Secondary metabolites of the endophytic fungi isolated from the root of *Anthriscus sylvestris*

Thesis Research Work
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1. Introduction

The use of active ingredients of plant origin is as old as mankind, in many folk remedies different plant parts - roots, leaves, even flowers - are used for medicinal purposes (Habtemariam, 2019). In addition, a significant part of officially approved drugs used in the medicine are plant or fungal metabolites or their derivatives (Newman and Cragg, 2020). Plants, which may even be medically significant, can live together with various microbial organisms, including specialized fungi (Yu et al., 2023). Another interesting and potential field of use is examining the active ingredient content of certain fungi living in symbiosis with plants. In many cases, endophytic fungi found in the tissues of plants produce the same or similar active substances as plants, but many times these metabolites are only tested in a smaller proportion for the fungi (Zhang et al., 2022). In my Thesis, I examine the already extensively researched cow parsley (*Anthriscus sylvestris* (L.) Hoffm.) and the endophytic fungi living in its roots, aiming to determine the secondary metabolites they produce together and separately.

The work process was built around a group of major research objectives. One of the most important of these was to isolate the endophytic fungi living in the roots of the above-mentioned plant species and identify them based on a barcode sequence of the DNA through DNA. In addition to the species identification, the following important task was to determine the active ingredients of the different plant parts and the fungal partners using high-performance liquid chromatography-mass spectrometry, HPLC-MS method. Using the data obtained in this way, we could compare the active ingredient contents of different species, examining and comparing the relationships between them. It is also possible to examine the exchange, accumulation, transfer, or just the presence and absence of metabolites between species.

2. Literature Review

2.1 The plant partner, *Anthriscus sylvestris*

2.1.1 Placement of *Anthriscus sylvestris* in the flora

Cow parsley (*Anthriscus sylvestris*) belongs to the genus *Anthriscus* and is located in the Apioideae subfamily of the Apiaceae family. Within the subfamily, we can distinguish several groups of subgenera, so-called tribes, of which the genus I am discussing belongs to the Scandiceae group. Several clades can be distinguished within the mentioned and monophyletically well-derived Scandiceae or Scandicinae group (Downie et al., 2001). Among the clades, it is worth mentioning the so-called 'crown clade' and the genera belonging to it, which are well derived based on the morphology of their fruits and can thus be easily identified

and separated. The genera in the clade are the *Anthriscus*, the *Kozlovia*, the *Geocaryum*, the *Myrrhis*, and the *Osmorhiza* (Fig. 1) (Spalik et al., 2001).

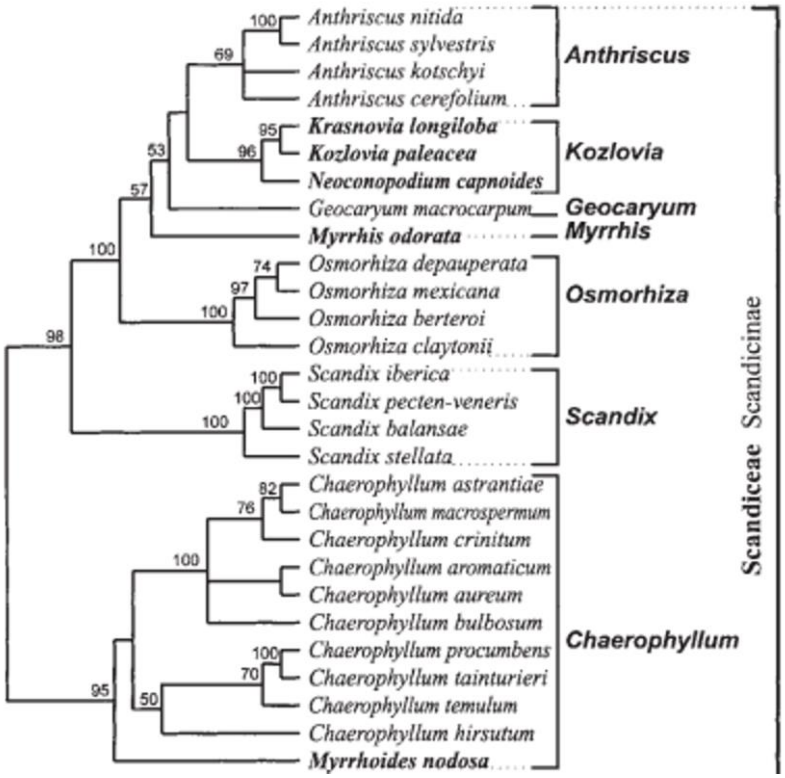

Figure 1. The figure shows a part of the strictly interpreted phylogenetic branching of *Scandiceae* subtribe *Scandicinae*, based on maximum parsimony analysis of combined morphological, anatomical and ITS sequence data. The genus *Anthriscus* and some related taxa are observed, including the five groups belonging to the 'crown clade'. Bootstrap numbers at branch points indicate values equal to or greater than 50%. Monotypic and bitypic genera are highlighted in bold (Spalik et al., 2001 edited).

Within the genus *Anthriscus*, three larger clades or sections, can be distinguished, which are *sect. Anthriscus*, *sect. Cacosciadium* and *sect. Caroides*. The separation of the three larger groups is mainly based on the life cycles and habitats of the species belonging to them. *Anthriscus sylvestris* belongs to the less well-known *sect. Cacosciadium* clade, containing robust species in terms of its lineage. Also belonging to this clade, is the so-called *Anthriscus nitida* (Wahlenb.) Hazsl., which is also native to Hungary, for a long time could not be clearly separated from the morphologically similar cow parsley, the *A. nitida* was related as its subspecies (Spalik, 1996).

2.1.2 Morphological and botanical presentation of *A. sylvestris*

For the morphological presentation of the species, it is worthwhile to go through the properties of the plant family first. Members of the Apiaceae family generally annual, biennial, or even perennial herbaceous plant species, their inflorescence is compound umbel. The flower stalks of the simple umbel inflorescence are the so-called rays, with an involucre of bracts, while the secondary rays (pedicels) have an involucel of bractlets at their base. Its flowers are usually small and white. Their general flower formula is the following, K 5 C 5 A 5 G (2) (lower position). The leaves are often alternate, divided and in many cases, they have a sheathing base. Their roots are usually strong and suitable for storage (see taproot for carrots). Their fruit is the characteristic cremocarp. Many species contain schizogenic essential oil passages, so many fragrant species belong to this family (Fazekas and Szerényi, 2002 & 2015, page 249).

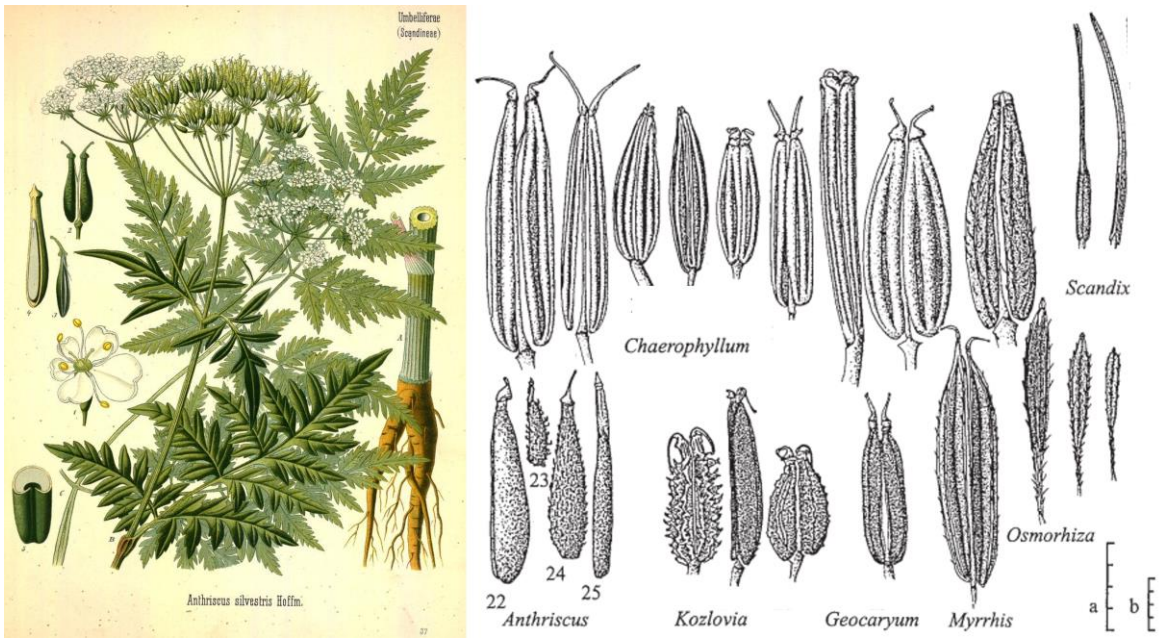

Figure 2. Botanical illustration of *Anthriscus sylvestris* (Köhler, 1887, left). Fruits of *Anthriscus* and some related genera. The bottom row shows the genera belonging to the 'crown clade'. 22, *A. lamprocarpa*; 23, *A. caucalis* var. *caucalis*; 24, *A. sylvestris* subsp. *nemorosa*; 25, *A. cerefolium* var. *cerefolium* (Spalik et al., 2001 edited, right).

The species belonging to the *Anthriscus* genus all have black, smooth, and shiny surfaces, and elliptically elongated or ovoid cremocarps. The basis for the determination between related taxa and species within the genus is provided by the morphology of the fruits. As it was mentioned above, *A. sylvestris* is closely related to *A. nitida*, but its pistils are less separated. Mostly perennial, in some cases biennial, its height can reach two meters. In addition to the height, it has the longest leaf blade among the Hungarian species, which is approximately

40 cm in length. Its stem is hairy at the nodes and at the parts closer to the ground. After the flowering period from May to August, it ripens in early autumn (Tekin and Civelek, 2017).

2.2 The endophytic fungal partners of *Anthriscus sylvestris*

2.2.1 The significance of the endophytic fungi in the ecosystem

Plants are complex multicellular organizations, which have evolved only a few times in some groups of organisms, such as metazoans, some groups of algae, or in the case of fungi (Nagy et al., 2018). The different belowground microorganisms, that are influenced by the biodiversity of plants can include fungi, bacteria, archaea, or even protists (Dassen et al., 2017). The symbiotic fungi living with plants can be grouped according to the place of colonization, so they can be epiphytic, close to or on the surface of the plant (rhizosphere or phyllosphere), and endophytic, within the plant tissue (Huang et al., 2024). The first definition of "endophyte" comes from Bary (1866), according to which all organisms growing inside plant tissue are endophytes. Later, over the years, many other, more and more precise definitions were created, e.g. Saikkonen et al., 1998. An endophyte is essentially an organism that colonizes, grows, and survives within plant tissues at a certain period of its life cycle without causing any harmful symptoms by its presence (Gouda et al., 2016).

2.3 The secondary metabolites in general

2.3.1 The main groups of the secondary metabolites

The different publications divide secondary metabolites into a variable number of main groups. The most important groups are 1) terpenoids (including steroids), 2) phenolics, 3) polyketides (including fatty acid derivatives), and 4) nitrogen-containing compounds (including alkaloids) (Thirumurugan et al., 2018; Rahman et al., 2023). The first one is the group of terpenoids, which includes also plant essential oils important in chemical communication and defense among others. The second group is the phenolics, which are extremely diverse and have many molecules with different structures. The larger, more complex molecules include tannins and lignins, which can be produced by many organisms such as plants, bacteria, or even fungi (Chomel et al., 2016). These compounds can reach up to 25% of the dry plant matter (Hättenschwiler and Vitousek, 2000). Phenolics also include lignans with significant physiological effects or flavonoids known for their antioxidant properties (Kessas et al., 2024). Nitrogen-containing substances include among others many toxic plant alkaloids. In many cases, these have deterrent properties of grazing produced against herbivorous animals (Faeth and Saari, 2012), but they can also play a role in protection against infections (Chomel et al.,

2016; Rahman et al., 2023). In the following, I will present some families of compounds that may be worth mentioning in terms of the examined plant of the Thesis (*A. sylvestris*) and possible endophytic fungi living with it.

2.3.2 Terpenoids

The terpenes are lipophilic compounds that are built up from five-carbon isoprene (2-methylbuta-1,3-diene) units. Based on the number of isoprene units involved in the formation of their structure, terpenes can be classified into different groups (Nagegowda and Gupta, 2020). Based on these, we can distinguish monoterpenes (C_{10}) formed from two isoprene molecules, or sesquiterpenes (C_{15}) containing three units. The compounds belonging to these groups are in many cases volatile substances and components of the essential oil of some plants. In addition, there are diterpenes (C_{20}), triterpenes (C_{30}), tetraterpenes (C_{40}), or polyterpenes $(C_5)_n$ formed from n-numbered isoprene units (Dubey et al., 2003).

The formation of terpenes is preceded by two biosynthetic pathways, in which processes many enzymes are involved and intermediate products are created (Tholl, 2015). One is the cytosolic mevalonate (MVA) pathway, which results in isopentenyl diphosphate (IPP). The other pathway is the 2-C-methylerythritol-4-phosphate (MEP) pathway, taking place in the plastid, in which dimethylallyl diphosphate (DMAPP) and IPP are produced. These compounds can be considered as the precursors for the formation of terpenes (Nagegowda, 2010). In the following steps, the basic compounds of each terpene family are formed by connecting different numbers of IPP molecules. These basic compounds are transformed by terpene synthases (TPS) into terpenes, which in many cases will be transformed into biologically functioning (active) products by secondary enzymatic steps such as hydroxylation, peroxidation, or methylation (Tholl, 2015).

Terpenes have several significant physiological and outstanding effects in medicine. An example of this is the antibacterial effect of terpenes containing oxygen (terpenoids) against Gram-negative species (Guimarães et al., 2019). It may be worth mentioning the antiviral effect of some terpenes, as they can inhibit the replication of SARS (severe acute respiratory syndrome) viruses (Loizzo et al., 2008). In addition, antimalarial (Nogueira and Lopes, 2011), anti-depressant (Saki et al., 2014), or tumor-inhibiting (Sobral et al., 2014) properties are also known and widely researched.

2.3.3 Phenolics in general

Phenolic compounds can be grouped according to several criteria, but one of the basic characteristic features is the presence of at least one hydroxylated aromatic ring (Chomel et al., 2016). Based on their chemical structure, they can be classified into several subgroups, including phenolic acids, flavonoids, coumarins, tannins, or lignans. It is worth mentioning the two subgroups of phenolic acids, hydroxybenzoic acids (C_6-C_1) and hydroxycinnamic acids (C_6-C_3), the latter group providing the phenylpropanoid backbone (Neelam and Sharma, 2020).

