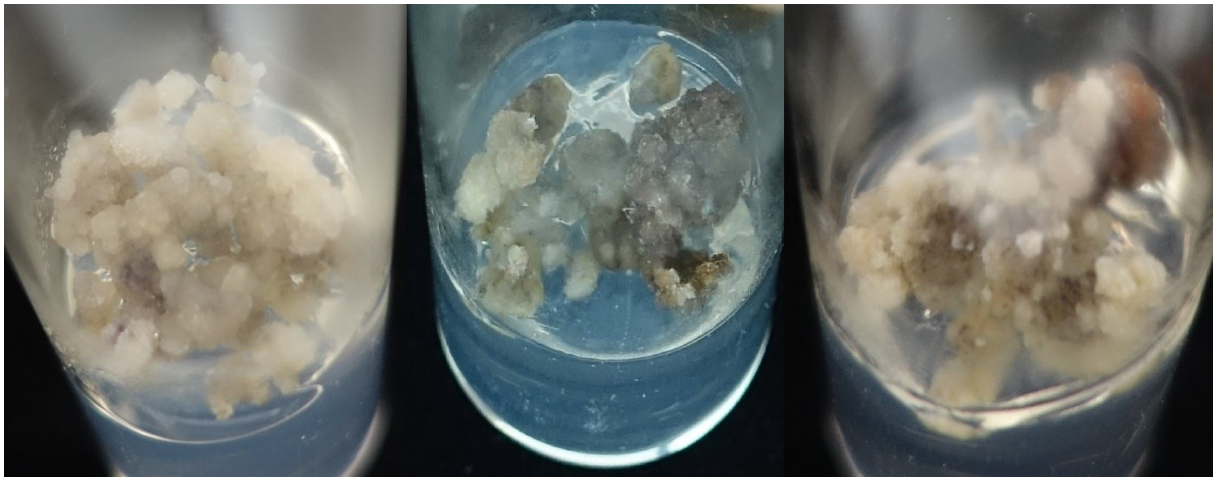

Figure 14: Callus lines on media BP 2 (left), BP 5 (middle) and BP 5 (right) cultivated at 28 °C.

After some six months test tubes were deemed too small for further propagation, which is why callus cultures were moved to petri dishes (92 x 16 mm; figure 15, page 55).

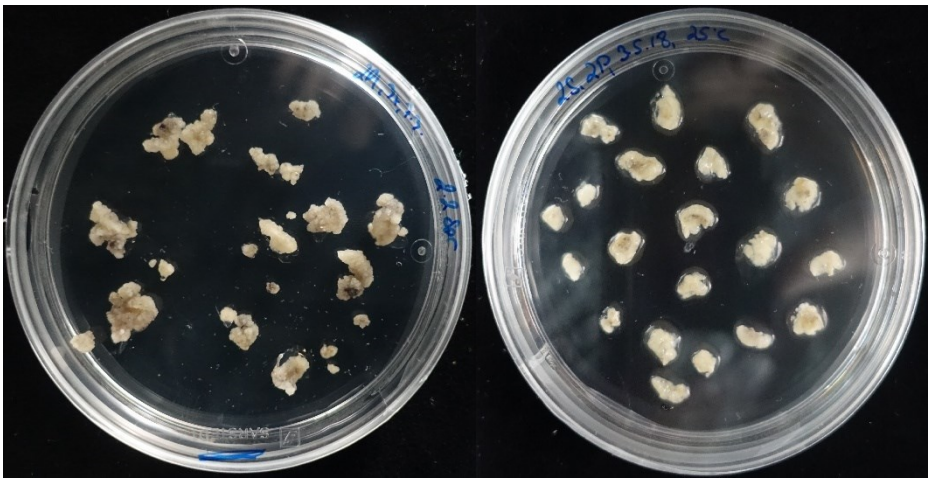

Figure 8: Callus on media BP 2 (lamina, left) and 2 (petiole, right).

At this stage, callus originating from medium BP 5 showed a much darker colour compared to callus on medium BP 2 (figure 16). To prevent the loss of these lines they were transferred to medium BP 2 in HIPP® jars.

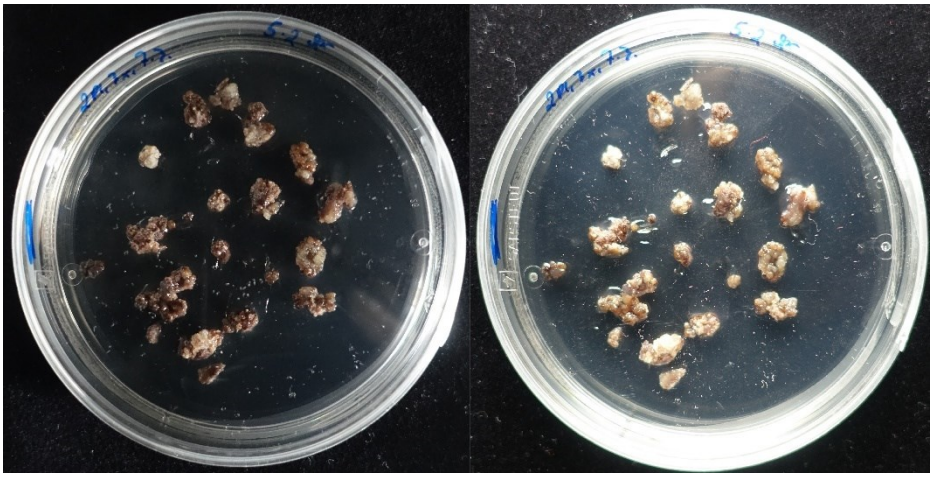

Figure 9: Callus formed on lamina and petiole explants on medium BP 5.

After 5 weeks of propagation in petri dishes, all callus cultures were moved to HIPP®-jars with medium BP 2. Every subsequent step of subculturing was performed using HIPP® jars as well (figure 17).

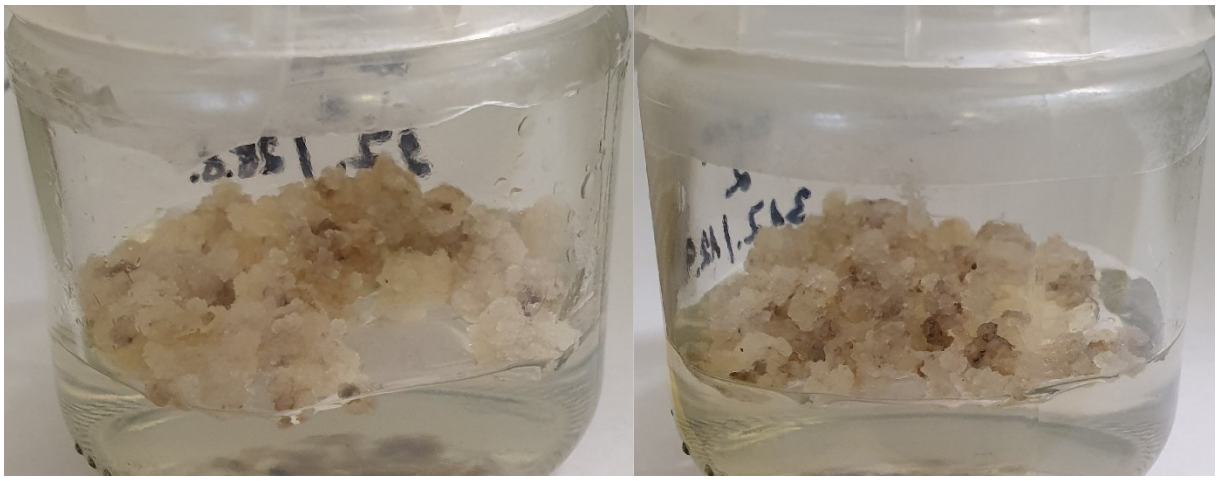

Figure 10: Callus formed on midrib and petiole explants on medium BP 2 in HIPP®-jars.

5.2. HPLC method optimization

Method 1 is a quick and easy way to determine arbutin contents in bearberry leaves. However, since this method only uses aqueous extracts it wasn't applicable to determine bergenin contents, since bergenin is hardly water soluble (Yuan Huang et al., 2008). Arbutin at 1000 µg/ml was tested (figure 18), as well as an aqueous bergenia leaf extract (figure 19, page 58).

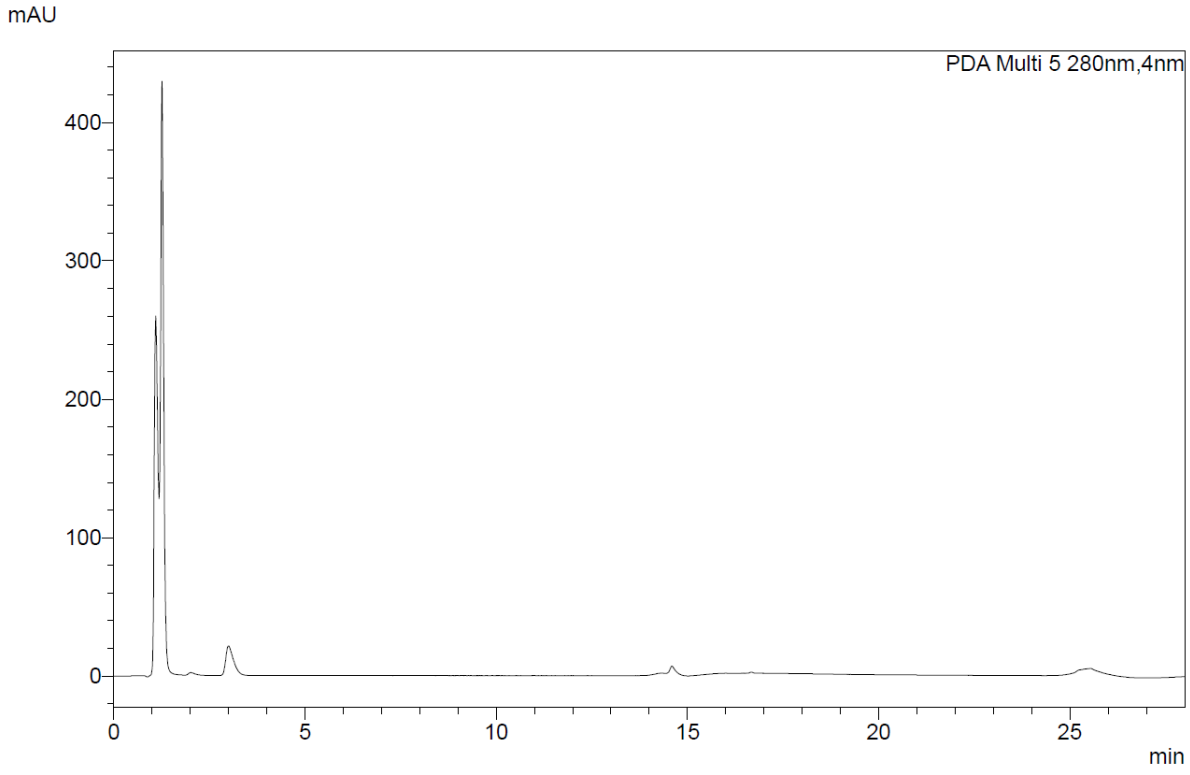

Figure 11: HPLC chromatogram of a standard solution of 1000 µg/ml arbutin analysed with method 1.

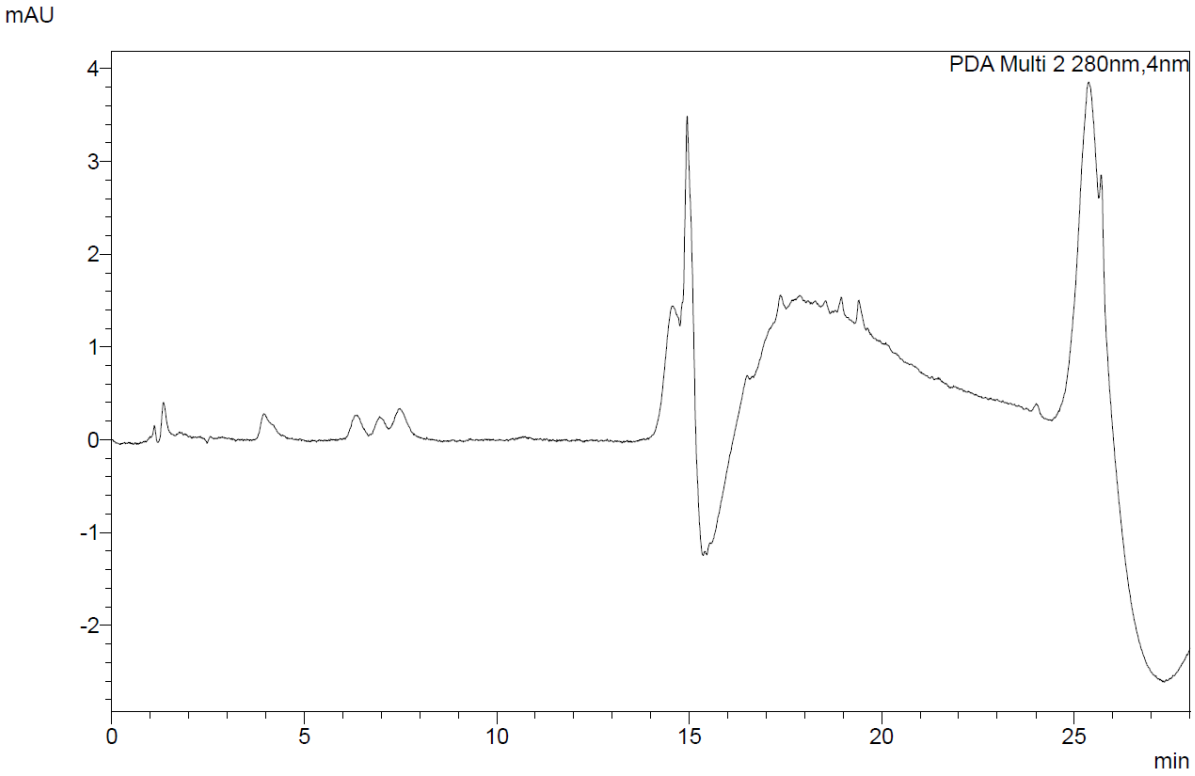

Figure 12: Chromatogram of an aqueous *B. pacumbis* leaf extract analysed with method 1.