2.3.4 Lignan subgroup of phenolics

Lignans are phenylpropanoid dimers linked together by their propyl side chains (ß-ß' linkage). In the joining molecules, the degree of oxidation on the side chains and the substitution patterns of the phenyl rings can be different and highly variable. According to one classification, lignans can be divided into eight subgroups: furofurans, furans, dibenzylbutanes, dibenzylbutyrolactones, aryltetralins, arylnaphthalenes, dibenzocyclooctadienes and dibenzylbutyrolactols (Fang and Hu, 2018). For some groups, an important aspect is the absence of oxygen at C9 (C9') or the presence of additional hydroxyl groups, such as in the case of podophyllotoxin at the C7 (C7') position (Umezawa, 2003), which is the most important molecule among the aryltetralin lignans in terms of human health (Federolf et al., 2007).

The basic units of lignan-type compounds are the so-called phenylpropane units, which are produced in the phenylpropanoid pathway. Through the biosynthetic pathway, after several intermediate steps, coniferyl alcohol is formed (Umezawa, 2003). The first lignan-type compound is pinoresinol biosynthesized by the dimerization of two coniferyl alcohols (Davin et al., 1997). The process in which pinoresinol is formed involves an oxidation reaction catalyzed by the pinoresinol synthase (PS) enzyme. In the general biosynthetic pathway of plant-derived lignans, pinoresinol is further reduced first to lariciresinol and later to secoisolariciresinol. Finally, secoisolariciresinol is oxidized to matairesinol in the presence of secoisolariciresinol dehydrogenase (SDH) enzyme (Khaled et al., 2013). Matairesinol is then converted to (-)-deoxypodophyllotoxin (DPT) through several intermediate steps by cytochrome P450s (CYPs), *O*-methyltransferases (OMTs) and 2-oxoglutarate/Fe-dependent dioxygenase (2-ODD). The 2-ODD enzyme catalyzes the transformation of (-)-yatein to (-)-deoxypodophyllotoxin (Federolf et al., 2007; Chen et al., 2021). For examination of the previous process, mayapple (*Podophyllum hexandrum*) was used to study the biochemical pathway (Lau and Sattely, 2015). The C7 hydroxylation of (-)-deoxypodophyllotoxin to (-)-podophyllotoxin (PTOX) is not fully examined (Federolf et al., 2007), but research in this

direction has already demonstrated the accumulation of some epimers of PTOX, such as (-)-epipodophyllotoxin (Lau and Sattely, 2015).

It is important to mention the anti-inflammatory effect of DPT, as it can inhibit the activity of two enzymes, cyclooxygenase-2 (COX-2) and arachidonate 5-lipoxygenase (5-LOX), thus inhibiting the production of prostaglandin D_2 in a dose-dependent manner (Lee et al., 2004). The PTOX is a component of many medicinal products as an antiviral agent. Among others, it can be used in the treatment of *Condyloma acuminatum* caused by the HPV papillomavirus, and in the case of other venereal warts (Gordaliza, 2007). The cell division inhibiting effect of DPT has been known for a long time. Nakano et al., 1998 found that the IC_{50} values of the raw root and herb extracts of *A. sylvestris* were below 2 μg/mL for all the investigated cancer cell lines. However, it should be highlighted that DPT is the most cytotoxic component of *A. sylvestris* (Lim et al., 1999) and that DPT and its derivatives have extremely low selectivity between various healthy and cancer cells (Orčić et al., 2021). PTOX binds to tubulin and inhibits the formation of the mitotic spindle, thus subsequently causing cell death. The semi-synthetic preparations derived from it, such as etoposide and teniposide (chemotherapeutics), can bind to the DNA-topoisomerase II complex responsible for DNA cleavage, thus inhibiting mitosis and cell division (Orčić et al., 2022).

2.3.5 Flavonoid subgroup of phenolics

Flavonoids are an extremely diverse group of secondary metabolic products with thousands of known chemical structures (Martens and Mithöfer, 2005). Polyphenol type compounds, their basic structure consists of a C_6-C_3-C_6 arranged ring system containing 15 carbon atoms (Chomel et al., 2016). Based on the modifications of the central carbon ring, we can distinguish several subgroups, these are flavanones, flavones, isoflavones, flavonols, flavanonols, flavan-3-ols and anthocyanins. It is a known fact that members of the Apiaceae family store flavonoids, mainly in the form of flavones and flavonols (Gebhardt et al., 2005).

The biosynthesis of flavonoids begins with the condensation reaction of one *p*-coumaroyl-CoA and three malonyl-CoAs, a process that leads to the formation of the first flavonoid-type compound, naringenin, through a chalcone intermediate (Martens and Mithöfer, 2005). Naringenin belongs to the group of flavanones from which flavones (such as apigenin) are formed, catalyzed by the flavone synthase (FNS) enzyme. Flavanonols or dihydroflavonols are also formed from flavanones in the presence of the flavanone 3ß-hydroxylase (FHT) enzyme. A compound that belongs to this group is dihydrokaempferol (Gebhardt et al., 2005). Another side branch is flavonols formed from dihydroflavonols with the contribution of the

flavonol synthase (FLS) enzyme, such as kaempferol. Continuing the descent of compounds, the dihydroflavonol-4-reductase (DFR) enzyme reduces dihydroflavonols to leucoanthocyanidins, which are further synthesized by the anthocyanidin synthase (ANS) enzyme to anthocyanidins (Gebhardt et al., 2005; Liu et al., 2021). This type of pigment is pelargonidin (Martens and Mithöfer, 2005; Liu et al., 2021).

Flavonoids have many different physiological effects, they have many pharmacological and medicinal uses (Karak, 2019). Among others, their antioxidant effect is one of the most well-known (Mira et al., 2002). This also includes the ability of free radical scavenging, which protects cells from the destructive effects of ROS (Dorta et al., 2008). The antiproliferative effects of certain flavonoids on cancer cell lines have been demonstrated (Zhang et al., 2014; LeJeune et al., 2015). In addition, the anti-inflammatory effects through the nitric oxide (NO) scavenging (Derouich et al., 2020) or the antiviral effects of certain bioflavonoids should be mentioned (Zandi et al., 2011).

2.3.6 Secondary metabolites and medicinal importance of *A. sylvestris*

Among the secondary metabolic products found in the cow parsley, it is worth mentioning the various lignan derivatives. Combining the liquid chromatography, electrospray ionization mass spectrometry (LC–ESI-MS/MS), and nuclear magnetic resonance spectroscopy (NMR) techniques, many significant compounds were detected in the root extract of the plant samples. In a publication, nine of the fourteen detected metabolites were lignans. Among these lignans, it is worth mentioning the dibenzylbutyrolactone anhydropodorhizol and yatein, and the aryltetralin deoxypodophyllotoxin. Deoxypodophyllotoxin is considered much more widespread in plants than the podophyllotoxin, which was also detectable (Hendrawati et al., 2011). Aryltetralin-type lignans have cytotoxic and cell growth inhibitory properties against certain cell lines, thus proving effective in the fight against various types of cancer (Wang et al., 2019). In addition, deoxypodophyllotoxin can be used as a precursor in the semi-synthetic production of the anticancer drugs etoposide and teniposide.

It is also worth mentioning the presence of essential oil components of *Anthriscus sylvestris* in the tissues of the plant, a group of compounds that is very common in other members of the Apiaceae family as well. Fresh leaf and root samples of the plant were examined together, after hydrodistillation, using gas chromatography-mass spectrometry (GC-MS) technology, result in the identification of many terpene-type compounds. The proportion of monoterpenes in the examined fractions was the highest in both leaf and root samples, the most dominant compound was beta-phellandrene. In addition, it is worth mentioning the beta-

myrcene and sabinene content of the leaf or the beta-ocimene and alpha-pinene content of the root (Bos et al., 2002). Many physiological effects and medicinal significance of volatile components have been described in various scientific publications over the years. In the case of the aforementioned phellandrene, for example, the pesticide effect of the compound has been demonstrated, a process that based on a genetic level toxicity, gene mutation and chromosome aberration (Cheng et al., 2017).

Interesting results were also obtained by examining the flavonoid-type compounds of the plant. The isoflavonoid and flavonoid contents of several species of the Apiaceae family were investigated together using high-performance liquid chromatography-mass spectrometry (HPLC-MS) method. In the case of *Anthriscus sylvestris* leaf extracts, isoformononetin was the most abundant isolated compound, but daidzin and genistin were also detected. In the case of flavonoids, rutin was the main component of the leaves (Abdulmanea et al., 2012). In a recent study, rutin was shown to have many positive physiological effects. In the examination, the neurotoxic effect of the molecule was investigated, but in addition, the antioxidant, anti-inflammatory, and antimicrobial properties of rutin should also be mentioned (Kessas et al., 2024).

2.3.7 Secondary metabolites and medicinal importance of endophytes

The study of compounds of endophytes with biologically interesting activity has greatly increased in the last few decades. Several publications deal with the ability of endophytes to produce secondary metabolic products. The compounds produced can be grouped into different classes, such as alkaloids, polyketides, terpenoids, and phenolics (e.g., flavonoids, and lignans) (Guo et al., 2008). In a publication highlighted that the podophyllotoxin compound produced by two *Fusarium* species, important in cancer research. These two species were isolated from the different parts of the Chinese medicinal plant, *Dysosma versipellis*, also known as *Podophyllum versipelle* (Tan et al., 2018; Biswas et al., 2020). Podophyllotoxin, which compound is also a well-known, researched metabolite of *Anthriscus sylvestris* (Hendrawati et al., 2011). In addition, many other research deals with the metabolite-producing effects of endophytes, such as taxol produced by the endophyte *Taxomyces andreanae*. The endophyte capable of producing taxol and related compounds was isolated from the inner bark of *Taxus brevifolia* plant (Stierle et al., 1993). The metabolites of *Fusarium* species have shown additional medicinal importance through their immunosuppressive activity or their use against intravascular thrombosis (Lee et al., 1995; Wu et al., 2009). Metabolites of endophytes with antiproliferative effects are investigated in many studies. An example is vermelhotin, a

compound that has shown considerable effects against several cancer cell lines, and which is also produced by the *Flavomyces fulophazii* (grass root endophyte), native to Hungary (Berek-Nagy et al., 2021). Endophytes belonging to the family Clavicipitaceae have already been shown to be capable of producing various ergot alkaloids, compounds that are significant from the point of view of LSD (Ashfaq et al., 2024, pages 241-257).

3. Objectives of the Diploma Work

- Isolation of the endophytic fungi species living within the root of *Anthriscus sylvestris*, and identification by ITS barcoding.
- Determination of the secondary metabolites of the root and leaves tissue extracts of *A. sylvestris*, using HPLC-MS technology.
- Determination of the secondary metabolites of the endophytic fungi species, isolated from the root of *A. sylvestris*, using HPLC-MS technology.

4. Materials and Methods

4.1 Preparation of biological samples prior to examinations

4.1.1 The plant samples, preparation of *A. sylvestris*

The root and leaf samples of *Anthriscus sylvestris* were collected in Farkasvölgy (Budapest, Hungary) on October 29, 2023. The preparation work was carried out at the Department of Plant Anatomy (Eötvös Loránd University, Budapest, Hungary). Four independent plants could be separated in the collected biological material. The roots and leaves were cut into a few cm long pieces (this step was preceded by the selection and separation of some root samples for fungal isolation, see in Chapter 4.1.2), and then spread on a metal tray, which is used to place the samples in the lyophilizer, freeze-drying process. In the study to examine the active ingredients of *Anthriscus sylvestris*, the LC-MS examination of the two plant extracts (leaf and root sample, respectively) was carried out on November 10, 2023.

4.1.2 The fungal samples, preparation of endophytic fungi

Both thinner and thicker root pieces from the *A. sylvestris* plant were used to isolate the different endophytic fungi species. The selected healthy segments were surface sterilized to remove all the other microbes of the roots, in order to only the endophytic fungi could be presented during the isolation. Four thinner (filamentous-like) and four thicker (several mm in diameter) pieces of the roots of all four plants were used. The procedure was performed under

a laminar-flow chamber, during which, in the first step, the roots were soaked in hydrogen peroxide (30%) for 30 seconds and then in ethanol (96%) for 60 seconds. After that, in two additional steps, the roots were washed in distilled water. The treated samples were cut into four, a few mm long pieces, and then placed on the opposite sides of the surface of the medium, at roughly equal distances from each other. For the media potato dextrose agar (VWR Internation, Belgium) with 10 μg/mL streptomycin was used in (90 mm diameter) Petri dishes.

After a few weeks, the morphologically identical or different fungal colonies with different growth rates appeared on the culture media. The fungi were transplanted onto 60 mm diameter Petri dishes of PDA. This methodology has resulted in 86 colonies, making sure, by several more transferation that the dishes contain one species. The final number of the examined isolates/colonies were 33, which were named in a uniform code system (P001 - P039). The morphologically uniform samples containing one species could be used to investigate the secondary metabolite content. In the case of endophytes, several LC-MS measurements (adjusted to the growth of morphologically identical fungi) were examined on January 25, 2024 and on March 7, 2024.

4.2 Preparation of biological samples for metabolite examination

Following the preparation procedures discussed in the previous Chapter 4.1, the biological samples (*A. sylvestris* root and leaf samples, endophytic fungal colonies) were used in the preparation process before metabolite determination. The freeze-drying procedure was preceded by pre-freezing at -20°C for one or two days. After lyophilization, the samples were pulverized using an electric coffee grinder. Using an analytical balance, 20.0 mg of homogenized samples were measured from the powdered samples into 4 mL glass screw-thread vials (separately examining the root and leaf parts, and the endophytes coded P001 … P039). After that, 3 mL of methanol was added to the pulverized samples, and then they were heated at 60°C on a heating plate for 30 minutes. The extraction of the active ingredient accelerated by heating was followed by a centrifugation step on 3600 r.p.m for 2 minutes, after which 200 μL of the supernatants were pipetted into the 1.5 mL sampling vials (equipped with restrictors), compatible with the vial holders of the HPLC automatic sampling unit. During the LC-MS step (the detailed structure and settings of the equipment can be seen in Chapter 4.3), the Gemini15cm20DIA method was used.

4.3 General experimental procedures for metabolic examination

Analytical HPLC with UV and high-resolution Orbitrap mass spectrometry: A Dionex Ultimate 3000 UHPLC system (3000RS diode array detector (DAD), TCC-3000RS column thermostat, HPG-3400RS pump, SRD-3400 solvent rack degasser, WPS-3000TRS autosampler) connected to an Orbitrap Q Exactive Focus Mass Spectrometer equipped with electrospray ionization (ESI) (Thermo Fisher Scientific, Waltham, MA, USA) was used. Column: Gemini NX-C18 column (150 × 3 mm; 3 μm) (Phenomenex, Torrance, CA, USA). Eluents: eluent A, 0.1% v/v formic acid, eluent B, acetonitrile:0.1% v/v formic acid (80:20, v/v). Gradient program 1: 0.0 min, 20% B; 15.0 min, 90% B (linear gradient); 18.0 min, 90%B (isocratic); 19.0 min, 20% B (linear gradient); 21.0 min 20% B (isocratic). Flow rate: 0.4 mL/min; column temperature: 25 °C; injected volume: 1.0–10.0 μL. The ESI source was operated in the positive and negative ionization mode (switching mode) and operation parameters were optimized automatically using the built-in software. Working parameters: spray voltage, 3500 V (+), 2500 V (-); capillary temperature 256 °C; sheath-, auxiliary-, and spare-gases (N_2): 47.50, 11.25, and 2.25 arbitrary units, respectively. The resolution of the full scan was 70,000 and the scanning range was between 100–1500 *m/z* units. Fragmentations (MS/MS) were performed by data-independent acquisition (DIA) method at a resolution of 35,000 using isolation widths of 100–300 *m/z*, 295–500 *m/z*, 495–800 *m/z*, 795–1100 *m/z*, and 1095–1500 *m/z* and collision energy of 15, 30 and 45 eV. DAD spectra were recorded between 230 and 600 nm.

4.4 Preparation of biological samples for molecular identification

The DNA extraction step was preceded by a preparatory procedure. Reserve samples of each of the isolates ready for extraction were stored in 1 mL of diluted glycerol (50%) at -80°C. In the same step, a conservation transfer was made from the isolates to new Petri dishes (60 mm), and the segments of the colonies intended for DNA extraction were transferred to 1.5 mL Eppendorf tubes. Sterile sand was later added to the samples to crush the hyphae and the media.

For molecular phylogenetic identification, total DNA isolation/extraction was performed on mature P001 ... P039 (morphologically uniform) isolates using the E.Z.N.A.® Fungal DNA Mini Kit (Omega Bio-Tek, Norcross, GA, USA) following the instructions of the manufacturers. The nuclear rDNA internal transcribed spacer (ITS) region of the samples was amplified with the primer pair ITS1F (Gardes and Bruns, 1993) and ITS4 (White et al., 1990), using the DreamTaq polymerase (Thermo Fisher Scientific, Vilnius, Lithuania).

Using the polymerase chain reaction (PCR) process, a specific DNA section is multiplied, and many copies are made, in an *in vitro* environment, on non-living organisms. PCR requires the use of a DNA polymerase such as Taq polymerase (heat-stable) and DNA primers specifically designed for the section to be amplified (Wilkin, 2016). The first step is to prepare the PCR master mix required for the process based on the ITS PCR recipe. The ingredients required for one tube, 25 µL volume, are: 16 µL mQ water; 2.5 µL DreamTaq buffer; 2.5 µL dNTP (2 mM); 1.25 µL ITS1F (10 µM) and 1.25 µL ITS4 (10 µM) primer pair (Gardes and Bruns, 1993; White et al., 1990) and 0.25 µL DreamTaq polymerase. For the master mix, the amount of each component must be multiplied by the number of samples required based on the recipe, including the positive (previous sample, with working results) and negative (not containing template DNA) controls. The next step was to measure out the master mix into 0.2 mL PCR strip tubes, the amount required for one tube based on the recipe, and the 1.25 µL template (target) DNA samples. After that, the ITS sequences were amplified with a Tianlong Genesy 96T PCR device, using the "its-1f-4" program, with the following settings: initiation at 94°C for 5 minutes, in 1 cycle; denaturation at 94°C for 30 seconds, annealing at 52°C for 30 seconds, extension at 72°C for 40 seconds, the last 3 steps in 35 cycles; final extension at 72°C for 10 minutes and storage at 10°C for unlimited time and cycles. The finished samples are then stored at -20°C.

Gel electrophoresis is a technique suitable for separating different DNA fragments according to their size. The DNA samples (PCR products) have to be filled into the pockets created in the agarose gel (or polyacrylamide), and then by conducting current into the system, the negatively charged DNA sections will move towards the positive electrode and separate according to size (Wilkin, 2024). After performing the PCR technique, the successful amplification of the ITS sequences was checked on a 1.5% electrophoresis gel made of SeaKem® LE Agarose (Lonza, Rockland, ME, USA) and TBE buffer. ECO Safe Nucleic Acid Staining Solution was used to stain the gel, which is suitable for binding to the DNA chain. 1 Kb Plus DNA Ladder was used as a gel band control, and DNA 6X Gel Loading Dye (Thermo Fisher Scientific, Vilnius, Lithuania) was used to insert the DNA samples into the pockets. The gel was finally checked with UV light in a NuGenius imaging device.

Sequencing of the positive samples was carried out with the primers used for the amplification by LGC GmbH (Berlin, Germany). The sequences were compiled from electrophoregrams using the Pregap4 and Gap4 of the Staden software packages (Staden et al. 2000). The sequences obtained were compared to sequences in public databases using BLASTn searches (http://blast.ncbi.nlm.nih.gov/Blast.cgi) (Altschul et al., 1990). The first 5-10 of the

best hits were checked in case of each isolate. The sequences of the isolates were aligned using the online version of MAFFT 7 (Katoh and Standley, 2013) and the E-INS-i method. The alignments were examined and edited using MEGA 7 (Kumar et al., 2016). Maximum likelihood (ML) phylogenetic analysis was carried out with RAXMLGUI 1.3 (Silvestro and Michalak, 2012; Stamatakis, 2014). The GTR + G nucleotide substitution model was also used for nucleotide partitions with ML estimation of base frequencies. ML bootstrap (BS) analysis with 1,000 replicates was used to test the support of the branches.

5. Results and Discussion

5.1 Identification of fungal isolates

In this work, a total of thirty-three fungal isolates were identified among the endophytes from the roots of the *Anthriscus sylvestris* sample collected on November 10, 2023. The isolates are grouped into 7 clades (Fig. 22). Clade-1 contains 10 isolates most probably belonging to the genus *Dactylonectria*. Clade-2 contains 6 isolates most probably belonging to the genus *Neonectria*. Clade-3 contains 7 isolates most probably belonging to the genus *Ilyonectria*. Clade-4 contains 2 isolates most probably belonging to the genus *Paracylindrocarpon*. These four genera belon to the order Sordariales. Clade-5 contains 4 isolates most probably belonging to the order Diaporthales, probably representing a new lineage. Clade-6 contains 3 isolates most probably belonging to the genus *Pyrenochaeta* within the order Pleosporales. Clade-7 contains one isolate probably belonging to genus *Tetracladium* within the order Helotiales. These orders are known to contain root endophytic species (Sieber and Grünig, 2013; Knapp et al., 2012 & 2019).

5.2 Metabolites of the root of *Anthriscus sylvestris*

In the methanolic root extract of *A. sylvestris*, the most abundant presumable compounds (lignan-type) were deoxypodophyllotoxin (RT: 13.79 min), yatein (RT: 14.43 min), anhydropodorhizol (RT: 14.79 min) and angeloylpodophyllotoxin (RT: 17.01 min). The presence of the four lignans was also detectable in the methanolic leaf extract, but with a much lower intensity than in the case of the root sample. The HPLC-UV chromatogram (total 230-600 nm) and the total ion chromatogram (obtained by positive ionization) of the plant extracts can be seen in Fig. 3 and the high-resolution mass spectra of the main compounds in Fig. 4.

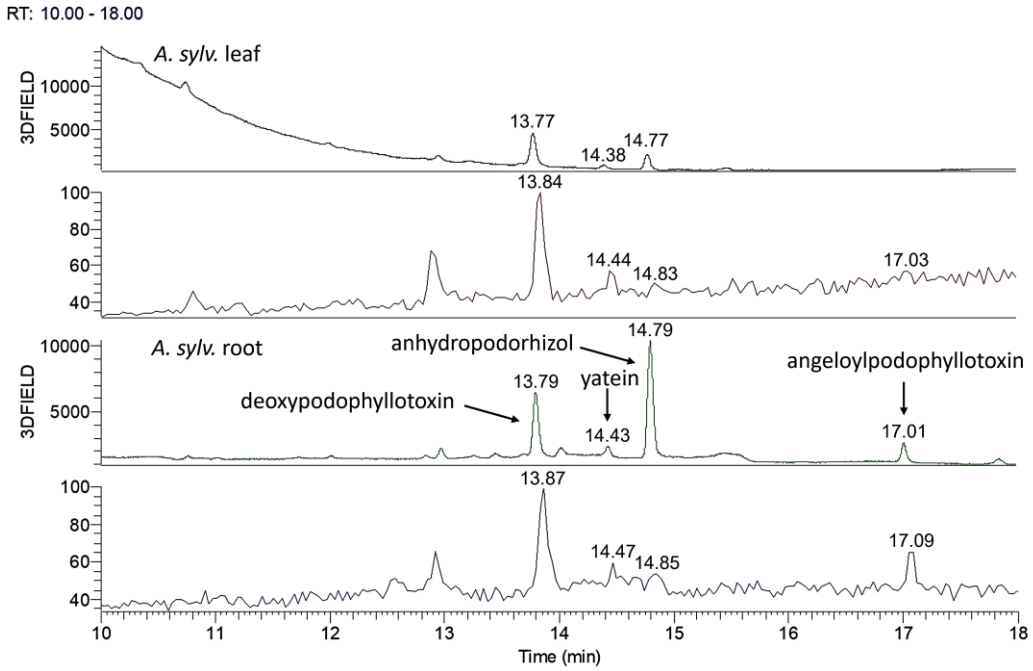

Figure 3. HPLC-UV chromatogram of the leaf and root extracts of *Anthriscus sylvestris*; total (230-600 nm) UV signal of the PDA detector, and total ion chromatograms obtained by positive ionization.

In the root extract, at the retention time of 13.79 min, the ions *m/z* 399.14 and *m/z* 416.17 can be detected by positive ionization (Fig. 4). These two ions correspond to the molecular formulas of $C_{22}H_{23}O_7$ and $C_{22}H_{26}O_7N$, respectively. These molecular formulas represent a protonated $[M+H]^+$ and an ammonium cationized $[M+NH_4]^+$ molecule having a molecular formula of $C_{22}H_{22}O_7$. Considering this molecular formula and the UV spectrum of this compound showing an absorption maximum of 292 nm (Fig. 5), we can conclude that this compound is the aryltetralin lignan deoxypodophyllotoxin (Kozawa et al., 1978).

At the retention time of 14.43 min and 14.79 min, the ions *m/z* 401.16 and *m/z* 399.14 resulted in the calculations of the molecular formulas of $C_{22}H_{25}O_7$ and $C_{22}H_{23}O_7$, respectively (Fig. 4). In addition to these ions, compounds at 14.43 min and 14.79 min retentions have ions *m/z* 418.18 and *m/z* 416.17, which correspond to the molecular formulas of $C_{22}H_{28}O_7N$ and $C_{22}H_{26}O_7N$ (Fig. 4). According to the calculated molecular formulas of compound at 14.43 min retention ($C_{22}H_{25}O_7$, $C_{22}H_{28}O_7N$) and those of compound at 14.79 min retention ($C_{22}H_{23}O_7$, $C_{22}H_{26}O_7N$), they can be detected as protonated $[M+H]^+$ and ammonium cationized $[M+NH_4]^+$ molecules. Consequently, the compounds at 14.43 min and 14.79 min retentions can be characterized with the molecular formulas of $C_{22}H_{24}O_7$ and $C_{22}H_{22}O_7$, respectively. The compound $C_{22}H_{24}O_7$ exhibits an absorption maximum of 288 nm in its UV spectra, while the compound $C_{22}H_{22}O_7$ exhibits an absorption maximum of 312 nm (Fig. 5). Comparing these

data of the compounds with those of the literature, the presence of two dibenzylbutyrolactone lignans yatein and anhydropodorhizol at 14.43 min and 14.79 min can be confirmed, respectively (Kozawa et al., 1978; Ikeda et al., 1998).

At the retention time of 17.01 min, the ions *m/z* 497.18 and *m/z* 514.21 can be detected by positive ionization, corresponding to the molecular formulas of $C_{27}H_{29}O_9$ and $C_{27}H_{32}O_9N$, respectively, which confirm a protonated and ammonium cationized molecule having a molecular formula of $C_{27}H_{28}O_9$. In addition, this compound exhibits an absorption maximum of 290 nm in its UV spectra. Based on this data of the compound at 17.01 min retention can be identified as the aryltetralin lignan angeloylpodophyllotoxin (Lim et al., 1999; Orčić et al., 2022). As further evidence of its identity, a high-intensity signal of the ion *m/z* 397.13 can be observed in the mass spectra of angeloylpodophyllotoxin due to the in-source fragmentation (i.e., fragmentation in the ion source) of this compound, providing evidence of the existence of the angeloyl unit in angeloylpodophyllotoxin (Fig. 4).

These four lignans can be considered as characteristic compounds of *A. sylvestris* plant (Orčić et al., 2022).

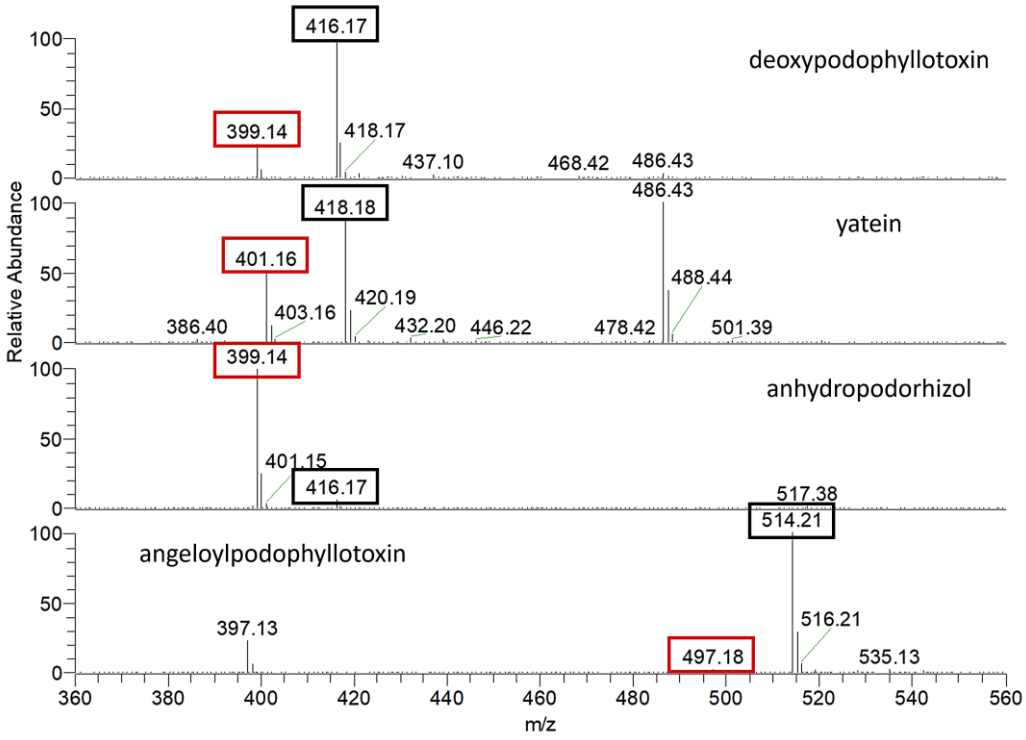

Figure 4. High-resolution mass spectra of the four assumed compounds of *Anthriscus sylvestris* root extract, obtained by positive ionization. Masses of the protonated molecules [M+H]+ given in Dalton, in the red, and the ammonium adduct ions [M+NH4]+ in the black frames.