Due to bad peak separation, overall poor quality and the aforementioned complications with the water solubility of bergenin this method was dropped and not further improved.

Method 2 was tested next, first with the same aqueous extract of bergenia leaves as before (figure 20), then with standards of arbutin and bergenin (figures 21 and 22, page 60). However, none of the peaks could be associated with arbutin or bergenin by comparing their UV spectra.

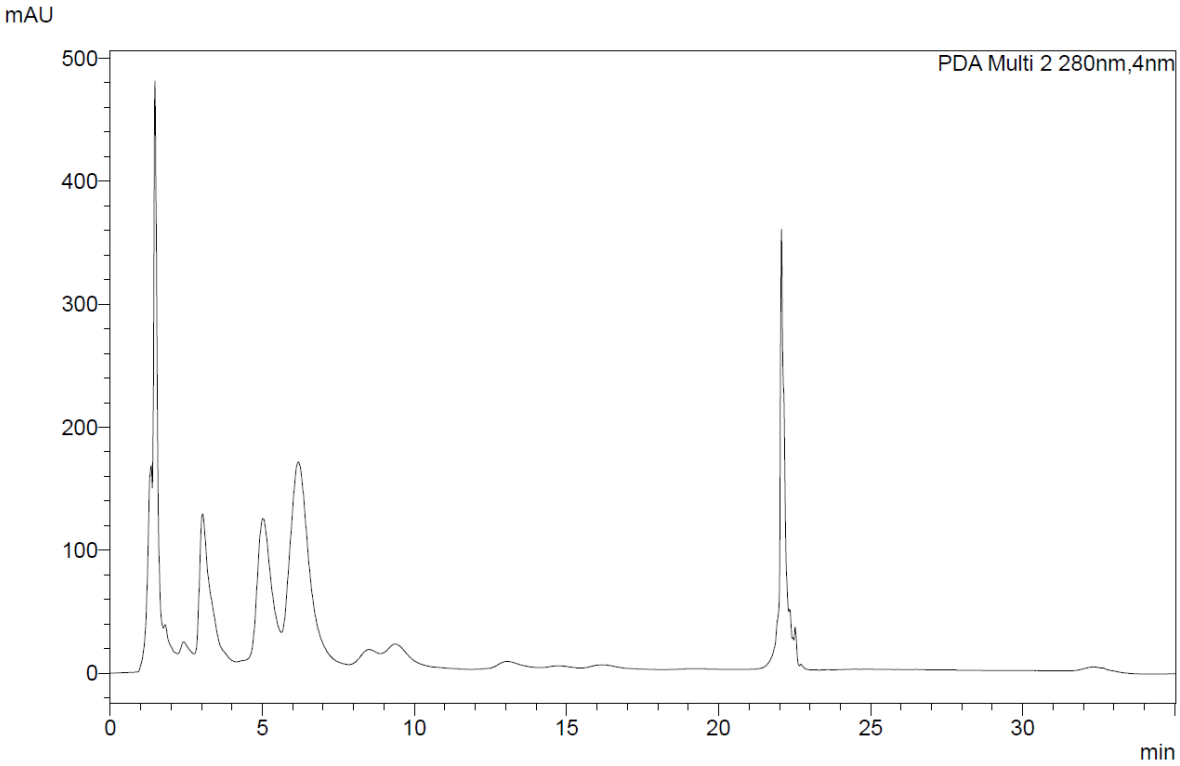

Figure 13: HPLC chromatogram of an aqueous bergenia leaf extract using method 2.

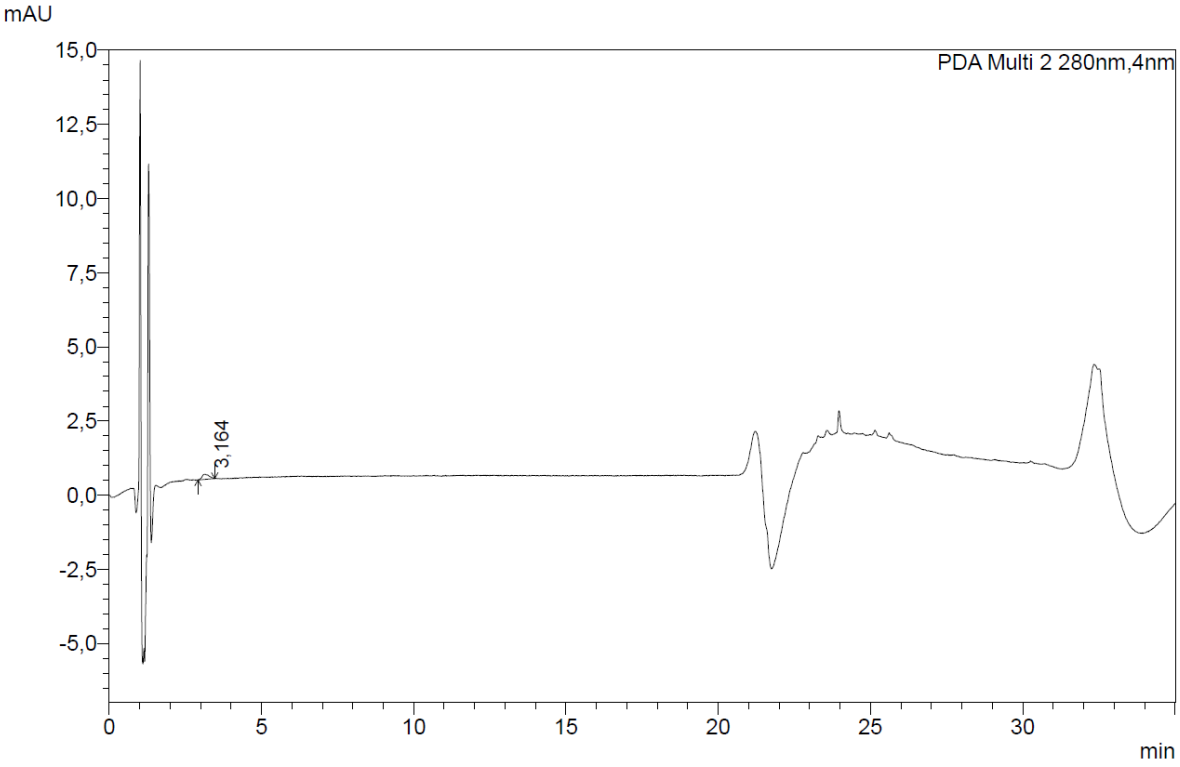

Figure 14: Chromatogram of arbutin standard, c = 1000 μg/ml using method 2.

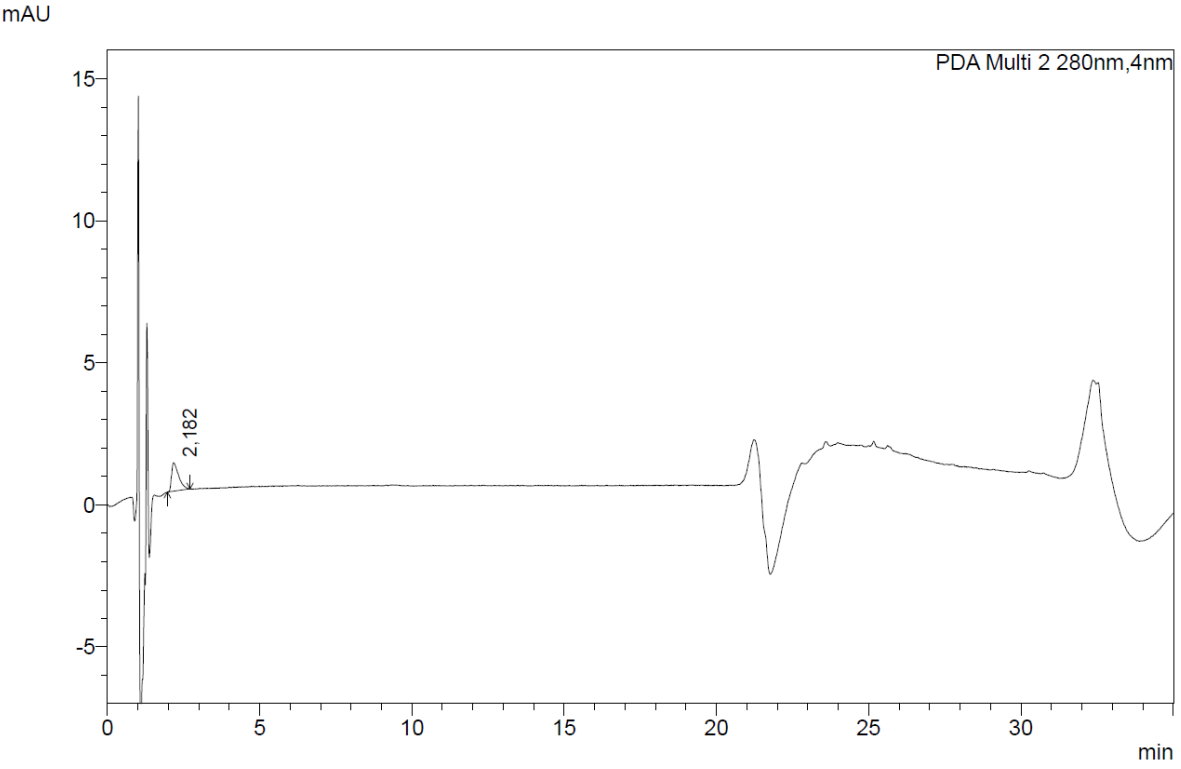

Figure 15: Chromatogram of bergenin standard, c = 1000 μg/ml using method 2.

The chromatograms of both standards displayed inexplicable peaks at retention times around one minute. Possible contamination with other substances could be excluded, since chromatograms of freshly prepared new solutions showed the same peaks again. Resolution was still very poor as well, and despite the high concentration of the standards the peak areas were very small. This method was therefore deemed not to be suitable for the determination of arbutin and bergenin and thus dropped and not further pursued.

Method 3 is a variation of the method established by Boros et al. (2014) which was customized to the equipment at hand. Since the original method was designed around a much shorter and narrower column the first step was to lengthen the runtime (table 11).

Table 11: Gradient profile of method 3.

Time (min)	A (%V/V)	B (%V/V)
0.00	100	0
10.00	100	0
10.10	30	30
40.00	30	30
40.10	5	95
55.00	5	95
55.10	100	0
60.00	Stop	Stop

The peaks thus obtained were sharp and pronounced (figure 23), making this the method of choice, the only downside being the long runtime of 60 minutes.

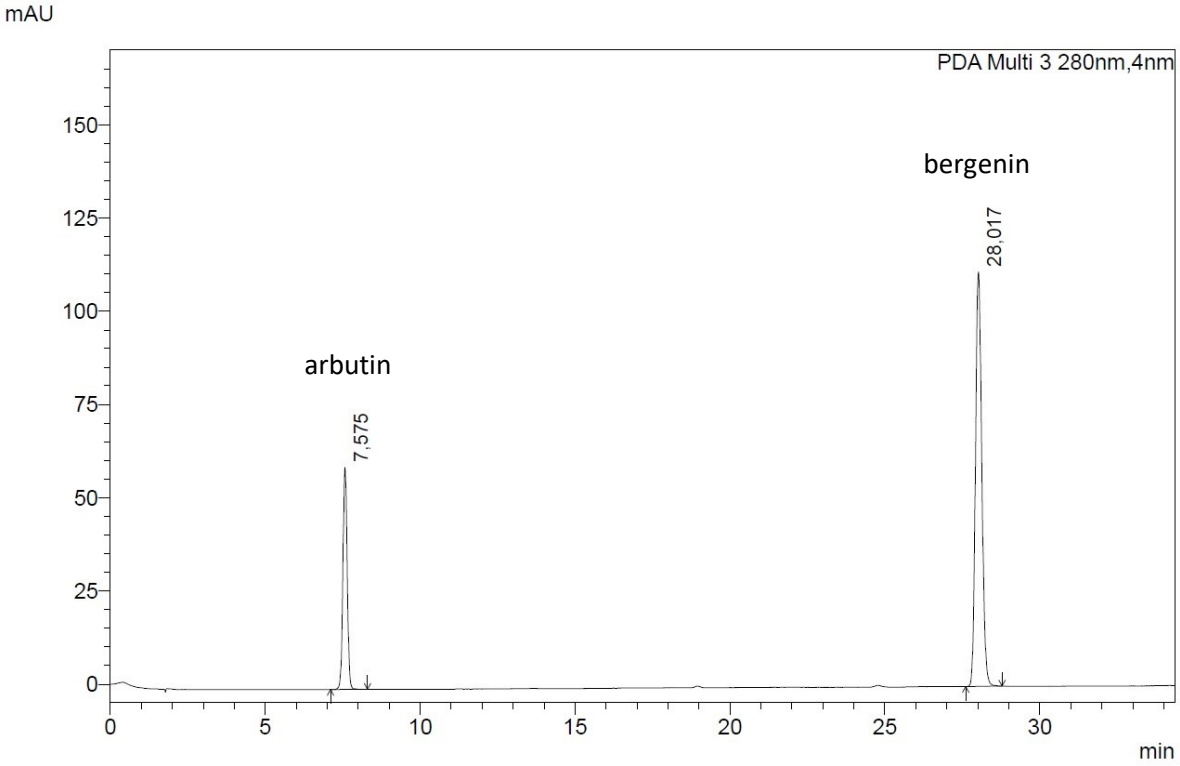

Figure 16: Chromatogram of a combined standard solution of arbutin and bergenin (500 µg/ml each) obtained with the first variation of method 3 (table 11). Arbutin eluted at 7.575 and bergenin at 28.017 minutes.

Thus, the overall runtime was shortened to 54 minutes. Instead of using isocratic elution, a gradient profile was introduced during the first 30 minutes of the run (table 12, next page).

Table 12: Gradient profile of modified method 3.

Time (min)	A (%V/V)	B (%V/V)
0.00	100	0
30.00	100	30
30.10	30	95
43.00	30	95
43.10	100	0
54.00	100	0
54.10	Stop	Stop

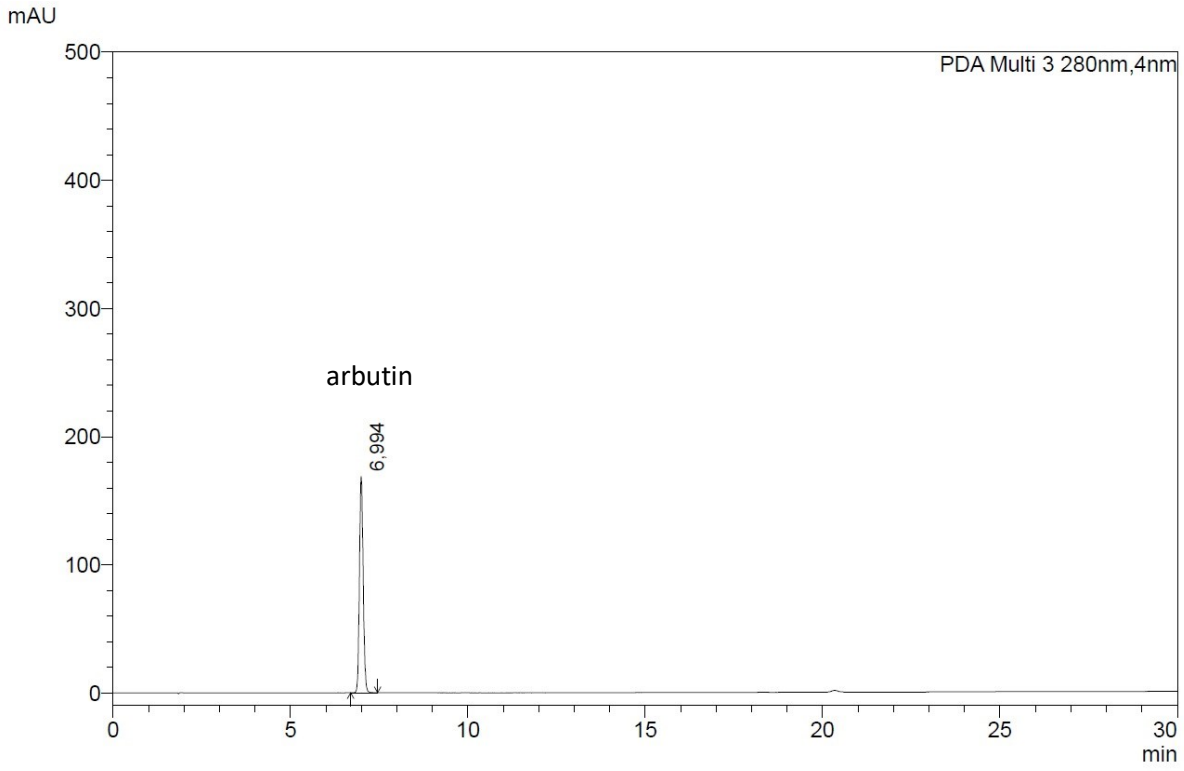

Figure 17: Chromatogram arbutin (1000 μg/ml) using the shortened version of method 3 (table 12).

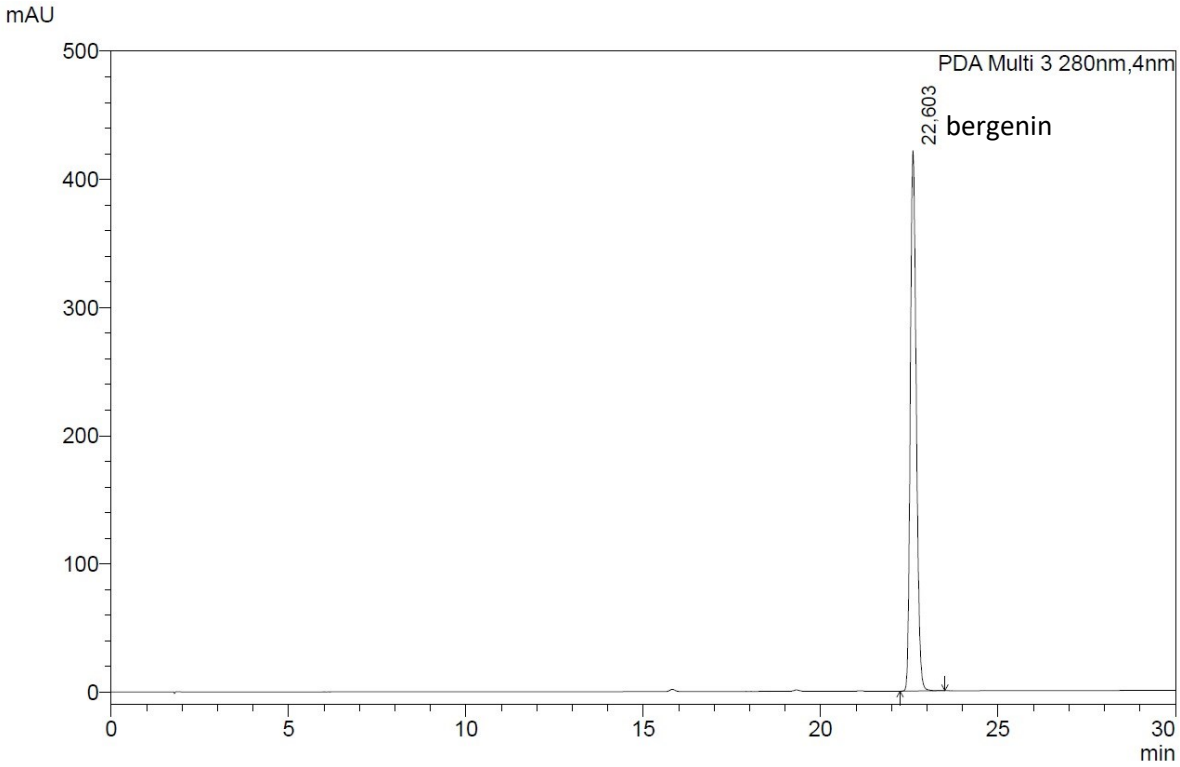

Figure 18: Chromatogram of bergenin (1000 μg/ml) using the shortened version of method 3 (table 12).

Since the results looked very promising, samples of lamina, rhizome and root extracts were analysed as well (figures 26 – 28, pages 60-61).

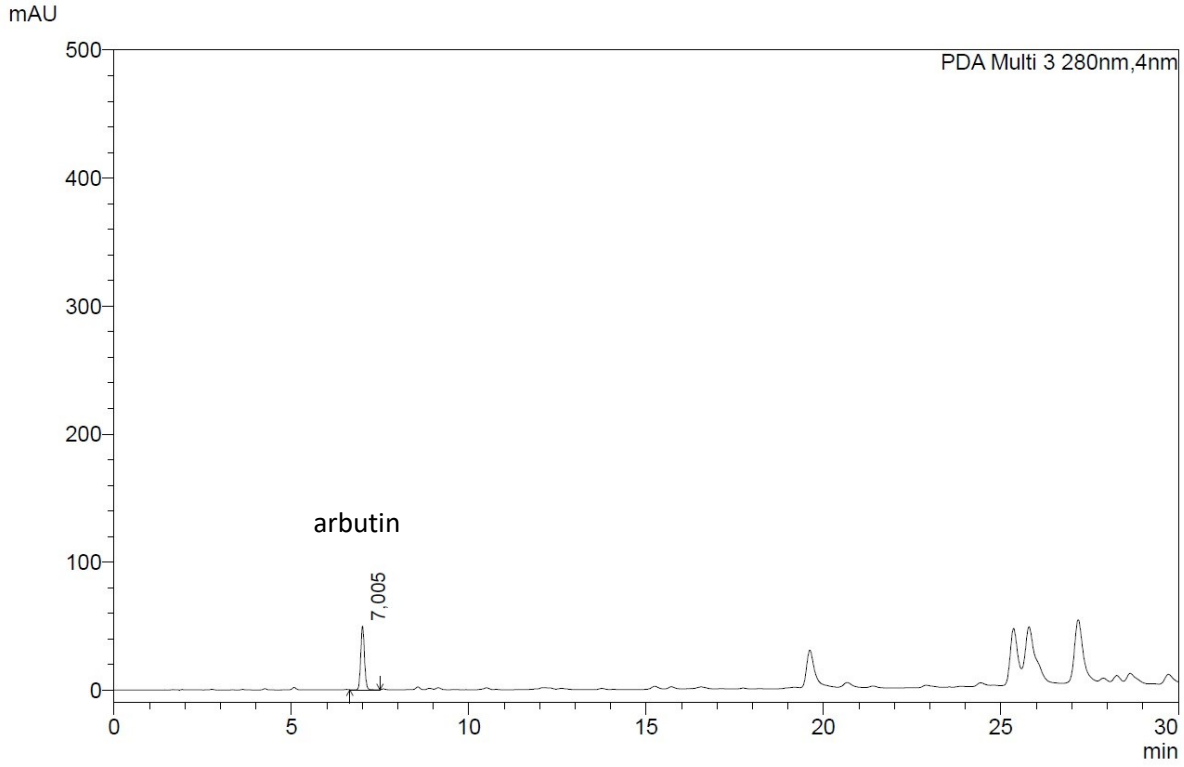

Figure 26: Chromatogram of a bergenia leaf sample (08/17).