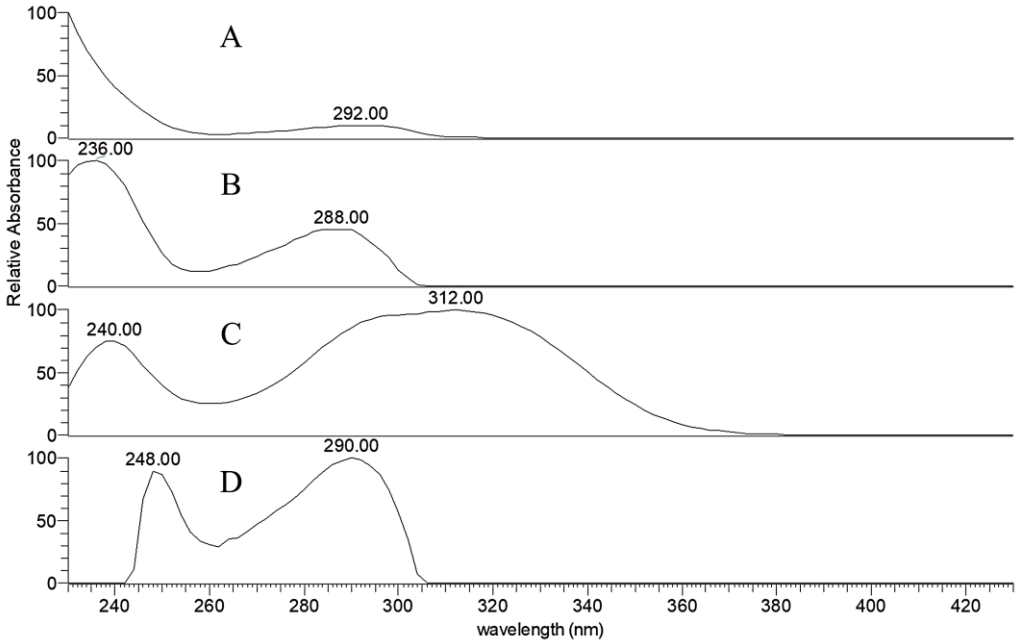

Figure 5. The UV spectra of compounds eluted at 13.79 min (A), 14.43 min (B), 14.79 min (C) and 17.01 min (D).

5.3 Metabolites of the isolates from clade-6

The most abundant presumable compounds detected in the methanolic extract of sample from P002 of the clade-6, probably belonging to genus *Pyrenochaeta* were monodictyochromone A and B (RT: 14.43 min and 14.94 min), phomopsidin (RT: 16.51 min). Monodictyochromone A and B (RT: 14.44 min and 14.95 min) also appeared in sample from P017, but phomopsidin was absent. In addition, nigrosporapyrone (RT: 6.67 min) and fusarpyrone B (RT: 6.80 min) were also detected. In sample from P039, all the previously mentioned compounds were detected. The HPLC-UV chromatograms (total 230-600 nm) of the clade-6 extracts can be seen in Fig. 6 and the high-resolution mass spectra (obtained by negative ionization) of the main compounds in Fig. 7.

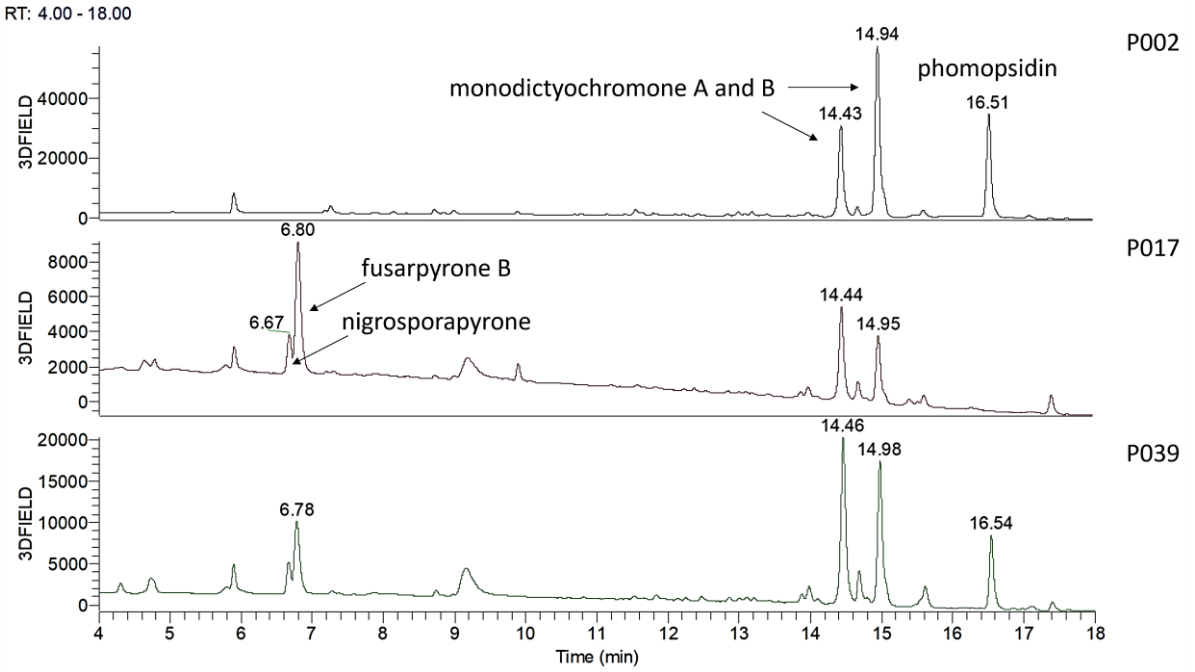

Figure 6. HPLC-UV chromatogram of the extracts of isolates: P002 (upper), P017 (middle), P039 (lower); total (230-600 nm) UV signal of the PDA detector.

In the extract of isolate P002, at the retention time of 14.43 min and 14.94 min, the ion *m/z* 565.17 by negative ionization (Fig. 7), and the ion *m/z* 567.19 by positive ionization can be detected. These two ions correspond to the molecular formulas of $C_{30}H_{29}O_{11}$ and $C_{30}H_{31}O_{11}$, respectively. These molecular formulas represent a deprotonated $[M-H]^-$ and protonated $[M+H]^+$ molecule having a molecular formula of $C_{30}H_{30}O_{11}$. Considering this molecular formula and the UV spectrum of these two compounds showing an absorption maximum of 350 nm (Fig. 8), we can conclude that these are the monodictyochromone A and B (Pontius et al., 2008). At the retention time of 16.51 min, the ion *m/z* 329.21 by negative ionization (Fig. 7), and the ion *m/z* 331.23 by positive ionization can be detected. These two ions correspond to the molecular formulas of $C_{21}H_{29}O_3$ and $C_{29}H_{31}O_3$, respectively. These two ions represent a deprotonated $[M-H]^-$ and protonated $[M+H]^+$ molecule having a molecular formula of $C_{21}H_{30}O_3$. Considering this molecular formula and the UV spectrum of this compound showing an absorption maximum of 266 nm (Fig. 8), we can conclude that this compound is the phomopsidin (Kobayashi et al., 2003).

In the extract of isolate P017, at the retention time of 6.67 min, the ion *m/z* 169.05 by negative ionization (Fig. 7), and the ion *m/z* 171.06 by positive ionization can be detected. These two ions correspond to the molecular formulas of $C_8H_9O_4$ and $C_8H_{11}O_4$, respectively. These two ions represent a deprotonated $[M-H]^-$ and protonated $[M+H]^+$ molecule having a molecular

formula of $C_8H_{10}O_4$,. Considering this molecular formula and the UV spectrum of this compound showing an absorption maximum of 260 nm (Fig. 8), we can conclude that this compound is the nigrosporapyrone (Trisuwan et al., 2009). At the retention time of 6.80 min, the ion *m/z* 167.03 by negative ionization (Fig. 7), and the ion *m/z* 169.05 by positive ionization can be detected. These two ions correspond to the molecular formulas of $C_8H_7O_4$ and $C_8H_9O_4$, respectively. These two ions represent a deprotonated $[M-H]^-$ and protonated $[M+H]^+$ molecule having a molecular formula of $C_8H_8O_4$. Considering this molecular formula and the UV spectrum of this compound showing an absorption maximum of 274 nm (Fig. 8), we can conclude that this compound is the fusarpyrone B (Trisuwan et al., 2013).

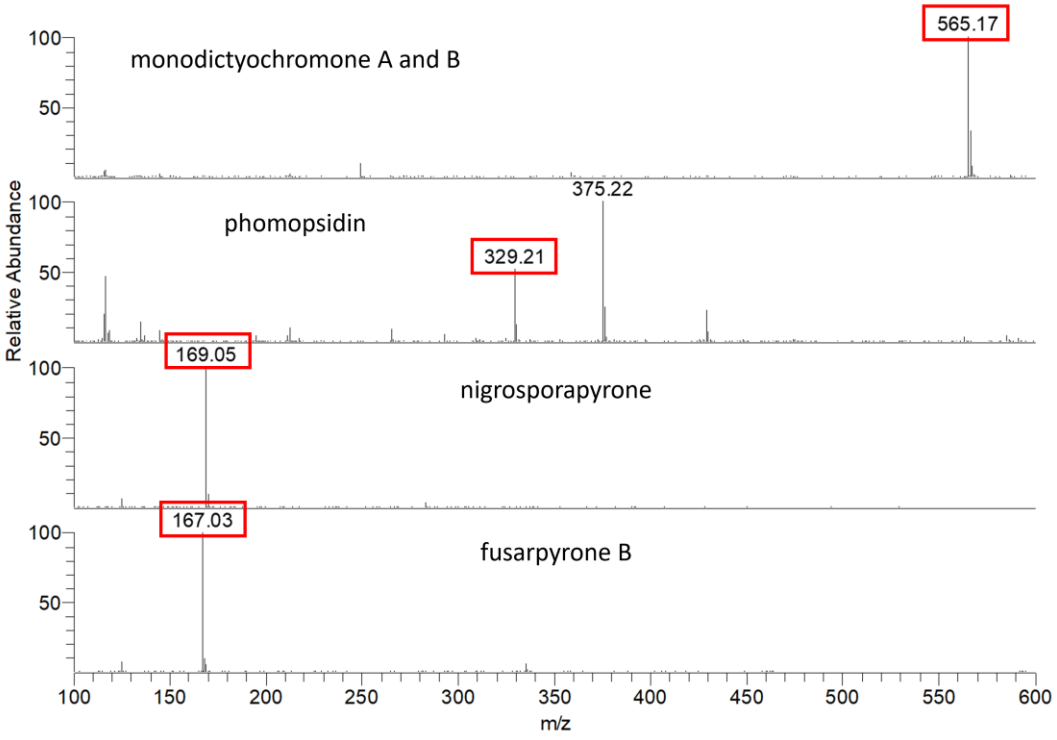

Figure 7. High-resolution mass spectra of the five assumed compounds of clade-6 extracts, obtained by negative ionization. Masses of the deprotonated molecules [M-H]- given in Dalton, in the red frames.

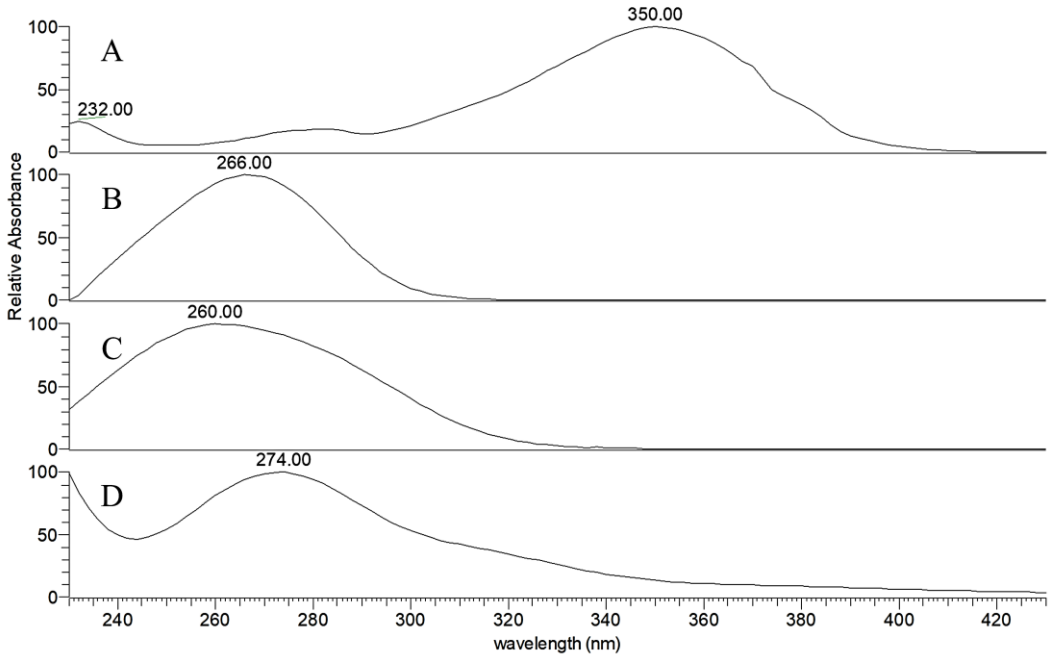

Figure 8. The UV spectra of compounds eluted at 14.43 min and 14.94 min (A), 16.51 min (B), 6.67 min (C) and 6.80 min (D).

5.4 Clade-1, clade-2, and clade-3

The three clades discussed in this chapter are grouped together in our phylogenetical analysis (ML-BS=79) (Fig. 22). Clade-1 contains two lineages (ML-BS=72) that probably belong to *Dactylonectria* represented by the isolates P003, P004, P019, P025-P028 and P038 in case of one lineage and P033, P009 in the other. The fully supported clade-2 contains P013, P014, P020, P021 and P031, where P013, P014, P020, P021 form a highly supported (ML-BS=99) group within the clade, that probably belongs to *Neonectria.* Clade-3 also contains two lineages: one is fully supported and is formed by isolates P011, P012, P032, P034, P036 and P037, the other lineage is represented by a single isolate: P010. The support of the latter isolate being part of this clade was low in the analysis, but based on the BLAST results all these isolates belong to *Ilyonectria,* therefore they were considered the same clade. All three genera are members of the Nectriaceae family.