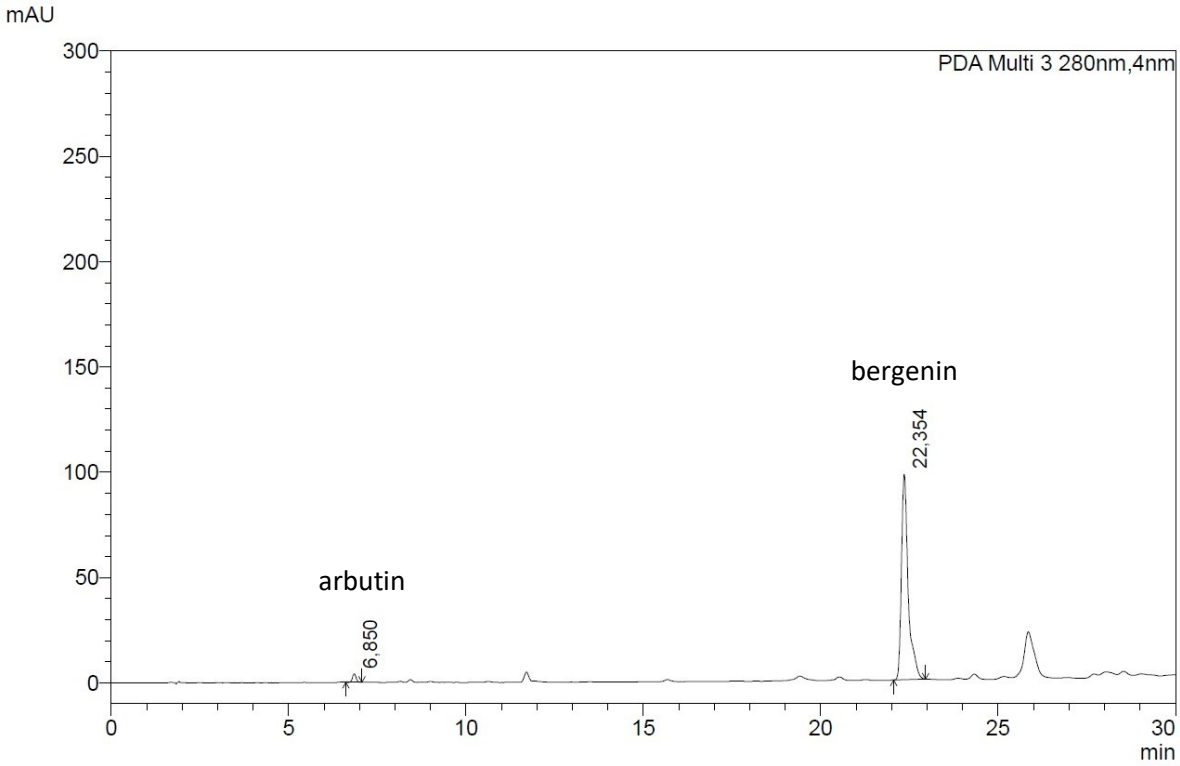

Figure 27: Chromatogram of a bergenia rhizome sample (05/18).

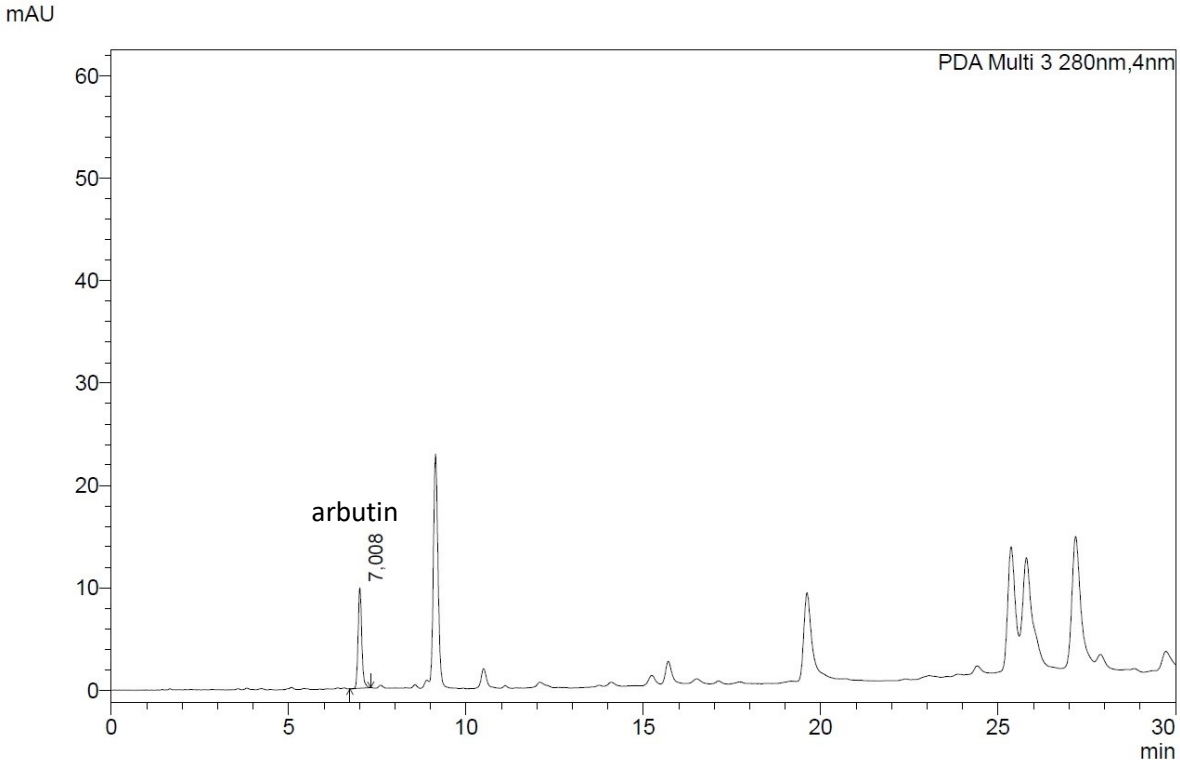

Figure 19: Chromatogram of a bergenia root sample (05/18).

Even though the chromatograms were evaluable, the runtime was still longer as needed be. The gradient elution in the beginning was maintained, however the individual flushing steps for the HPLC column were shortened (table 13). Thus, the retention times of both arbutin and bergenin remained the same. The following figures 29 to 34 (pages 62 to 65) show chromatograms of various samples obtained with this final variation of method 3.

Table 13: Gradient profile of the second modification of method 3.

Time (min)	A (%V/V)	B (%V/V)
0.00	100	0
30.00	100	30
30.10	30	95
38.00	30	95
38.10	100	0
46.00	100	0
46.10	Stop	Stop

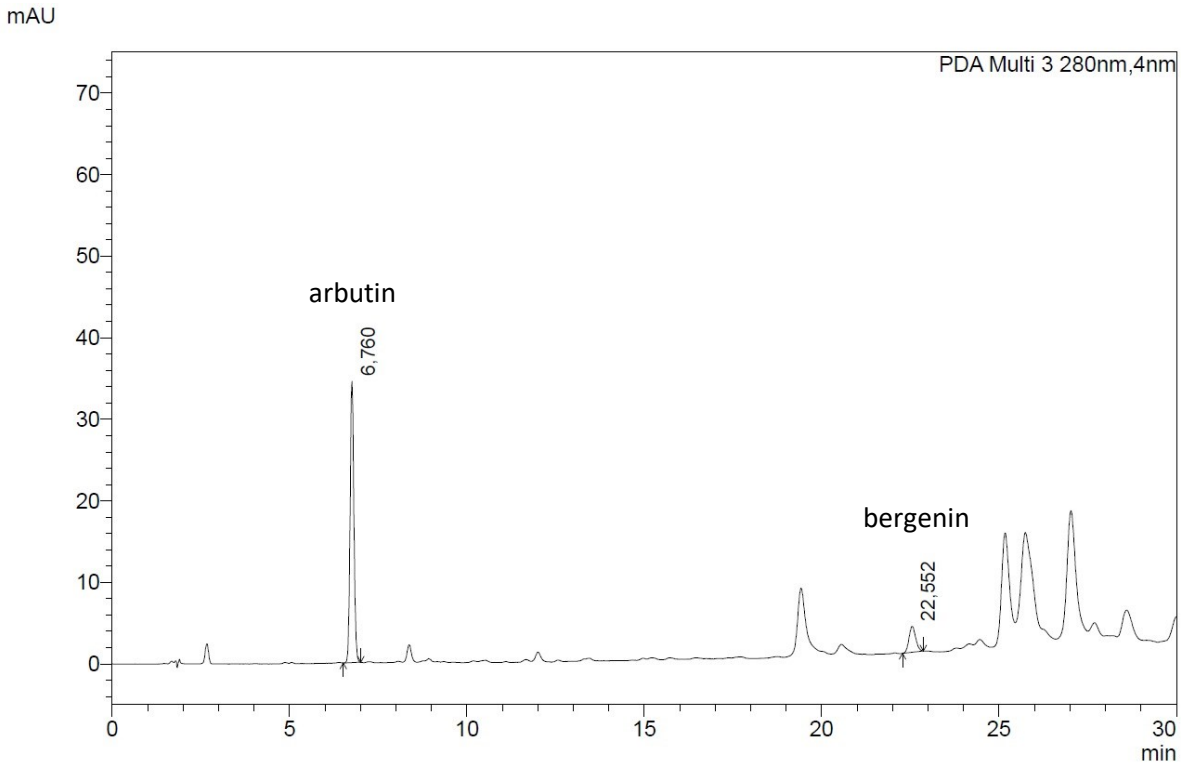

Figure 20: Chromatogram of a bergenia leaf sample (05/18)