5.4.1 Metabolites of the isolates from clade-3

The most abundant presumable compounds detected in the methanolic extract of sample from P010 of the clade-3 belonging to genus *Ilyonectria* were radicicol B (RT: 8.64 min), 8,9-dihydrogreensporone C (RT: 9.50 min) and radicicol (RT: 10.76 min). Radicicol B appeared with roughly equal intensity in all samples from this clade, the presence of 8,9-

dihydrogreensporone C was prominent only in sample from P010, while radicicol was also detected with equal intensity, except for sample from P011. The HPLC-UV chromatograms (total 230-600 nm) of the clade-3 extracts can be seen in Fig. 9 and the high-resolution mass spectra (obtained by negative ionization) of the main compounds in Fig. 10.

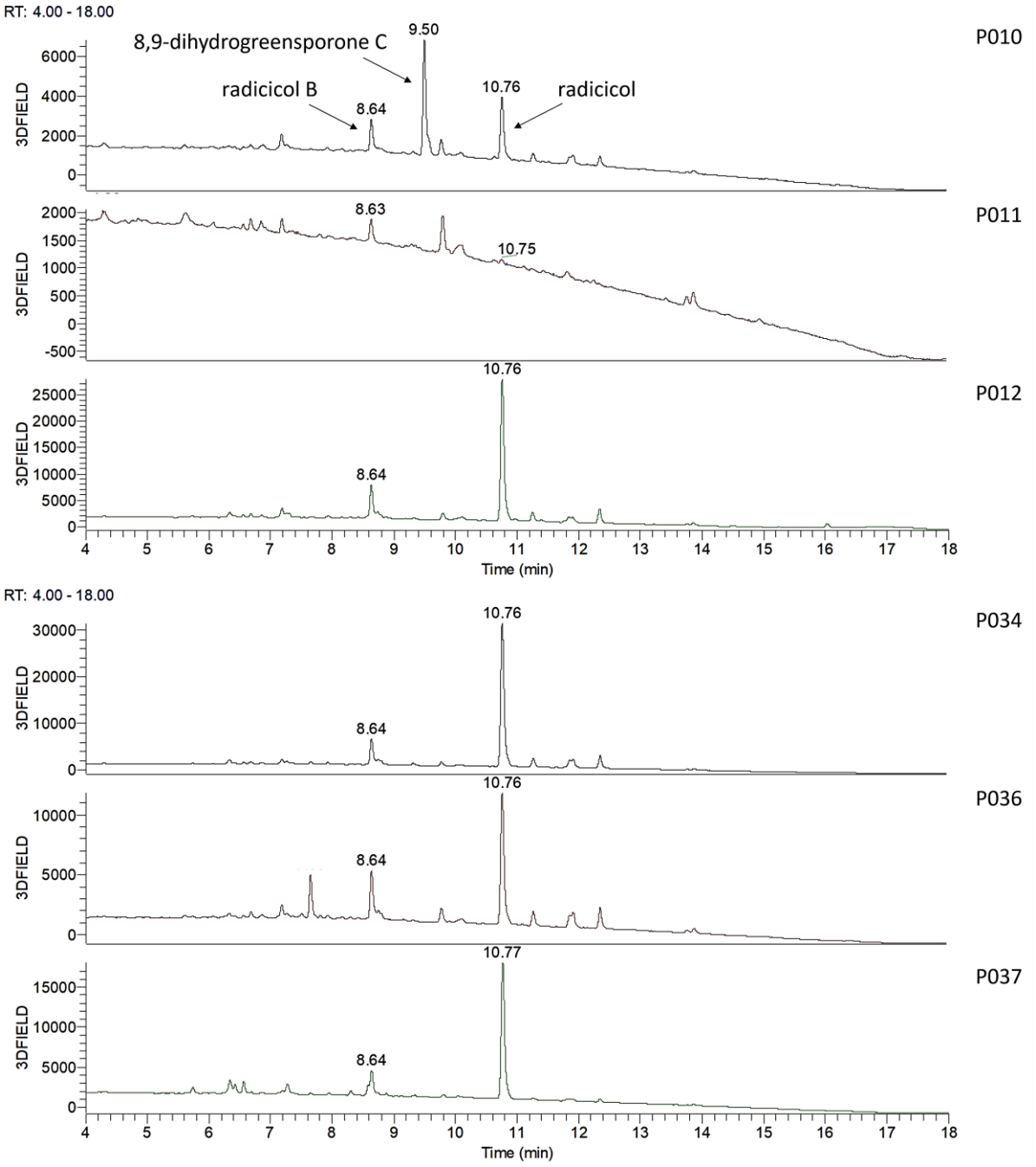

Figure 9. HPLC-UV chromatogram of the extracts of isolates: P010, P011, P012, P034, P036 and P037; total (230-600 nm) UV signal of the PDA detector.

In the clade-3 extracts, at the retention time of 8.64 min, the chloride-containing (because of the 3:1 intensity) ions *m/z* 381.08 and *m/z* 383.07 can be detected by negative ionization (Fig. 10), corresponding to the molecular formulas of $C_{18}H_{18}O_7Cl^{35}$ and $C_{18}H_{18}O_7Cl^{37}$, respectively, which confirm deprotonated $[MCl^{35}\text{-}H]^-$ and $[MCl^{37}\text{-}H]^-$ molecules. In addition to these ions, at the retention time of 8.64 min, the ions *m/z* 400.12, *m/z* 402.11, *m/z* 405.07 and *m/z* 407.07 can be detected by positive ionization, corresponding to the molecular formulas of $C_{18}H_{23}O_7NCl^{35}$, $C_{18}H_{23}O_7NCl^{37}$, $C_{18}H_{19}O_7Cl^{35}Na$ and $C_{18}H_{19}O_7Cl^{37}Na$, respectively, which confirm ammonium cationized $[MCl^{35}+NH_4]^+$, $[MCl^{37}+NH_4]^+$, and sodium cationized $[MCl^{35}+Na]^+$, $[MCl^{37}+Na]^+$ molecules. Consequently, the compound at 8.64 min retention can be characterized with the molecular formula of $C_{18}H_{19}O_7Cl$. The compound $C_{18}H_{19}O_7Cl$ exhibits an absorption maximum of 262 nm (Fig. 11) in its UV spectra. Based on the data of the compound at 8.64 min retention can be identified as the radicicol B (Mejia et al., 2014).

At the retention time of 9.50 min, the ion *m/z* 333.17 ion can be detected by negative ionization (Fig. 10), corresponding to the molecular formula of $C_{19}H_{25}O_5$, which confirm a deprotonated molecule having a molecular formula of $C_{19}H_{26}O_5$. In addition, this compound exhibits an absorption maximum of 238 nm (Fig. 11) in its UV spectra. Based on the data of the compound at 9.50 min retention can be identified as the 8,9-dihydrogreensporone C (Li et al., 2016).

At the retention time of 10.76 min, the chloride-containing ions *m/z* 363.06 and *m/z* 365.06 can be detected by negative ionization (Fig. 10), corresponding to the molecular formulas of $C_{18}H_{16}O_6Cl^{35}$ and $C_{18}H_{16}O_6Cl^{37}$, respectively, which confirm deprotonated $[MCl^{35}\text{-}H]^-$ and $[MCl^{37}\text{-}H]^-$ molecules. In addition to these ions, at the retention time of 10.76 min, the ions *m/z* 365.08 and *m/z* 367.08 can be detected by positive ionization, corresponding to the molecular formulas of $C_{18}H_{18}O_6Cl^{35}$ and $C_{18}H_{18}O_6Cl^{37}$, respectively, which confirm protonated $[MCl^{35}+H]^+$ and $[MCl^{37}+H]^+$ molecules. Consequently, the compound at 10.76 min retention can be characterized with the molecular formula of $C_{18}H_{17}O_6Cl$. The compound $C_{18}H_{17}O_6Cl$ exhibits an absorption maximum of 268 nm (Fig. 11) in its UV spectra. Based on the data of the compound at 10.76 min retention can be identified as the radicicol. Radicicol contains one less water (H_2O) molecule, compared to the radicicol B mentioned above (Mirrington et al., 1966; Torres Acosta et al., 2019).

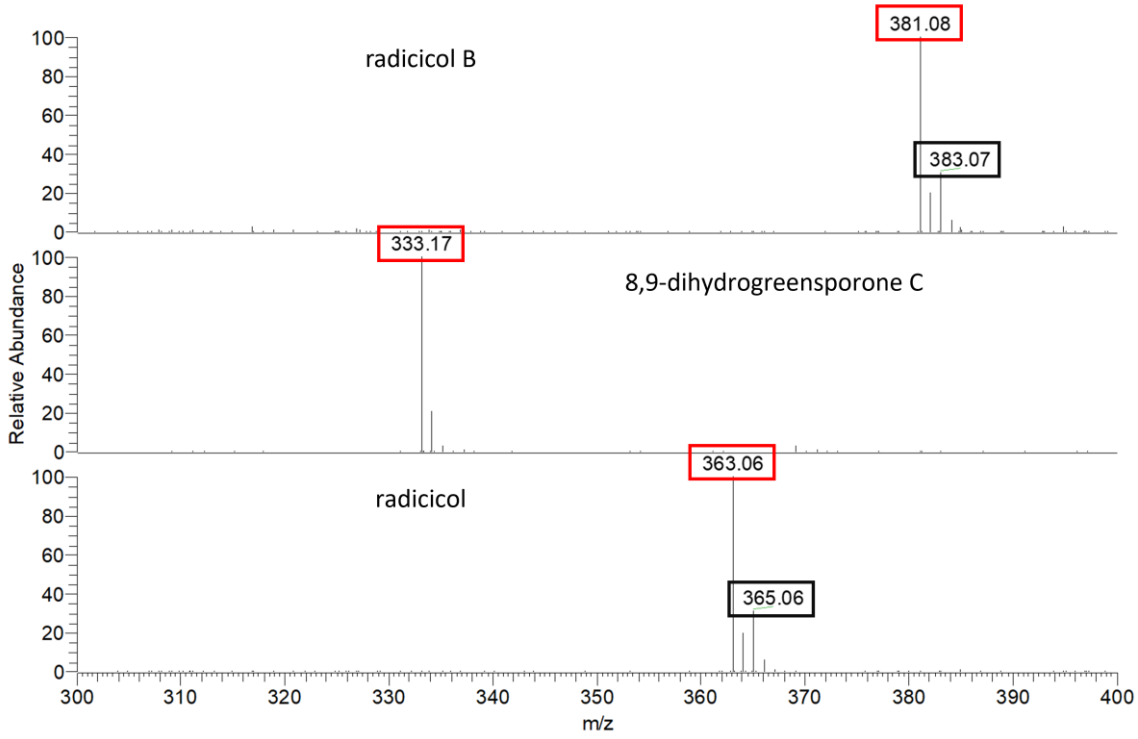

Figure 10. High-resolution mass spectra of the three assumed compounds of clade-3 extracts, obtained by negative ionization. Masses of the deprotonated, Cl^{35} isotope molecules $[MCl^{35}-H]-$ given in Dalton, in the red, and the Cl^{37} isotope molecules $[MCl^{37}-H]-$ in the black frames.

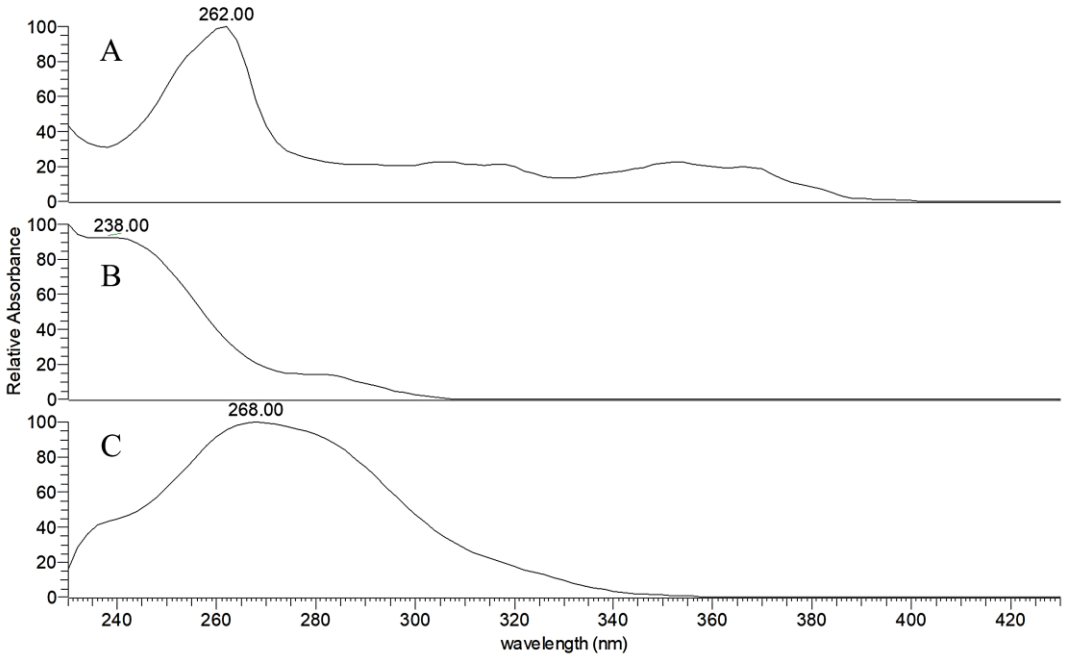

Figure 11. The UV spectra of compounds eluted at 8.64 min (A), 9.50 min (B) and 10.76 min (C).

5.4.2 Determination of brefeldin A in clade-1

The most abundant presumable compound detected in the methanolic extract of six isolates (P003, P004, P026, P027, P028 and P033) of clade-1 belonging to the genus *Dactylonectria* was brefeldin A (RT: 9.56 min). Brefeldin A appeared with roughly equal intensity in four isolates: P003, P004, P026 and P027, while it was detected with lower intensity in sample from P028. The HPLC-UV chromatograms (total 230-600 nm) of the clade-1 and clade-2 extracts can be seen in Fig. 12 and the high-resolution mass spectra (obtained by negative and positive ionizations) of brefeldin A in Fig. 13.

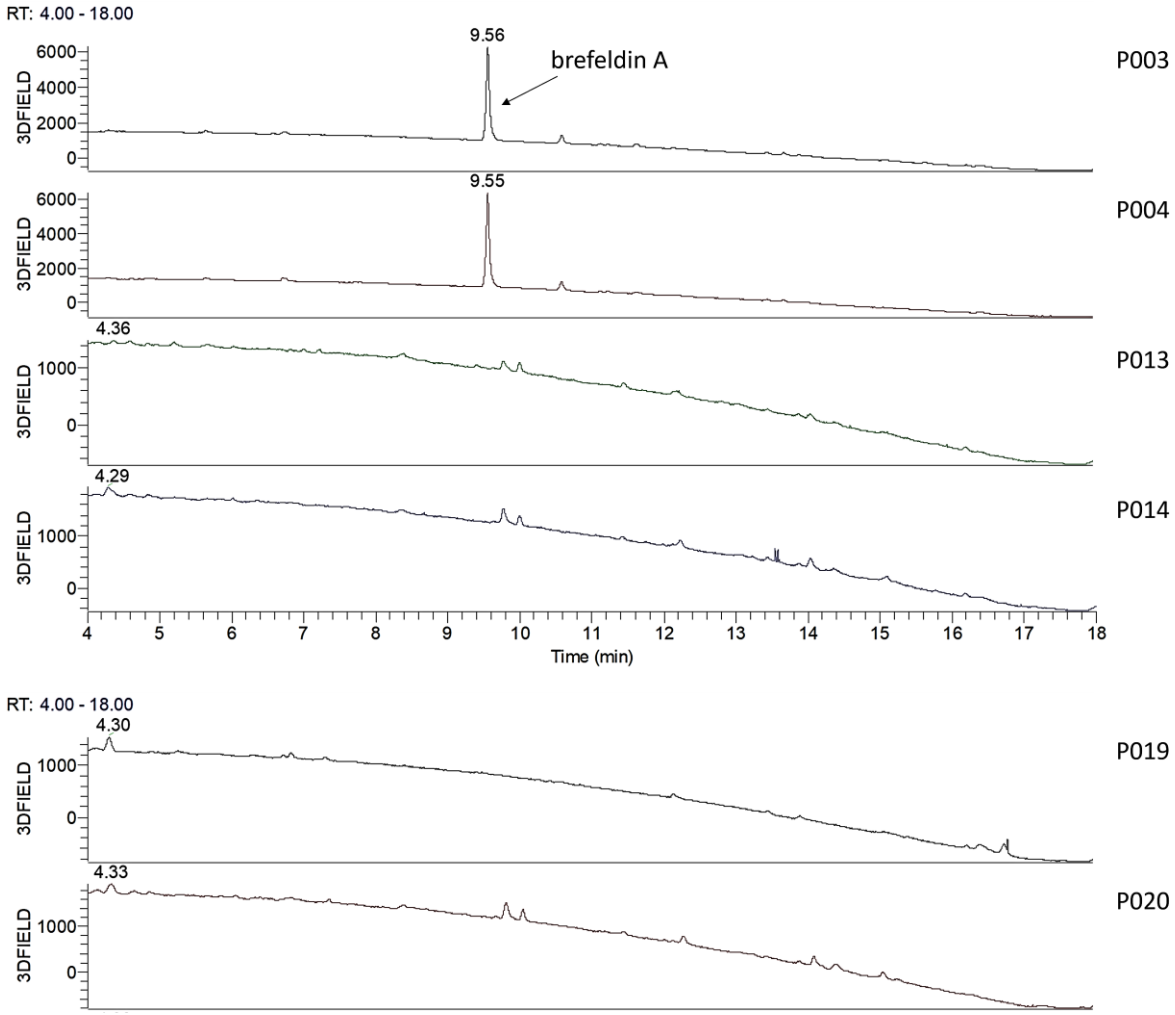

Figure 12. continues on the next page

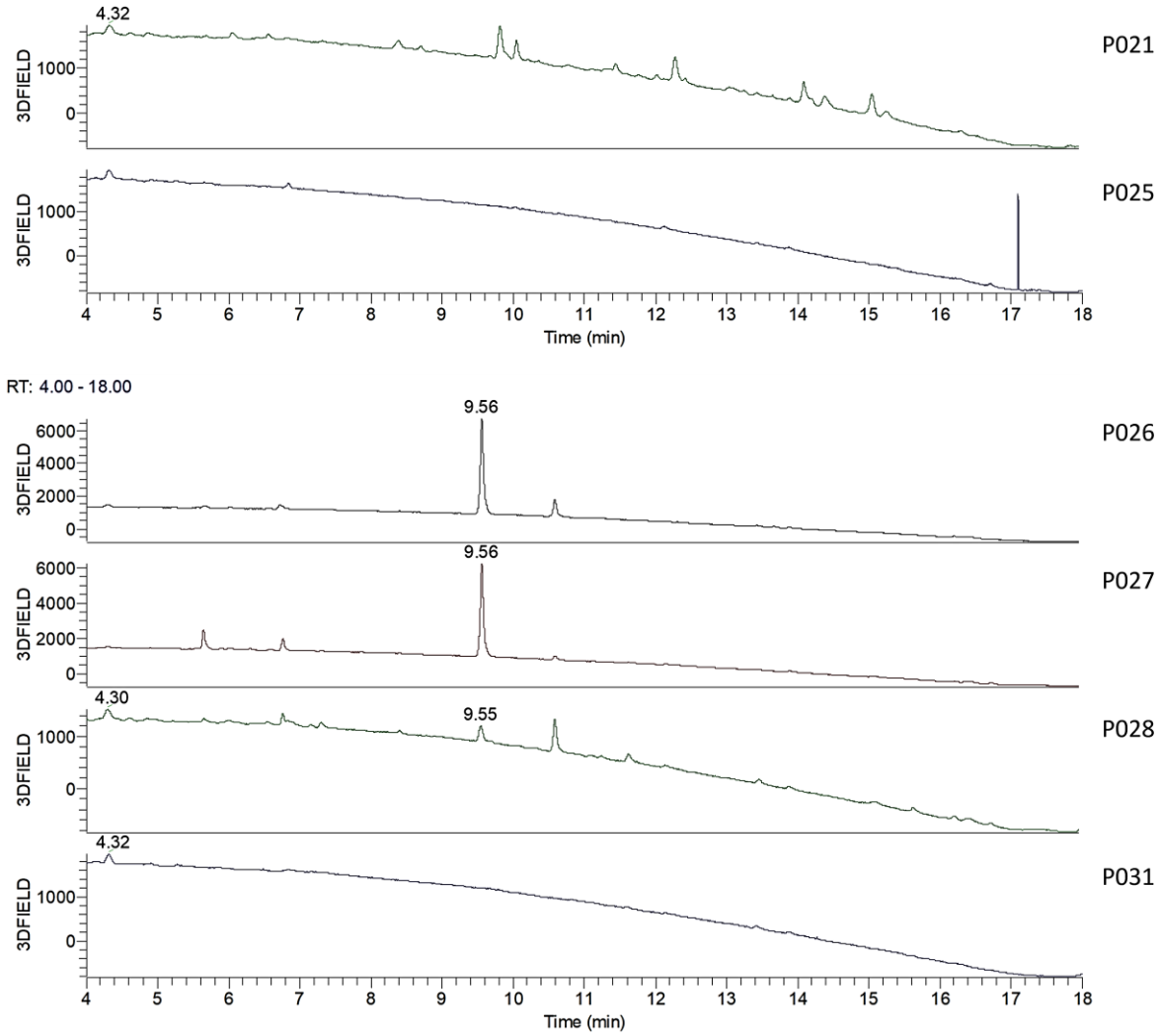

Figure 12. HPLC-UV chromatogram of the extracts of isolates: P003, P004, P013, P014, P019, P020, P021, P025, P026, P027, P028 and P031; total (230-600 nm) UV signal of the PDA detector.

In the extract of isolate P003, at the retention time of 9.56 min, the ion *m/z* 325.17 by negative ionization, and the ion *m/z* 281.17 by positive ionization can be detected (Fig. 13). These two ions correspond to the molecular formulas of $C_{17}H_{25}O_6$ and $C_{16}H_{25}O_4$, respectively. These molecular formulas represent a formate anionized $[M\text{-}HCOO]^-$ and protonated $[M+H]^+$ molecule having a molecular formula of $C_{16}H_{24}O_4$. The absorption maximum of brefeldin A falls in a too low wavelength range (comparing to total 230-600 nm) for detection, but according to the literature, the absorption spectrum of brefeldin A is roughly between 200-250 nm (Brüning et al., 1992). Based on the data of the compound at 9.56 min retention can be identified as the brefeldin A (Fang et al., 2006).

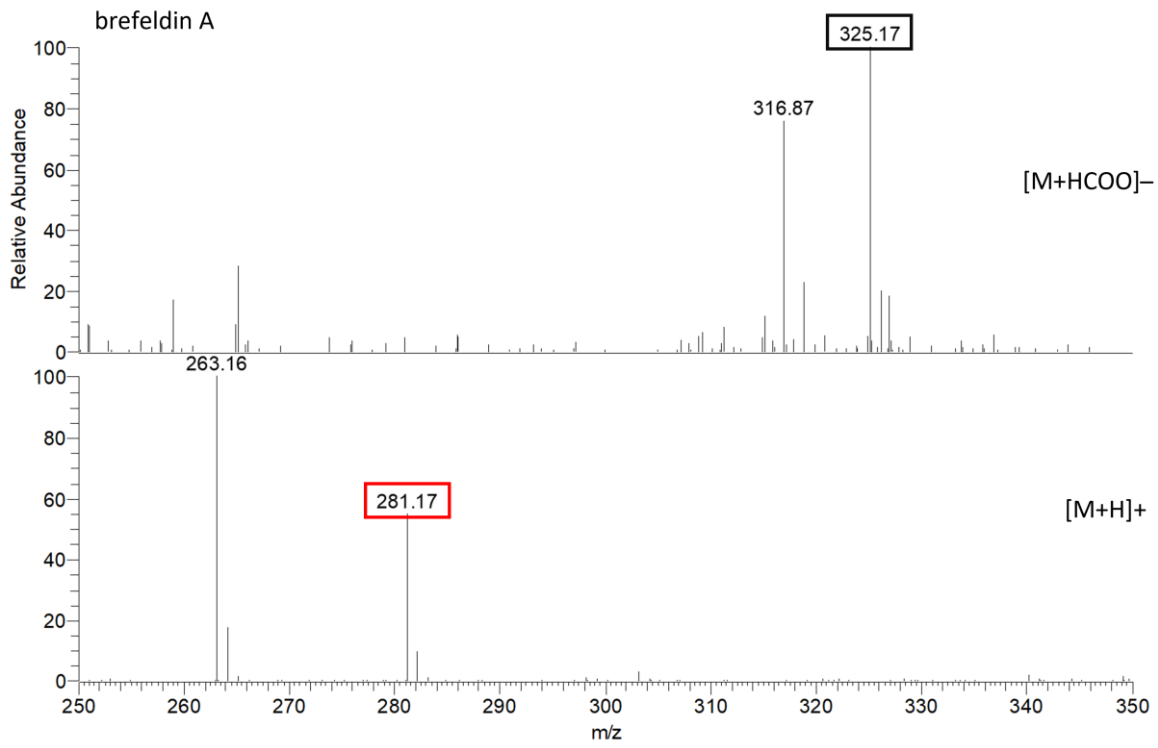

Figure 13. High-resolution mass spectra of the brefeldin A of clade-1 extracts, obtained by negative and positive ionizations. Masses of the deprotonated, formate adduct ion [M+HCOO]- given in Dalton, in the black, and the protonated molecule [M+H]+ in the red frame.

5.5 Determination of a new metabolite in clade-5

The most abundant presumable compound detected in the methanolic extract of four isolates (P005, P006, P007 and P008) of the clade-5, belonging probably to order Diaporthales was most probably a new metabolite (RT: 5.93 min). The new metabolite appeared with roughly equal intensity in samples from P005 and P006, while it showed a more prominent peak in sample from P008. The signal with the lowest intensity was detected in the case of sample from P007. The HPLC-UV chromatograms (total 230-600 nm) of the Diaporthales extracts can be seen in Fig. 14 and the high-resolution mass spectra (obtained by negative and positive ionizations) of the new metabolite in Fig. 15.

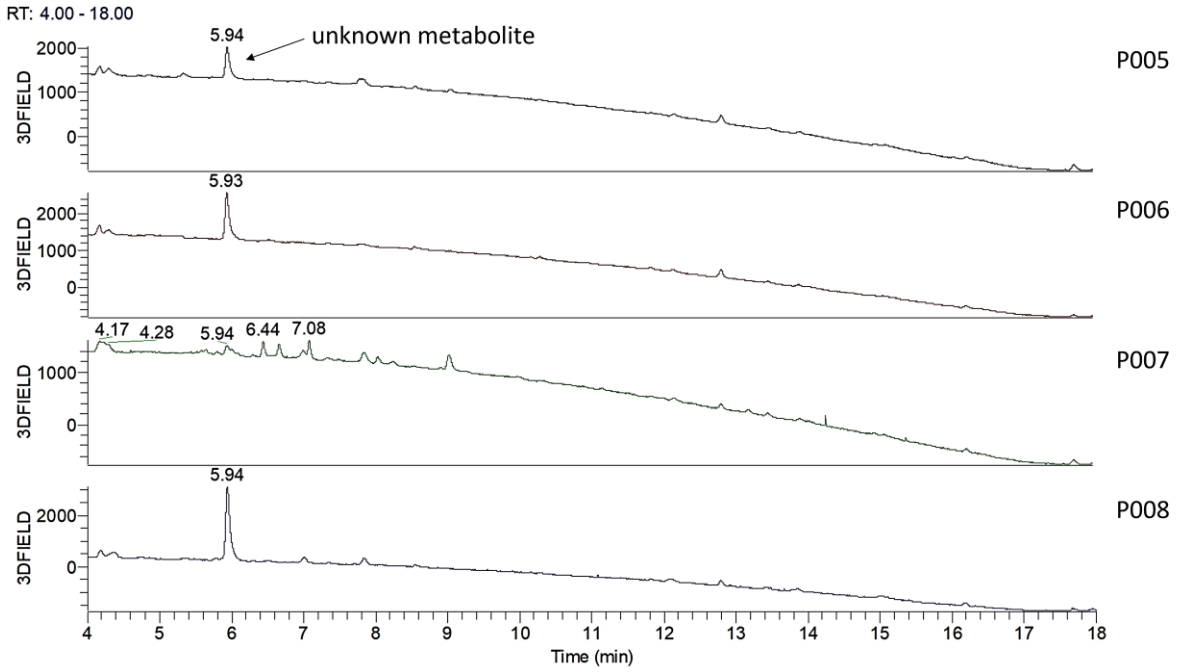

Figure 14. HPLC-UV chromatogram of the extracts of P005, P006, P007 and P008 isolates belonging to Diaporthales; total (230-600 nm) UV signal of the PDA detector.

In the clade-5 extracts, at the retention time of 5.94 min, the chloride-containing ions *m/z* 787.27 and *m/z* 789.27 can be detected by negative ionization (Fig. 15), corresponding to the molecular formulas of $C_{40}H_{48}O_{14}Cl^{35}$ and $C_{40}H_{48}O_{14}Cl^{37}$, respectively, which confirm deprotonated $[MCl^{35}\text{-}H]^-$ and $[MCl^{37}\text{-}H]^-$ molecules. In addition to these ions, at the retention time of 5.94 min, the ions *m/z* 789.28 and *m/z* 791.28 can be detected by positive ionization (Fig. 15), corresponding to the molecular formulas of $C_{40}H_{50}O_{14}Cl^{35}$ and $C_{40}H_{50}O_{14}Cl^{37}$, respectively, which confirm protonated $[MCl^{35}+H]^+$ and $[MCl^{37}+H]^+$ molecules. Consequently, the compound at 5.94 min retention can be characterized with the molecular formula of $C_{40}H_{49}O_{14}Cl$. The compound $C_{40}H_{49}O_{14}Cl$ exhibits an absorption maximum of 288 nm (Fig. 16) in its UV spectra. Based on the data of the compound at 5.94 min retention is most probably a new metabolite.