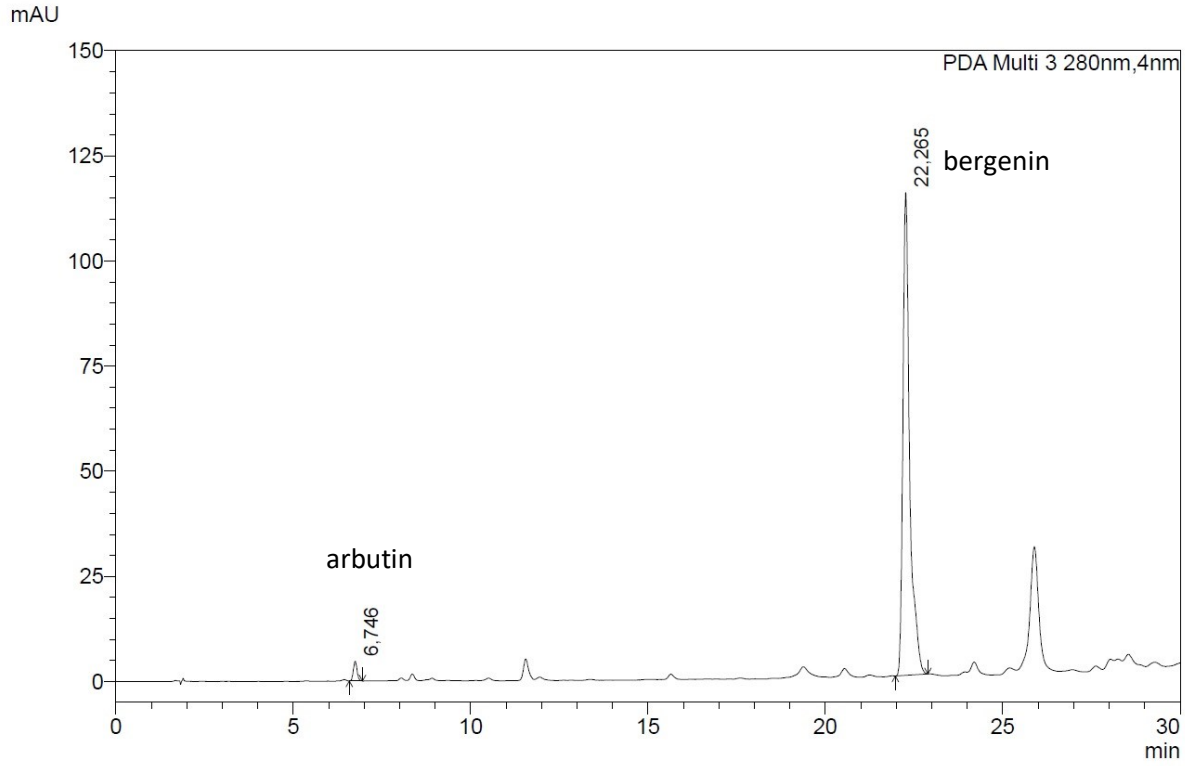

Figure 21: Chromatogram of a bergenia rhizome sample

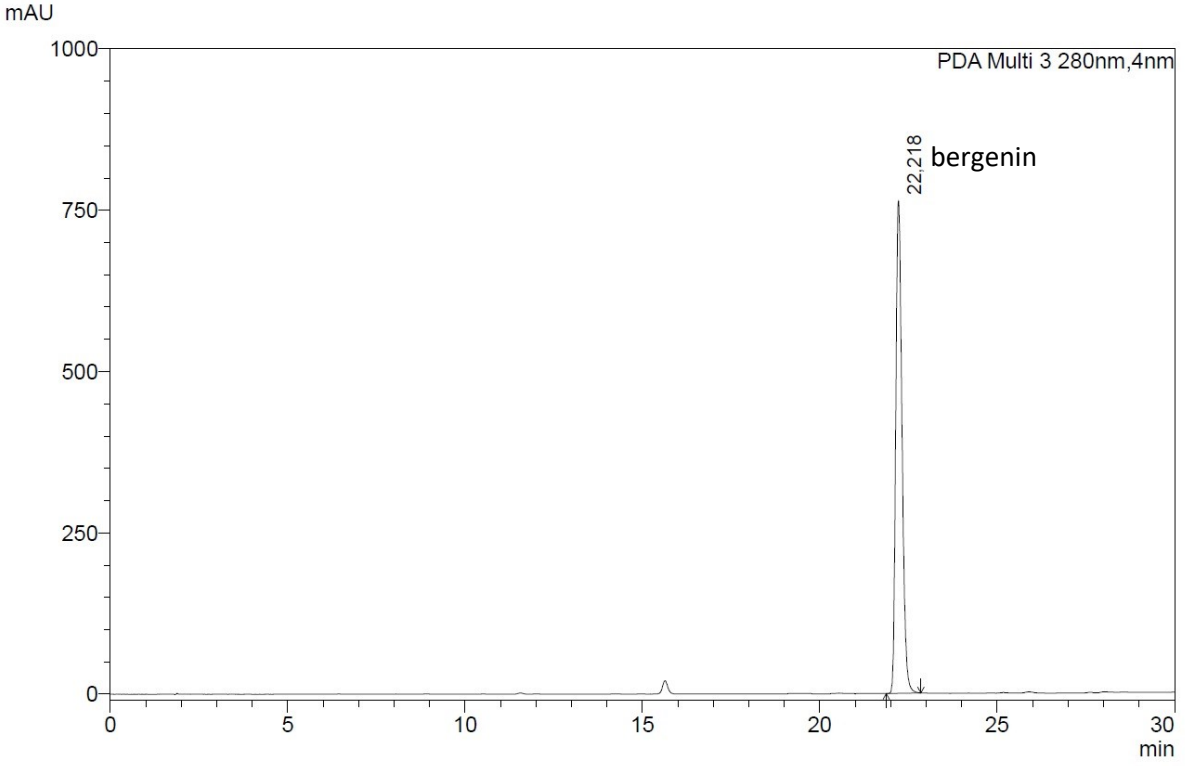

Figure 22: Chromatogram of a bergenia root sample

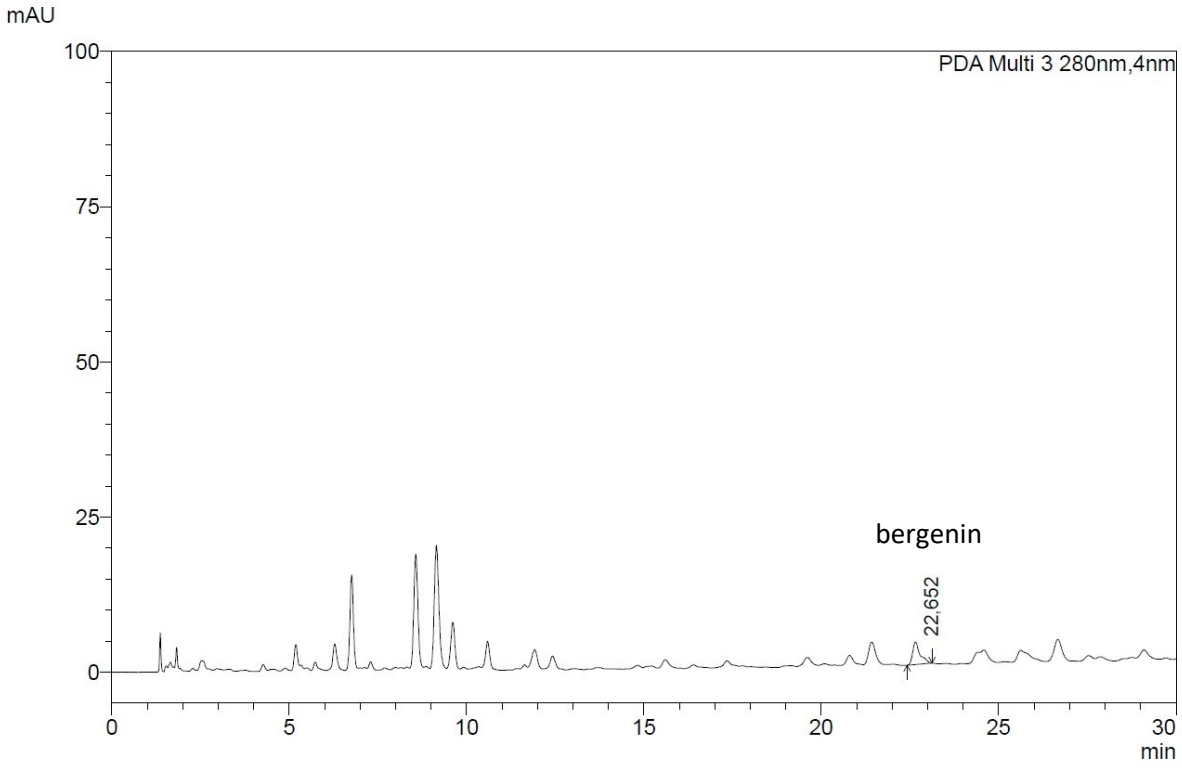

Figure 23: Chromatogram of callus line 2.1S

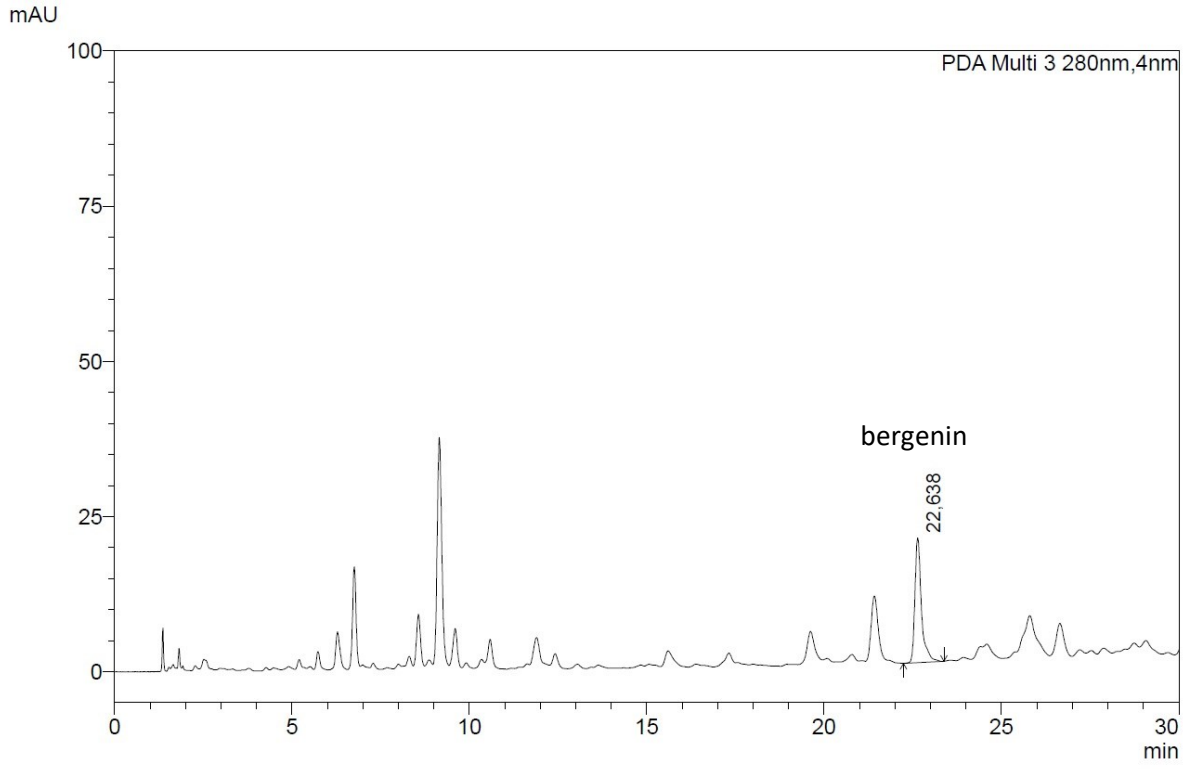

Figure 24: Chromatogram of callus line 2.2R

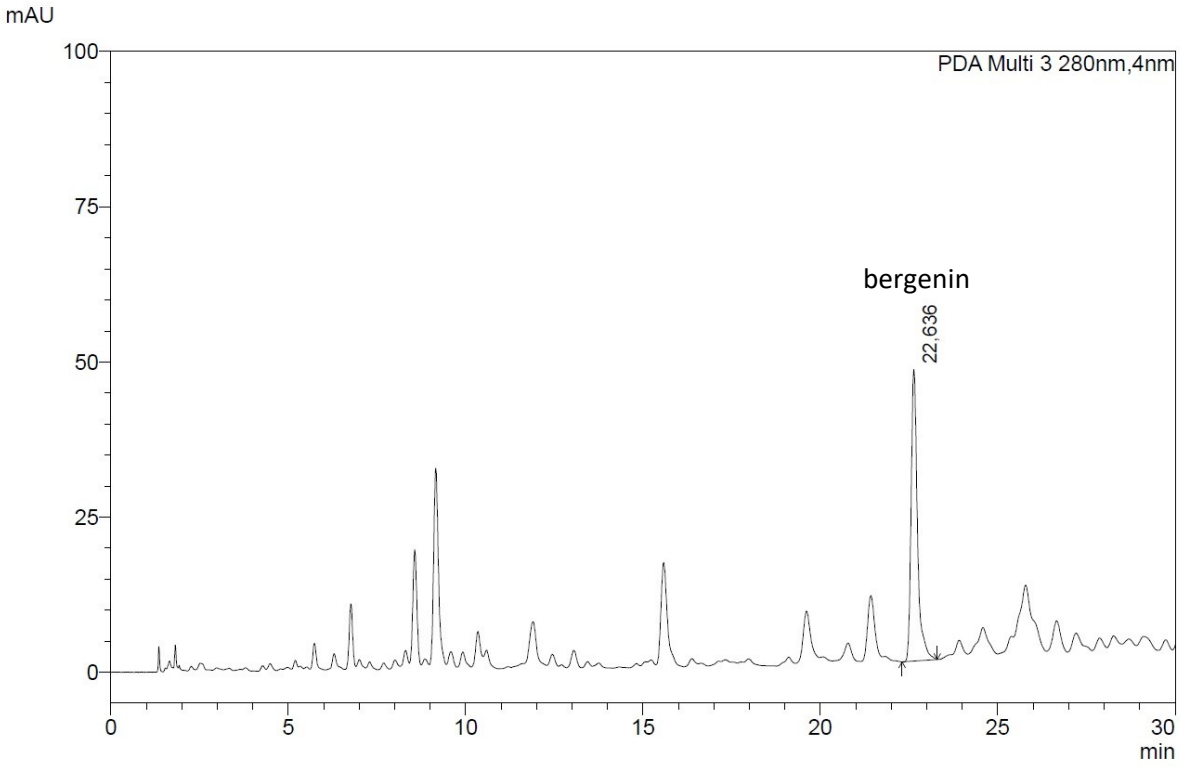

Figure 25: Chromatogram of callus line 5.2R

5.2.1. Recovery

Recovery of the extraction and HPLC methods was tested by spiking 3 different extracts of lyophilized and pulverized rhizome samples with pure bergenin. Values were determined at +50%, +100% and +150%. First, the pure extract was measured with the HPLC to determine the innate bergenin content, which turned out to be 12.321 mg/g. Pure bergenin was added to the methanol/water solvent mixture before the sonification of the extracts. The following table lists the results of the analyses.

Table 14: Results of the recovery experiment.

Sample + addition of bergenin (%)	Supposed amount (mg/g)	Found amount (mg/g)	Recovery[a] (%) ± R.S.D.
1 (+50%)	18.482	15.793	85.45 ± < 0.01
2 (+100%)	24.642	23.934	97.13 ± 0.01
3 (+150%)	30.803	29.190	94.76 ± < 0.01

According to SANCO-guidelines (SANCO/12571/2013) the recovery should be between 70 – 120%, while the standard deviation should be ≤ 20 %. Accordingly, the method utilized in this study was successfully validated.

5.3. Identification of peaks

The peaks of arbutin and bergenin were determined by spiking, i.e. the addition of a defined amount of pure arbutin and bergenin as methanolic solutions to the extract of callus line BP 1S. The chromatogram of the unspiked extract is shown in figure 36. After spiking only one peak (bergenin) increased considerably (figure 37).