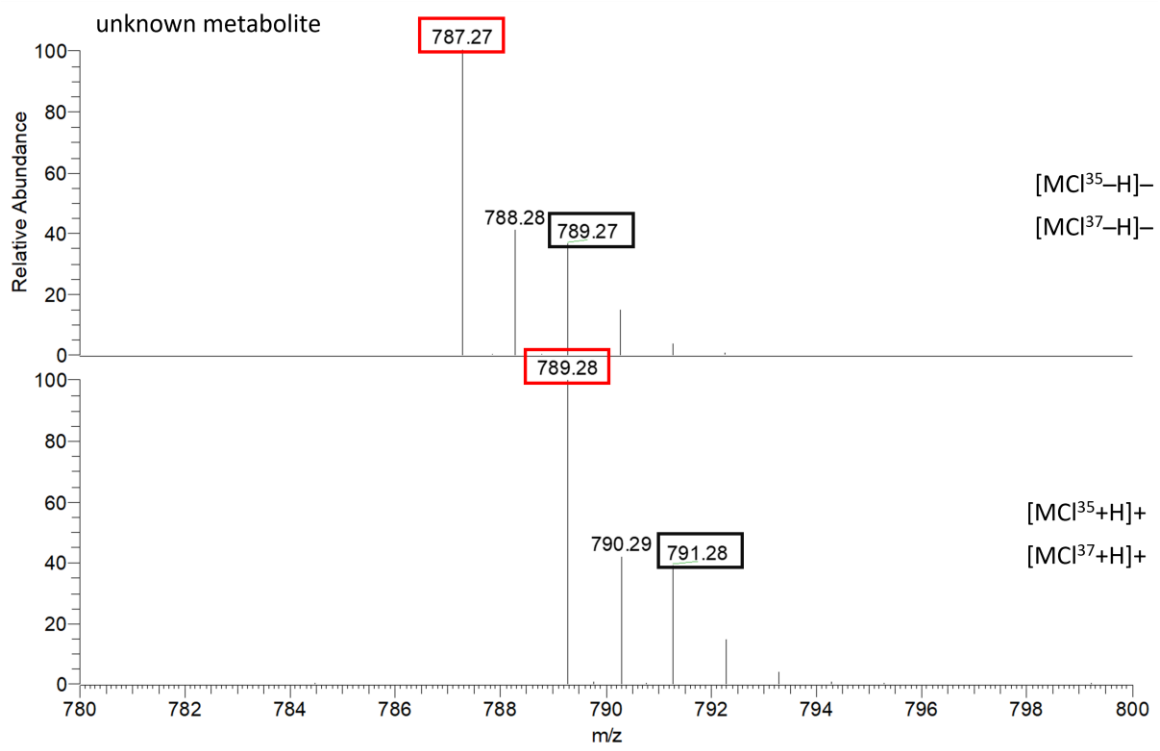

Figure 15. High-resolution mass spectra of a new metabolite of clade-5 extracts, obtained by negative and positive ionizations. Masses of the Cl^{35}-isotope containing [MCl^{35}-H]- and [MCl^{35}+H]+ ions given in Dalton, in the red, and the Cl^{37}-isotope containing [MCl^{37}-H]- and [MCl^{37}+H]+ ions in the black frames.

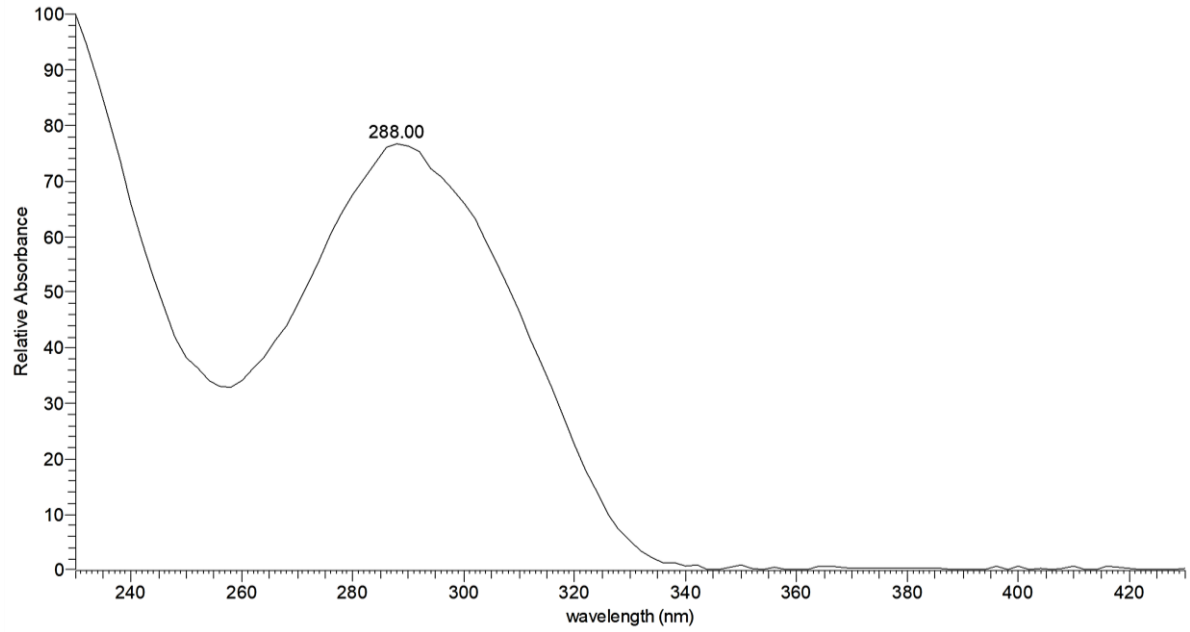

Figure 16. The UV spectra of compounds eluted at 5.94 min.

5.6 Consideration of further isolates

In the case of some samples, the molecular identification could not be conducted due to certain limiting factors (for example, too low or high concentration of the ITS sequence required for determination, or possibly mixed mycelia). This was the case with isolate P023, where the ITS region could not been amplified. However, at the retention time of 5.42 min, a compound shows a high intensity (unique among the tested samples), which is most probably the aspergillspin F. In some other cases, such as, the isolates P015 and P016 forming clade-4 could be identified, but did not show significant metabolite production. Isolates P033 and P009, which belong to the previously discussed clade-1 in genus *Dactylonectria* might represent a different lineage than the other isolates of this clade (Fig. 22). However, the electrophoregrams of the ITS regions sequenced for these isolates were of low quality. P033 produced the compound most probably determined as brefeldin A, supporting the claim that it belongs to clade-1 (Fig. 17). Based on the BLAST results, isolate P030 may belong to genus *Tetracladium*, or possibly a closely related taxon.

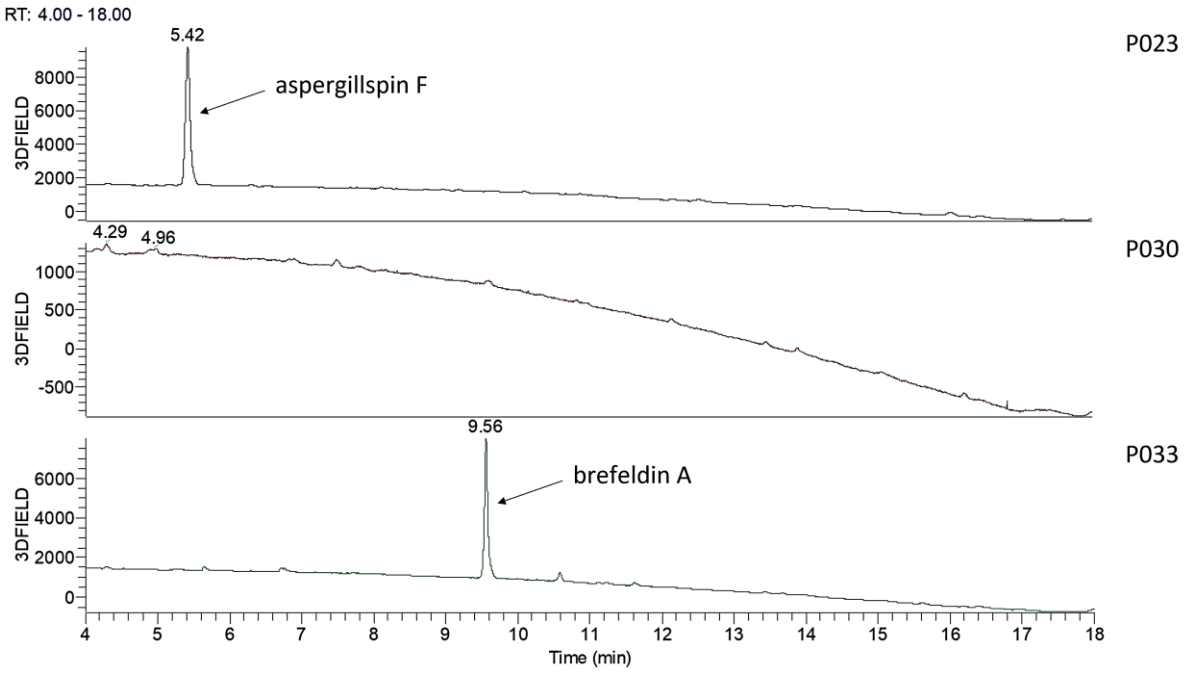

Figure 17. HPLC-UV chromatogram of the extracts of isolates: P023, P030 and P033; total (230-600 nm) UV signal of the PDA detector.

In the extract of isolate P023, at the retention time of 5.42 min, the ion *m/z* 237.04 by negative ionization can be detected (Fig. 18), corresponding to the molecular formula of $C_{11}H_9O_6$, which confirm deprotonated $[M\text{-}H]^-$ molecule. In addition to this ion, at the retention

time of 5.42 min, the ions *m/z* 239.05 and *m/z* 221.04 by positive ionization can be detected (Fig. 18), corresponding to the molecular formulas of $C_{11}H_{11}O_6$ and $C_{11}H_9O_5$, which confirm a protonated $[M+H]^+$ molecule and a water-loss $[M+H-H_2O]^+$ fragment ion. Consequently, the compound at 5.42 min retention can be characterized with the molecular formula of $C_{11}H_{10}O_6$. The compound $C_{11}H_{10}O_6$ exhibits an absorption maximum of 254 nm (Fig. 19) in its UV spectra. Based on the data of the compound at 5.42 min retention can be identified as the aspergillspin F (Ma et al., 2020).

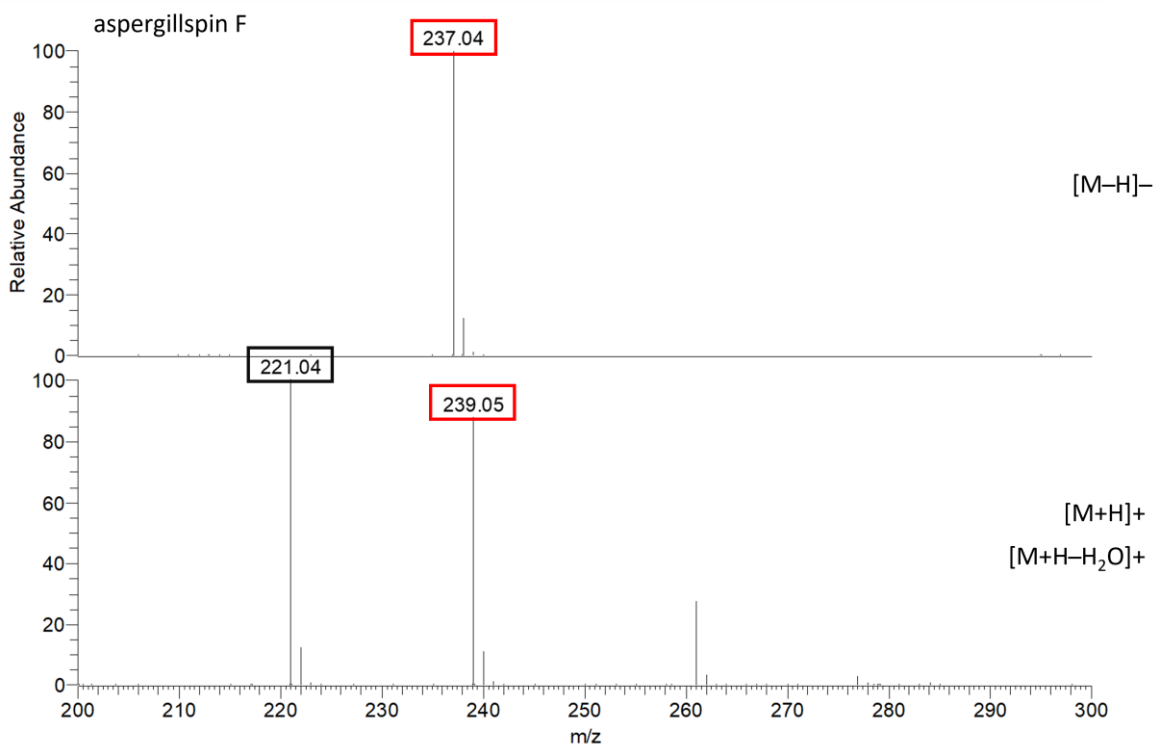

Figure 18. High-resolution mass spectra of sample P023, obtained by negative and positive ionizations. Masses of the deprotonated [M-H]- and protonated [M+H]+ ions given in Dalton, in the red, and the water-loss fragment $[M+H-H_2O]+$ ion in the black frame.