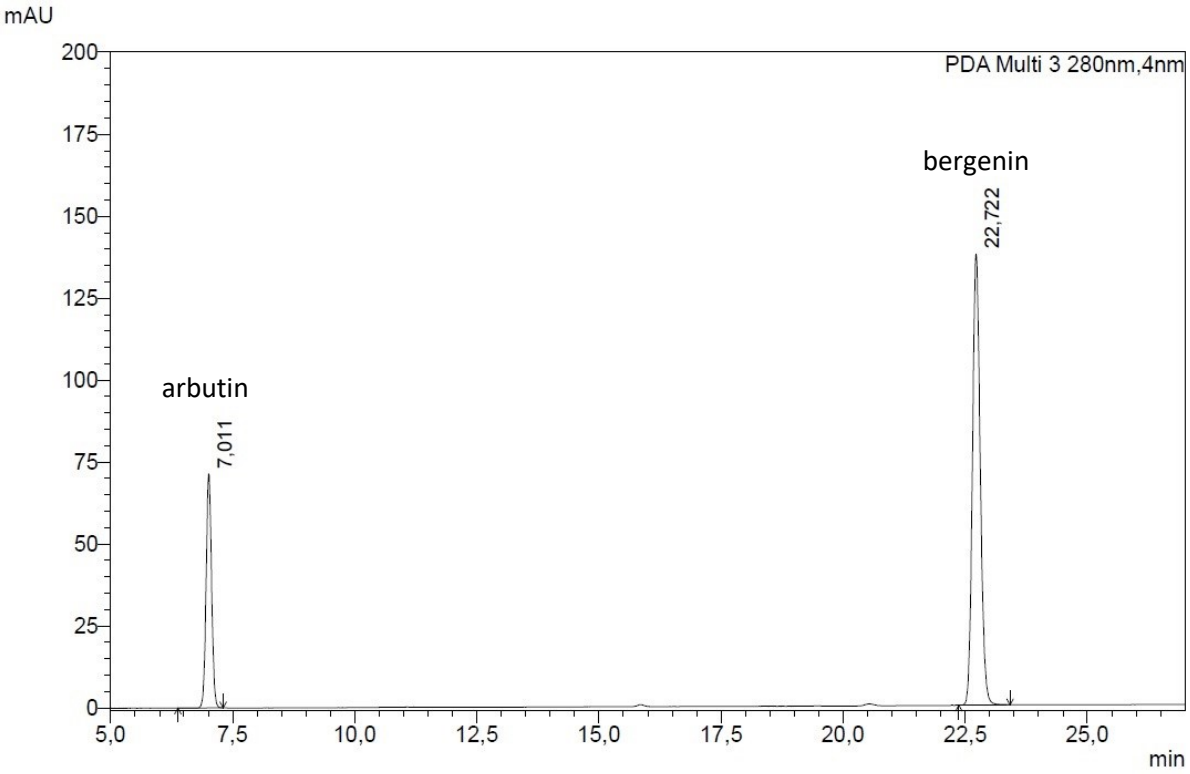

Figure 26: HPLC chromatogram of a combined methanolic standard solution of both arbutin and bergenin of 500 μg/ml each.

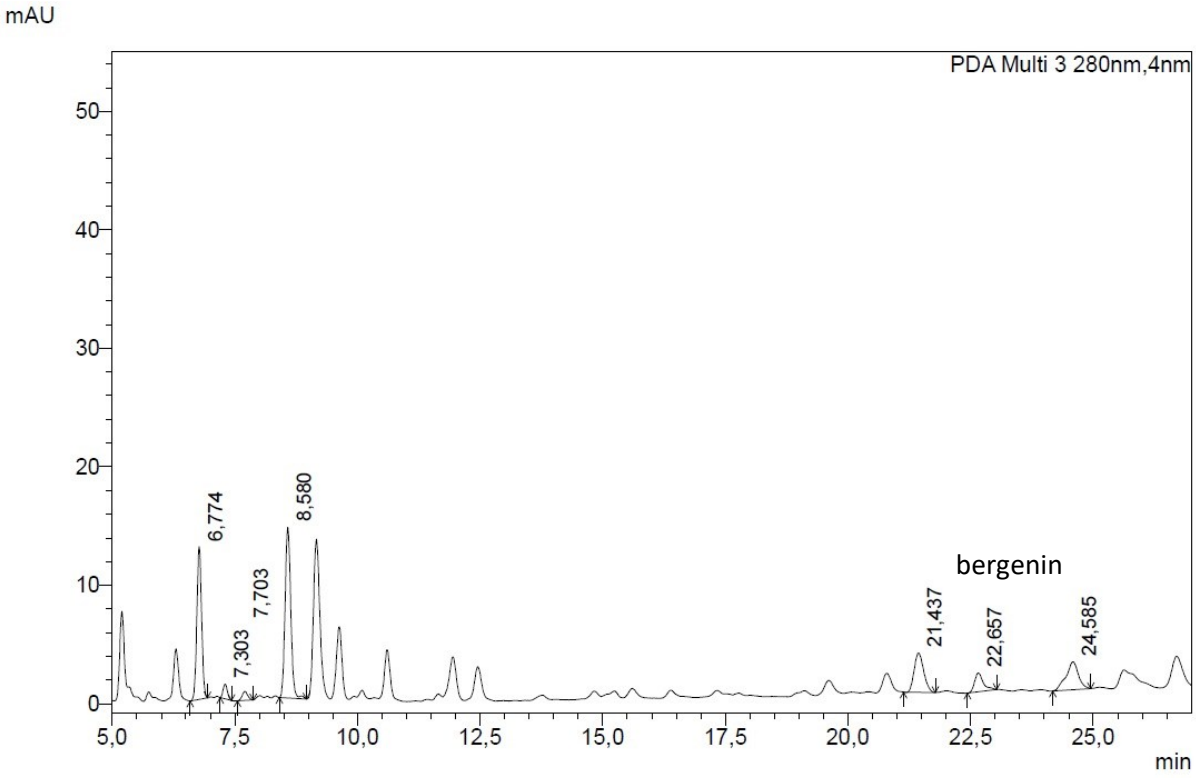

Figure 27: HPLC chromatogram of *B. pacumbis* callus extract BP 1S.

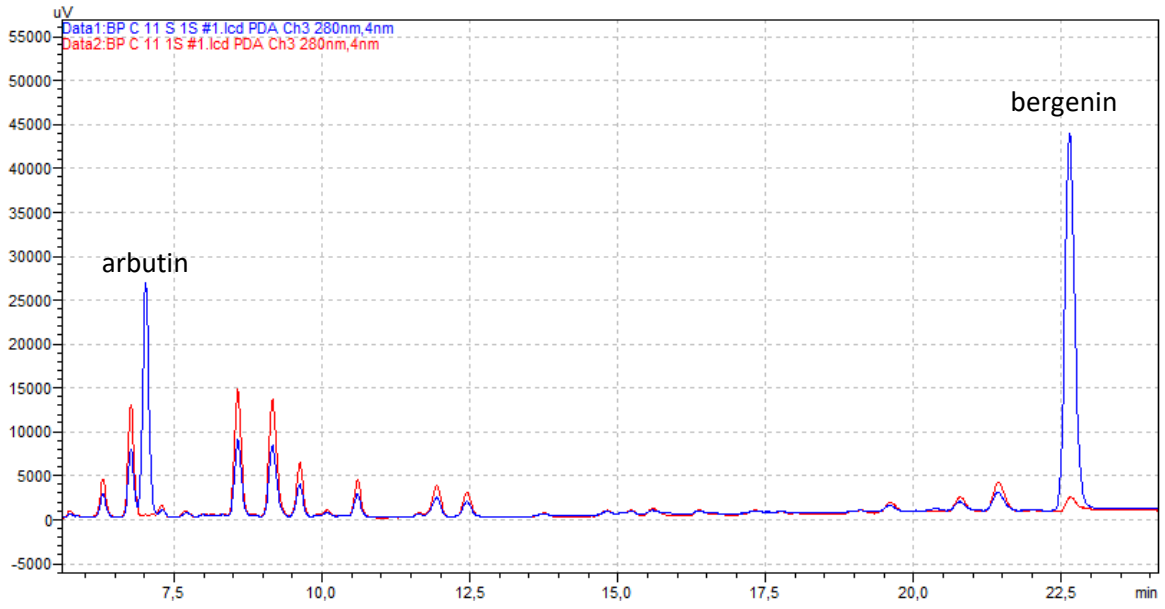

Figure 28: HPLC chromatogram overlay of *B. pacumbis* callus extract BP 1S (red) and the same extract that was spiked with methanolic solutions of both arbutin and bergenin at 500 mg/ml each (blue). At a retention time of 7.015, an additional peak appeared, representing the newly introduced arbutin. The peak at 22.634 grew largely in size compared to the pure extract, indicating the presence of bergenin in the callus extract.

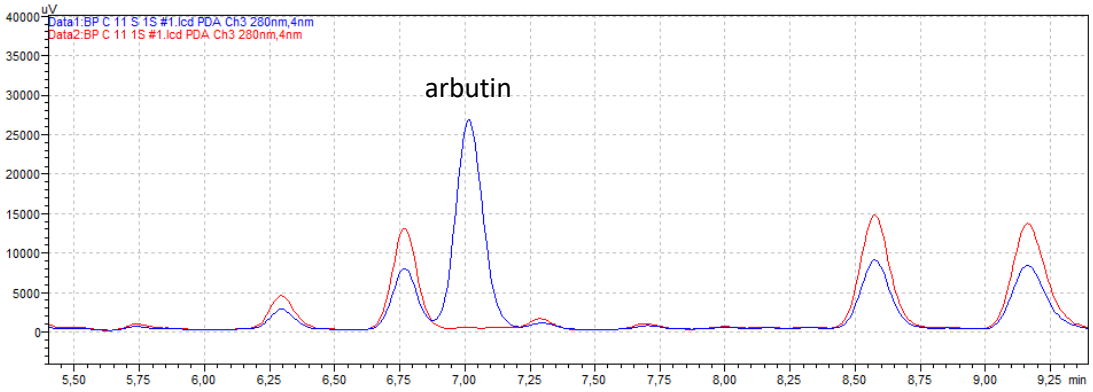

Figure 29: Close-up of the HPLC chromatogram overlay of *B. pacumbis* callus extract BP 1S (red) and the same extract that was spiked with methanolic solutions of both arbutin and bergenin at 500 mg/ml each (blue). Notice the lack of a peak at a retention time of around 7.0 in the pure callus extract (red), indicating the absence of arbutin.

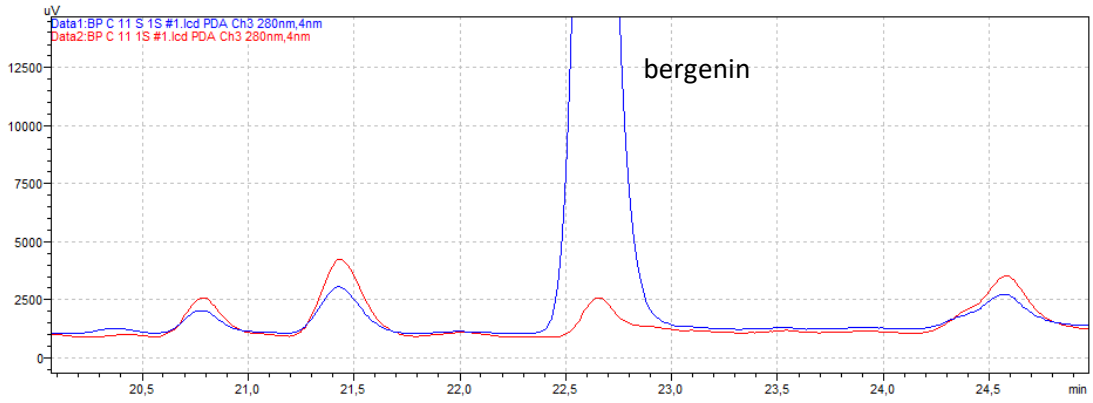

Figure 30: Close-up of the HPLC chromatogram overlay of *B. pacumbis* callus extract BP 1S (red) and the same extract that was spiked with methanolic solutions of both arbutin and bergenin at 500 mg/ml each (blue). The peak at 22.634 grew largely in size compared to the pure extract, indicating the presence of bergenin in the callus extract.

5.4. Calibration Curve

For creating the calibration curve, two methanolic stock solutions of both arbutin and bergenin (1 mg/ml each) were prepared. The exact concentrations for the arbutin and bergenin solutions were 1.11 mg/ml and 1.02 mg/ml respectively. Each subsequent dilution was prepared by mixing parts of the stock solutions with a solvent mixture [methanol:water = 1:1 (v/v)]. Both calibration curves showed correlation coefficients of >0.99 (figure 40 and 41).

For the calibration curve of arbutin, an equation of y = 942 076,59388x + 2 904,36629 and a correlation coefficient of 0,99870 was determined.

For the calibration curve of bergenin, an equation of y = 2 847 877,09042x - 1 252,64247 and a correlation coefficient of 0,99998 was determined. After the determination of the corresponding peak areas of arbutin and bergenin, their contents in all the samples could be calculated.

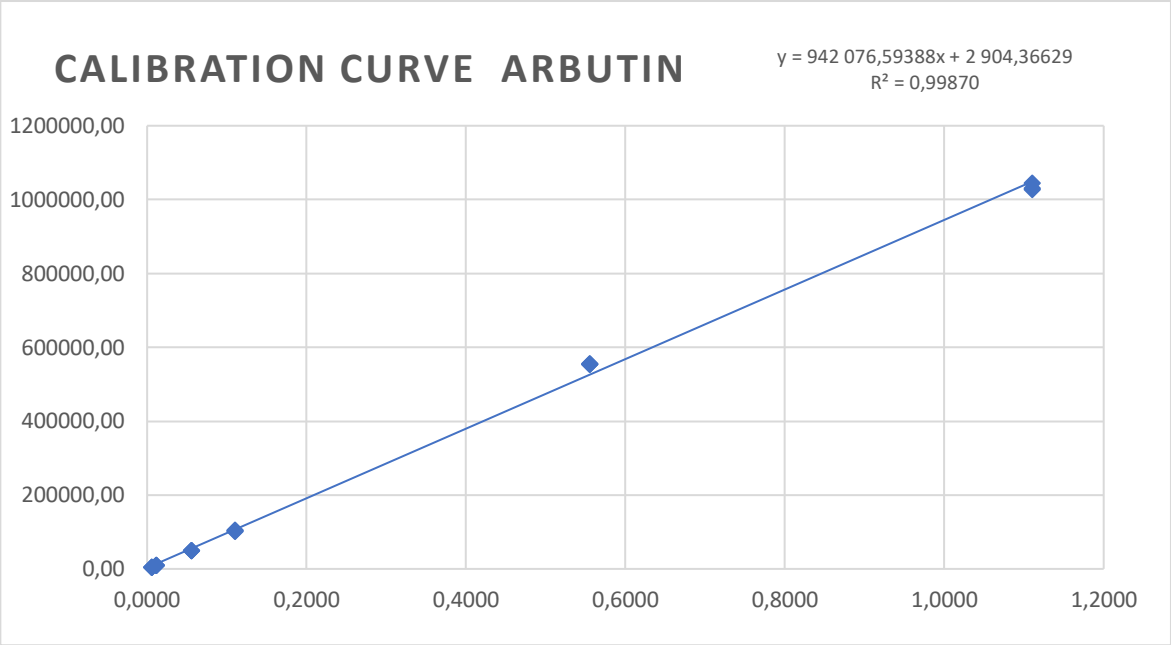

Figure 40: Calibration curve of arbutin, using concentrations of 1110.0, 555.0, 110.0, 55.5, 11.0 and 5.5 µg/ml.

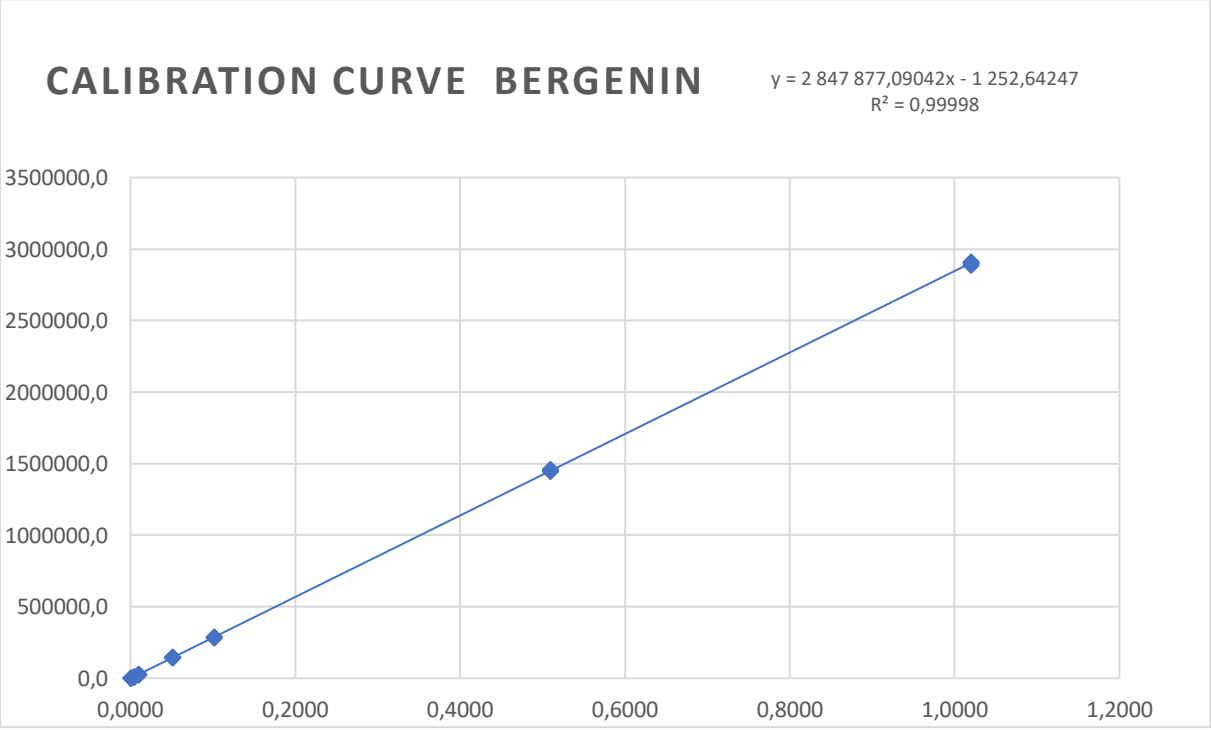

Figure 41: Calibration curve of bergenin, using concentration of 1020.0, 510.0, 102.0, 51.0, 10.2, 5.1 and 1.0 µg/ml.

5.5. Quantification

Table 15: Average arbutin and bergenin contents from various organs of the *B. pacumbis* specimens that served as source plants for the induction of callus culture ($\bar{x}^y$: average of y samples).

Sample	Avg. arbutin content w%	Avg. bergenin content w%
Leaf samples 08/17	1.59, $\bar{x}^1$	0.00
Leaf samples 12/17	1.84, $\bar{x}^1$	0.00
Leaf samples 05/18	1.26, $\bar{x}^3$	0.02, $\bar{x}^3$
Rhizome samples 05/18	0.13, $\bar{x}^3$	1.70, $\bar{x}^3$
Root samples 05/18	0.00, $\bar{x}^3$	13.58, $\bar{x}^3$

Table 16: Average bergenin content in various callus cell lines from media BP 1, 2 and 5.

None of the 23 tested callus cell extracts contained any arbutin whatsoever ($\bar{x}^y$: average of y samples).

Tissue source	Medium	Avg. bergenin content w%
Petiole	BP 1	0.01, $\bar{x}^2$
Petiole	BP 2	0.03, $\bar{x}^6$
Midrib	BP 2	0.08, $\bar{x}^6$
Lamina	BP 2	0.03, $\bar{x}^3$
Petiole	BP 5	0.16, $\bar{x}^1$
Midrib	BP 5	0.17, $\bar{x}^3$
Lamina	BP 5	0.27, $\bar{x}^2$

Table 17: Calculated limit of detection (LOD) and limit of quantification (LOQ) for both arbutin and bergenin.

Substance	LOD (mg/ml)	LOQ (mg/ml)
Arbutin	0,05	0,16
Bergenin	0,01	0,02

5.6. Identification of Endophytes

Table 18: List of the isolated endophytes from *Bergenia pacumbis* that were successfully identified with both forward and reverse primer using the modified protocol of the Wizard ® SV Genomic DNA Purification System.

Isolate Number	Plant tissue	Genbank Blast search identity	Plant individual	Replicate
BL 4	Root	*Kluyvera intermedia*	1	2
BL 9	Root	*Pseudomonas plecoglossicida*	2	1
BL 11	Root	*Raoultella terrigena*	1	3
BL 12	Root	*Raoultella terrigena*	1	3
BL 15	Root	*Raoultella terrigena*	1	3
BL 18	Root	*Rahnella aquatilis*	1	1
BL 24	Root	*Raoultella terrigena*	2	1
BL 34	Petiole	*Acinetobacter lwoffii*	1	3
BL 34a	Petiole	*Agrobacterium tumefaciens*	1	3
BL 35b	Petiole	*Pseudomonas umsongensis*	2	3
BL 39	Root	*Rhizobium tibeticum*	2	3
BL 42	Root	*Pseudomonas alcaligenes*	2	3
BL 44b	Root	*Agrobacterium tumefaciens*	2	1
BL 47	Leaf	*Xanthomonas theicola*	1	3
BL 54	Root	*Pseudomonas alcaligenes*	2	1
BL 58	Petiole	*Pseudomonas kilonensis*	2	3
BL 63	Petiole	*Micrococcus aloeverae*	Pooled samples	Pooled samples
BL 64	Petiole	*Haematobacter massiliensis*	Pooled samples	Pooled samples

BL 69	Petiole	*Bacillus sp. / Acinetobacter sp.*	Pooled samples	Pooled samples
BL 70	Petiole	*Acinetobacter lwoffii*	Pooled samples	Pooled samples
BL 76	Petiole	*Pseudomonas alcaligenes*	2	3
BL 81	Root	*Bacillus mycoides*	3	2
BL 82	Petiole	*Acinetobacter lwoffii*	Pooled samples	Pooled samples
BL 84	Petiole	*Lelliottia amnigena*	Pooled samples	Pooled samples
BL 86b	Petiole	*Acinetobacter lwoffii*	Pooled samples	Pooled samples
BL 87	Petiole	*Bacillus mycoides*	Pooled samples	Pooled samples
BL 88	Petiole	*Acinetobacter lwoffii*	Pooled samples	Pooled samples
BL 89	Petiole	*Acinetobacter lwoffii*	Pooled samples	Pooled samples
BL 91	Petiole	*Micrococcus yunnanensis*	Pooled samples	Pooled samples
BL 94	Petiole	*Bacillus cereus*	Pooled samples	Pooled samples
BL 97	Petiole	*Acinetobacter lwoffii*	Pooled samples	Pooled samples
BL 98	Petiole	*Acinetobacter lwoffii*	Pooled samples	Pooled samples
BL 99	Petiole	*Janibacter indicus*	Pooled samples	Pooled samples
BL 100	Petiole	*Micrococcus aloeverae*	Pooled samples	Pooled samples
BL 102	Petiole	*Bacillus sp. / Acinetobacter sp.*	Pooled samples	Pooled samples
BL 106	Petiole	*Acinetobacter lwoffii*	Pooled samples	Pooled samples
BL 109	Petiole	*Bacillus mycoides*	Pooled samples	Pooled samples
BL 111	Petiole	*Acinetobacter lwoffii*	Pooled samples	Pooled samples
BL 112	Petiole	*Acinetobacter lwoffii*	Pooled samples	Pooled samples
BL 114	Petiole	*Paracoccus yeei*	Pooled samples	Pooled samples
BL 115	Petiole	*Bacillus mycoides*	Pooled samples	Pooled samples
BL 120	Petiole	*Acinetobacter lwoffii*	Pooled samples	Pooled samples
BL 121	Petiole	*Acinetobacter lwoffii*	Pooled samples	Pooled samples
BL 127	Petiole	*Bacillus mycoides*	Pooled samples	Pooled samples
BL 130	Petiole	*Massilia timonae*	Pooled samples	Pooled samples
BL 131	Petiole	*Prolinoborus fasciculus*	Pooled samples	Pooled samples
BL 147	Petiole	*Pseudomonas alcaligenes*	2	3

Table 19: List of the endophytes identified by unidirectional sequences using forward primer. The sequence of the reverse primer could not successfully be sequenced and lacked quality.

Isolate Number	Plant tissue	Genbank Blast search identity	Plant individual	Replicate
BL 5	Root	*Acinetobacter lwoffii*	*1*	*3*
BL 62	Petiole	Paracoccus sp. CECT 8481	Pooled samples	Pooled samples
BL 92	Petiole	*Acinetobacter lwoffii*	Pooled samples	Pooled samples

Table 20: List of the endophytes that were identified using only the reverse primer. The sequence of the forward primer could not successfully be sequenced and lacked quality.

Isolate Number	Plant tissue	Genbank Blast search identity	Plant individual	Replicate
BL 21	Root	Pseudomonas cremoricolorata	2	1
BL 79	Petiole	*Acinetobacter lwoffii*	-	-
BL 117	Petiole	*Acinetobacter lwoffii*	Pooled samples	Pooled samples

Isolates were aquired using two different methods: Isolates numbered 1-61 and 72-81 were isolated from surface sterilized plant tissues that were cut into small pieces using 3 replicates per tissue and plant individual. Isolates with number 62-71, 82-134 and 138-143 originated from tissue samples that were pooled regarding plant tissues and contained each 3 plant individuals (Dr. Oberhofer, personal communication).

5.6.1. Phylogeny

Taxonomic relations of the isolates were inferred using the maximum likelihood method and the most probable base substitution model by Tamura and Nei (1993). The tree with the highest log likelihood (-20392.60) is shown (figure 42, next page). The percentage of trees in which the associated taxa clustered together is shown next to the branches (bootstrap values). Initial tree(s) for the heuristic search were obtained automatically by applying Neighbor-Join and BioNJ algorithms to a matrix of pairwise distances estimated using the Maximum Composite Likelihood (MCL) approach, and then selecting the topology with superior log likelihood value. A discrete Gamma distribution was used to model evolutionary rate differences among sites (5 categories (+G, parameter = 0.5715)). The rate variation model allowed for some sites to be evolutionarily invariable ([+I], 30.12% sites). The tree is drawn to scale, with branch lengths measured in the number of substitutions per site. This analysis involved 70 nucleotide sequences. Codon positions included were 1st+2nd+3rd+Noncoding. There were a total of 1349 positions in the final dataset. Phylogentic analyses were conducted in MEGA X (Kumar et al., 2018).

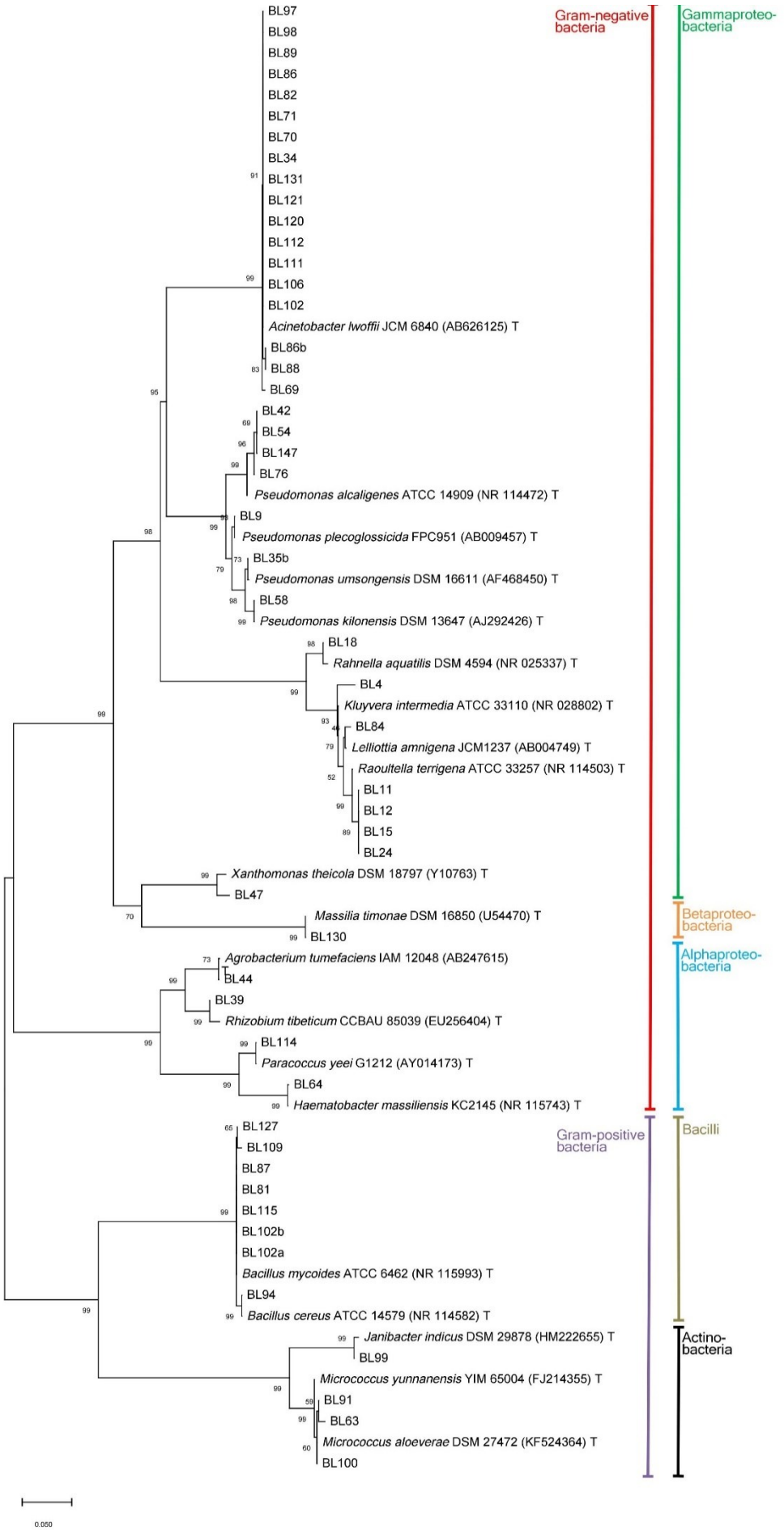
BL97
BL98
BL89
BL86
BL82
BL71
BL70
BL34
BL131
BL121
BL120
BL112
BL111
BL106
BL102
Acinetobacter lwoffii JCM 6840 (AB626125) T
BL86b
BL88
BL69
BL42
BL54
BL147
BL76
Pseudomonas alcaligenes ATCC 14909 (NR 114472) T
BL9
Pseudomonas plecoglossicida FPC951 (AB009457) T
BL35b
Pseudomonas umsongensis DSM 16611 (AF468450) T
BL58
Pseudomonas kilonensis DSM 13647 (AJ292426) T
BL18
Rahnella aquatilis DSM 4594 (NR 025337) T
BL4
Kluyvera intermedia ATCC 33110 (NR 028802) T
BL84
Lelliottia amnigena JCM1237 (AB004749) T
Raoultella terrigena ATCC 33257 (NR 114503) T
BL11
BL12
BL15
BL24
Xanthomonas theicola DSM 18797 (Y10763) T
BL47
Massilia timonae DSM 16850 (U54470) T
BL130
Agrobacterium tumefaciens IAM 12048 (AB247615)
BL44
BL39
Rhizobium tibeticum CCBAU 85039 (EU256404) T
BL114
Paracoccus yeei G1212 (AY014173) T
BL64
Haematobacter massiliensis KC2145 (NR 115743) T
BL127
BL109
BL87
BL81
BL115
BL102b
BL102a
Bacillus mycoides ATCC 6462 (NR 115993) T
BL94
Bacillus cereus ATCC 14579 (NR 114582) T
Janibacter indicus DSM 29878 (HM222655) T
BL99
Micrococcus yunnanensis YIM 65004 (FJ214355) T
BL91
BL63
Micrococcus aloeverae DSM 27472 (KF524364) T
BL100
0.050
Gram-negative bacteria
Gammaproteo-bacteria
Betaproteo-bacteria
Alphaproteo-bacteria
Gram-positive bacteria
Bacilli
Actino-bacteria

Figure 31: Maximum likelihood phylogeny of endophyte and closest related type strain references. This unrooted tree consists of two major clades: The first one containing 38 strains of Gram-negative bacteria (red), of which 18 could be identified as *Acinetobacter lwoffii*. The second one consists 12 gram-positive strains (purple), of which 8 belong to *Bacillus sp*. The tree can be further divided into 32 isolates belonging to gammaproteobacteria, a single one belonging to betaproteobacteria and 16 belonging to alphaproteobacterial.

6. Discussion

This Diploma thesis aimed at laying the groundwork for future studies on the impact of endophytes on SM production in callus cultures of *Bergenia pacumbis*. The induction of callus formation on leaf explants proved to be a tedious process. Apart from their slow growth, callus cells were also darker in colour and of harder consistency, which are both undesirable attributes. Browning in callus cultures is a sign of heavy oxidative stress, which has been credited as one of the agents causing cell damage as well as slow growth (Foyer et al., 1994; Tang et al., 2004). Furthermore, it's associated with cell disorganization and eventual cell death (Laukkanen et al., 1999; (Laukkanen et al., 2000), with might be the reason the overall darker cultures on BP 5 showed a slower growth then the brighter callus cells on BP 2.

This study focused solely on using MS (as well as ½ MS) as a basal medium. Murashige and Skoog devised this medium specifically while working with tobacco tissue cultures, which belongs to the family of *Solanaceae* (Murashige and Skoog, 1962). This might explain the slow growth of bergenia callus cells, which belongs to the family of *Saxifragaceae*. An adjustment of constituents might have accelerated overall growth. For example, studies showed that lowering the sucrose concentration from the initial 3% to only 1% vastly improved callogenesis when working with conifers on LP medium (Webb et al., 1989).

A completely different basal medium for cultivation to test might be Gamborg's B5 basal medium (Gamborg et al., 1968). Rafi et al. (2018) successfully grew callus cells of *B. ciliata* on ½ B5 medium, which comprises different concentrations of macro and micro elements, with very promising results. One of the very first studies on the *in vitro* culture of the genus *Bergenia* was performed by Furmanowa and Rapczewska in 1993. They germinated seeds from *B. crassifolia* on MS medium and used the hypocotyls as explants for the initiation of

callus formation on MS medium supplemented with 0.3 mg/L NAA, 1 mg/L BAP and 80 mg/L adenine sulphate, which produced a friable and light green callus. This indicates that the type of explant plays a vital role as well.

Media BP 3 and BP 4 were quickly discarded as being unsuitable for growing callus cells in this study since there wasn't any noticeable growth even after several weeks. Medium BP 1 and BP 2 both induced callogenesis, however, since callus formation on medium BP 1 was very limited compared to medium BP 2, the former medium was not used further. Media BP 3 and BP 4 were soon discarded as being unsuitable for growing callus cells in this study since there wasn't any noticeable growth even after several weeks of propagation. Medium BP 1 and BP 2 both induced sufficient callogenesis, however, since callus formation on medium BP 1 was very limited compared to medium BP 2, the former medium was not studied any further. Explants on medium BP 5 did grow callus tissue of suitable size, though with every subculturing step the cell aggregates turned darker in colour and growth almost halted completely over time. Browning in plant tissue culture is a severe problem, often ending in the death of affected cultures (Tang et al., 2004). It manifests itself with the accumulation and oxidation of phenolic compounds, to which bergenin belongs (Singh et al., 2007). HPLC analysis proved that bergenin contents of these callus lines were ten times higher when compared to callus lines stemming from medium BP 2. Thus, the excessive browning in cultures on medium BP 5 might have occurred due to overall higher bergenin contents compared to other cell lines. An addition of 0.1% PVP in all subsequent preparations of BP 5 might have helped here, although absorption into the media would possibly result in lower bergenin contents in callus cells. Since the medium was visibly affected by browning as well, HPLC analysis of lyophilised medium might have showed bergenin as well. Although bergenin contents were low in callus cells on medium BP 2 when compared to callus on medium BP 5, growth, colour and

consistency proved to be the most desirable out of all tested media. Since bergenin contents were well within LOD and LOQ as well, these cell lines were kept in propagation for future investigation concerning suspension culture. This decision was supported by the fact that callus agglomerates turned slightly more friable after months of subculturing as well, which is indispensable when establishing suspension cultures (Dunstan and Short, 1977) .

HPLC analyses of 23 callus cell lines revealed that none of the analysed cultures produced detectable amounts of arbutin. This might be because of a lacking precursor, namely hydroquinone. Arbutin is the glycosylated version of hydroquinone, and if a tissue culture is unable to produce the former, it won't be able to synthesize the latter. Several studies performed by Kittipongpatana proved that varying tissue cultures of different Solanaceae species weren't able to synthesize arbutin on their own.After the addition of hydroquinone there was a significant amount of arbutin detectable (Kittipongpatana et al., 2007b, 2007a, 2007c). However, this might have been the effect of some unspecific glycosyl transferases. Tissue cultures of *Arctostaphylos uvae-ursi* lacked both hydroquinone and arbutin as well (Jahodar et al., 1982). Future research concerning the establishment of suspension cultures using bergenia callus cells could look into whether the addition of hydroquinone ignites the production of arbutin.

The extraction method developed by Boros et al. (2014) was suitable for quickly extracting a large batch of lyophilized callus samples. The HPLC method described by the same authors had to be adapted to the equipment at hand. However, due to lack of time the method could not be fully optimized, which resulted in a long runtime of 46 minutes. In future investigations a steeper gradient during the first minutes of runtime, as well as shorter washing steps during the end phase could be implemented. The flow rate of solvents could be increased as well, since according to manufacturer data the column used supports higher pressures.

Out of 156 isolated endophyte strains in total, 41 were already previously taxonomically determined by sequencing. During the course of this diploma thesis another 53 strains could be identified, however, for 6 of those only a unidirectional sequence (forward or reverse primer) was available. 18 of the strains revealed to be *Acinetobacter* sp., which implied possible contamination during one of the working steps as this bacterium is known to reside on human skin (Seifert et al., 1997). All the reagents used for extracting bacterial DNA were tested for contamination on petri dishes filled with L(A) medium and none of them showed any growth. Plating out the affected isolates revealed the source of the contamination. Somewhere during their preparation, the isolates were contaminated, which was proved by the appearance of different strains with different morphological attributes on a nutrient agar plate originating from one cryo-vial with a proposed pure bacterial culture. This means that those isolates are indeed not pure and cannot be successfully identified with the Sanger sequencing method. In order to achieve final results, each colony would have to be picked and plated out again, resulting in pure isolates again.

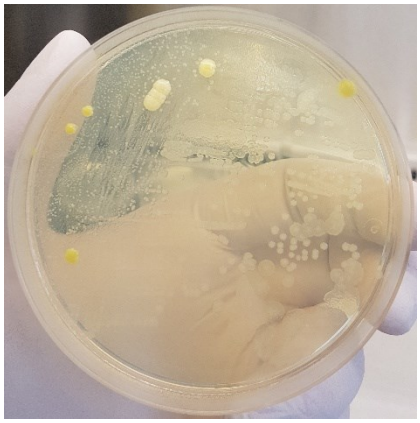

Figure 32: Plated out isolates showing more than 1 strain of bacteria

7. References

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