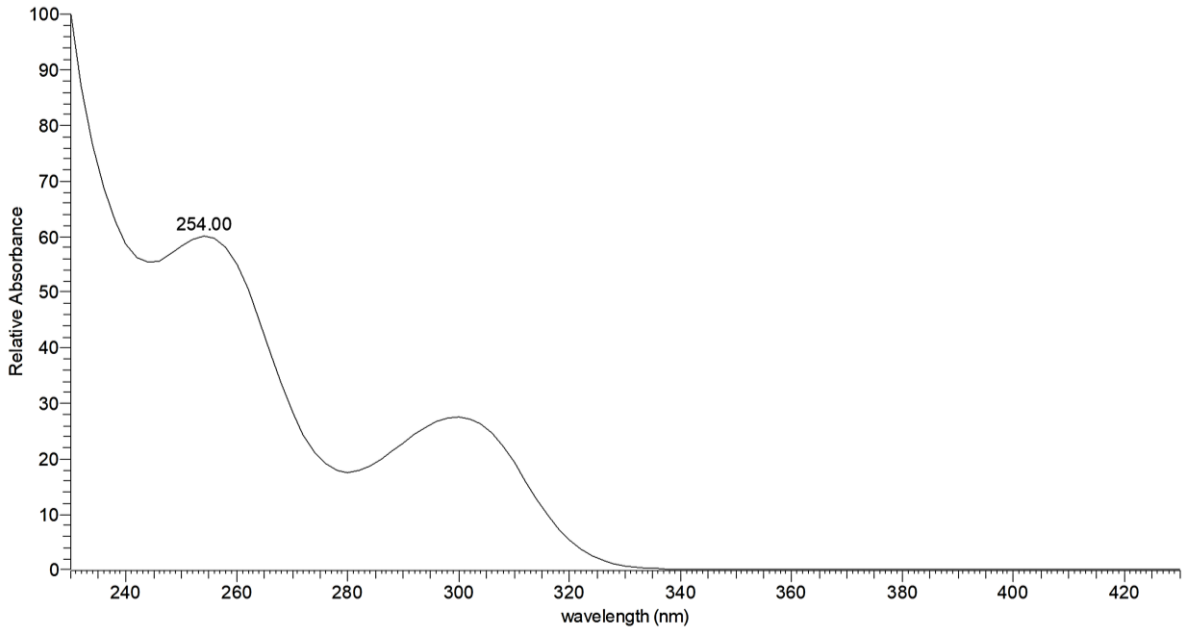

Figure 19. The UV spectra of compounds eluted at 5.42 min.

5.7 Discussion of the results of plant data

An important group of secondary metabolites is the lignans, which belong to the phenolic compounds, whose medical importance is also worth mentioning. They are also significant from the point of view of the Thesis Work. Four possible lignans were detected in the methanolic extracts of *A. sylvestris* leaves and roots using HPLC-UV-MS data. From the masses of the ions produced by positive ionization, it was feasible to determine the possible sum formulas, and from them the most likely originally present compounds. Based on these, the main lignans present in the *A. sylvestris* plant extracts are deoxypodophyllotoxin (Hendrawati et al., 2011), yatein (Kozawa et al., 1978), anhydropodorhizol (Suzuki et al., 2002) and angeloylpodophyllotoxin (Lim et al., 1999).

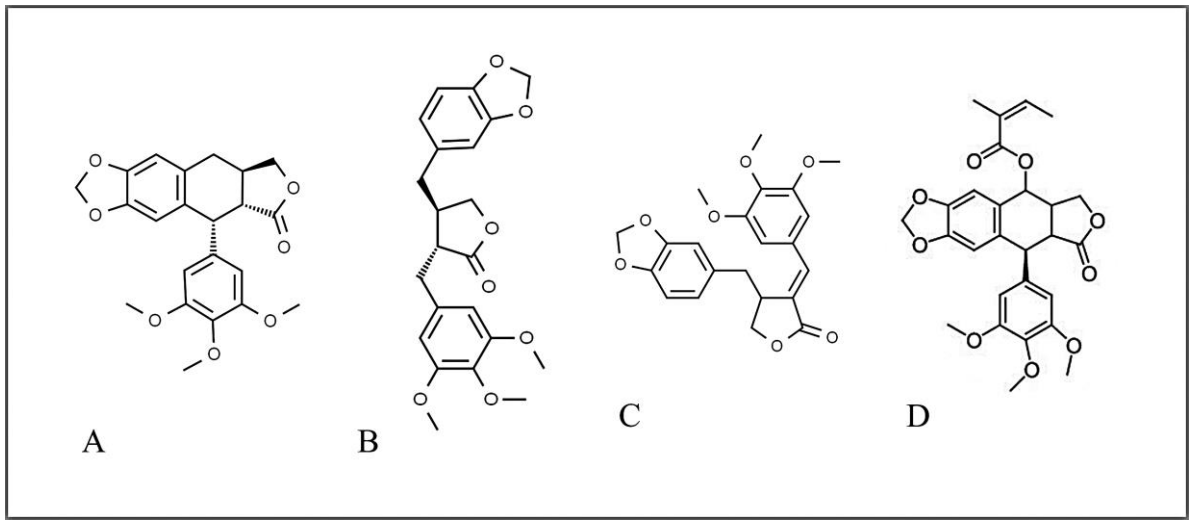

Figure 20. The main four detected lignans of *Anthriscus sylvestris*; deoxypodophyllotoxin (A), yatein (B), anhydropodorhizol (C) and angeloylpodophyllotoxin (D) (source of structures A-C: Royal Society of Chemistry, 2024; D: Mukhija et al., 2022).

Some publications also confirm that the root of *A. sylvestris* contains more lignans and at higher concentrations compared to the leaves (Hendrawati et al., 2011). This assumption is also confirmed by the data obtained during the metabolite examination part of Thesis Work. The deviations can be seen in chromatograms, the intensity differences of individual peaks of the two samples (see Fig. 3). In addition, quantitative differences can be inferred not only between plant parts, but also between individual types of compounds. It can also be seen in Fig. 3 that deoxypodophyllotoxin was present in the samples (for both leaves and roots) in the largest relative abundance.

Many publications deal with the medicinal importance of lignans (Fang and Hu, 2018). Among these, deoxypodophyllotoxin (DPT) is one of the most researched, many of its uses have been described in recent years (Khaled et al., 2013). Among others, the antiproliferative effect of DPT has been reported. As to the mechanism of action, the binding of tubulin and thus the inhibition of the formation of the mitotic spindle, thereby causing cell death, was confirmed (Wang et al., 2015). In addition, DPT has anti-inflammatory effect, as it can inhibit the production of prostaglandin D_2 in a dose-dependent manner (Lee et al., 2004). It can also be mentioned its antiviral effect, because of the inhibition of the herpes simplex virus (HSV) (Sudo et al., 1998). Finally, DPT is an important podophyllotoxin (PTOX) precursor. The semisynthetic derivatives of the cytotoxic aryltetralin PTOX are etoposide and teniposide used in cancer therapy (Khaled et al., 2013). Yatein, as a precursor molecule of PTOX, is also an important compound among anticancer drugs. In addition, due to its similar structure to the PTOX molecule, it may be able to bind to tubulin, causing the previously mentioned effects. In

addition, it has been revealed to inhibit the proliferation of P3X myeloma cells (Donoso-Fierro et al., 2015). A publication reports the anti-inflammatory effect of anhydropodorhizol, which activity is connected to the inhibition of 5-LOX. But in the same article, the antioxidant, and cytotoxic effects of anhydropodorhizol were also investigated (Golakoti et al., 2016). In the case of angeloylpodophyllotoxin, it was established that it can induce apoptosis through the activation of the caspase-3 enzyme and can cause DNA fragmentation (Jeong et al., 2007). A relatively potent antiproliferative activity of angeloylpodophyllotoxin was demonstrated, although it does not reach the typical extent of DPT, which is the most important cytotoxic compound in *A. sylvestris* plant (Orčić et al., 2022).

5.8 Discussion of the results of fungi data

The identification of fungi relies exclusively on the NCBI BLAST results, therefore the final fungal genera used in the Thesis are not proven in detail, and the determination of more precise lineages requires further investigation.

5.8.1 The evaluation of clade-6

The monodictyochromone A and B compounds were detected in all samples of the genus (see Fig. 6). The detected ions of the two compounds, the molecular formulas generated from them, and the UV absorption maximum do not show significant differences either. For this reason, the isomeric compounds obtained at the two retention times (RT: 14.43 min and 14.94 min) are interchangeable in terms of the detected peaks. The compounds were originally isolated from a marine-derived fungus *Monodictys putredinis* (Pontius et al., 2008). The compounds have not previously been detected in the genus *Pyrenochaeta* according to literature results. However, genus *Pyrenochaeta* and *Monodictys* are sistergroups in the order Pleosporales (Schoch et al., 2020). Monodictyochromone A and B were investigated for their cancer chemopreventive potential and found to inhibit cytochrome P450. Furthermore, it was found that they display moderate activity as an inducer of NAD(P)H, in cultured mouse Hepa cells, double the concentration of quinone reductase was required for its specific activity (Pinto et al., 2006).

The phomopsidin compound was detected in two members of clade-6 (isolates P002 and P039, see Fig. 6). The compound is a marine-derived fungal metabolite, isolated from the strain TUF 95F47 *Phomopsis* sp. (Kobayashi et al., 2003). The compound has been previously detected in the genus *Pyrenochaeta*, along with eight other metabolites (Lin et al., 2011).

Regarding the biological activity of phomopsidin, it has been described as a new inhibitor against the assembly of microtubules, purified from porcine brain (Suzuki et al., 2004).

The nigrosporapyrone compound was detected in two members of the genus (samples P017 and P039, see Fig. 6). The compound was originally isolated from a marine-derived fungus, the strain PSU-F18 *Nigrospora* sp. (Trisuwan et al., 2009). The compound has not previously been detected in the genus *Pyrenochaeta*. The different nigrosporapyrone isomers have been shown to have antibacterial activity against *Staphylococcus aureus* (SA) and methicillin-resistant *S. aureus* (MRSA) bacterial isolates (Trisuwan et al., 2009).

The fusarpyrone B was detected together with nigrosporapyrone in the same samples, with very close retention times (6.80 min and 6.67 min, respectively, see Fig. 6). The compound was originally isolated from a soil fungus, strain PSU-RSPG37 *Fusarium solani* (Trisuwan et al., 2013). The compound has not previously been detected in the genus *Pyrenochaeta*. Fusarpyrone B compound has been shown to be antimalarial (against *Plasmodium falciparum*) and antimycobacterial (against *Mycobacterium tuberculosis*). In addition, its cytotoxic properties have also been demonstrated against various human-derived cancer cells (e.g. human breast or lung cancer cells) (Trisuwan et al., 2013).

5.8.2 The evaluation of clade-1, clade-2, and clade-3

The radicicol (also known as monorden) and radicicol B were detectable in all of the clade-3 isolates (see Fig. 9), in some cases with different intensities. Radicicol was originally isolated from *Nectria radicicola* (Mirrington et al., 1966). Radicicol B was isolated for the first time from a *Humicola fuscoatra* extract, together with two other analogs (radicicol C and D) (Mejia et al., 2014). The compounds have been detected before, in the genus *Ilyonectria* discussed here (in the case of the ginseng-derived *Ilyonectria mors-panacis*) (Walsh et al., 2022). Radicicol has been shown to influence the expression of heat shock proteins (HSPs) and inhibit their activity. This effect appears specifically in the case of the HSP90 family (Torres Acosta et al., 2019).

The 8,9-dihydrogreensporone C compound was detected only in isolate P010 at a retention time of 9.50 min (see Fig. 9), further strengthening the theory of this isolate representing a different taxon than the others in this clade. The compound was originally isolated from a freshwater fungus, *Halenospora* sp. (El-Elimat et al., 2014). The compound has not previously been detected in the genus *Ilyonectria* discussed here. Several articles report on the cytotoxic effect of the compound on various human cancer cell lines (e.g. MDA-MB-435 and HepG2) (El-Elimat et al., 2014; Li et al., 2016).

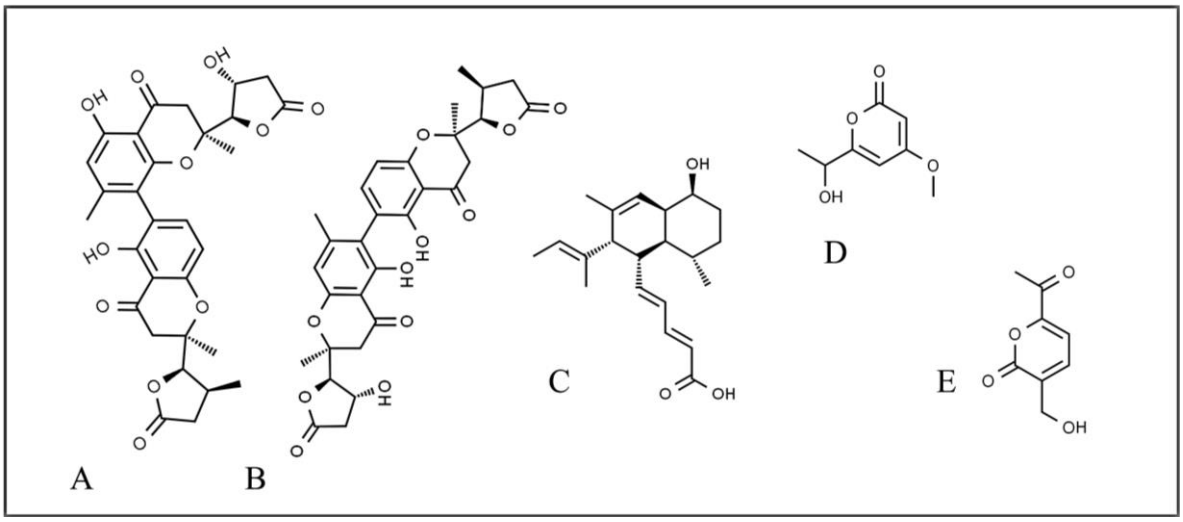

Figure 21. Some of the main detected secondary metabolic products of endophytic fungi; monodictyochromone A (A), monodictyochromone B (B), phomopsidin (C), nigrosporapyrone (D) and fusarpyrone B (E) (source of structures: Royal Society of Chemistry, 2024).

The compound brefeldin A (BFA) was detectable in isolates P003, P004, P026, P027 and P028, that are most likely members of the genus *Dactylonectria* (see Fig. 12). BFA has already been detected in several fungal genera, including *Alternaria*, *Penicillium*, *Curvularia* or *Cercospora* (Fang et al., 2006). Some *Cylindrocarpon*/*Ilyonectria* isolates have been shown to produce BFA (Whitelaw-Weckert et al., 2013), and it has been associated with the virulence of several *Ilyonectria* species (Manici et al., 2018). However, it was reported in publication that among the isolated secondary metabolites, BFA was the predominant one in *Dactylonectria alcacerensis* CT-6. It is also reported here that BFA is the most important component of *D. alcacerensis* CT-6 anti-tumor cell activity (Ming et al., 2023). In addition, many biological uses have been described, including antibiotic and antiviral effects (Fang et al., 2006).

5.8.3 The evaluation of further isolates

In the isolate P023 a compound called aspergillspin F was detected in this sample at the retention time of 5.42 min (see Fig. 17). Aspergillspin F was originally isolated from a marine gorgonian-derived fungi, *Aspergillus* sp. SCSIO 41501, expressing cytotoxic and antibacterial effects (Ma et al., 2020).

A probably new metabolite was detected in all four isolates (P005-P008) belonging to the order Diaporthales. The molecular formula generated from its ions is $C_{40}H_{49}O_{14}Cl$. There is no publication has been published about a compound with such a formula in fungi before. A more detailed study conforming the structure of this new metabolite is required. Several

encouraging results can be achieved by multiplying the sample colony, containing the compound, and then using preparative HPLC and nuclear magnetic resonance spectroscopy (NMR) techniques.

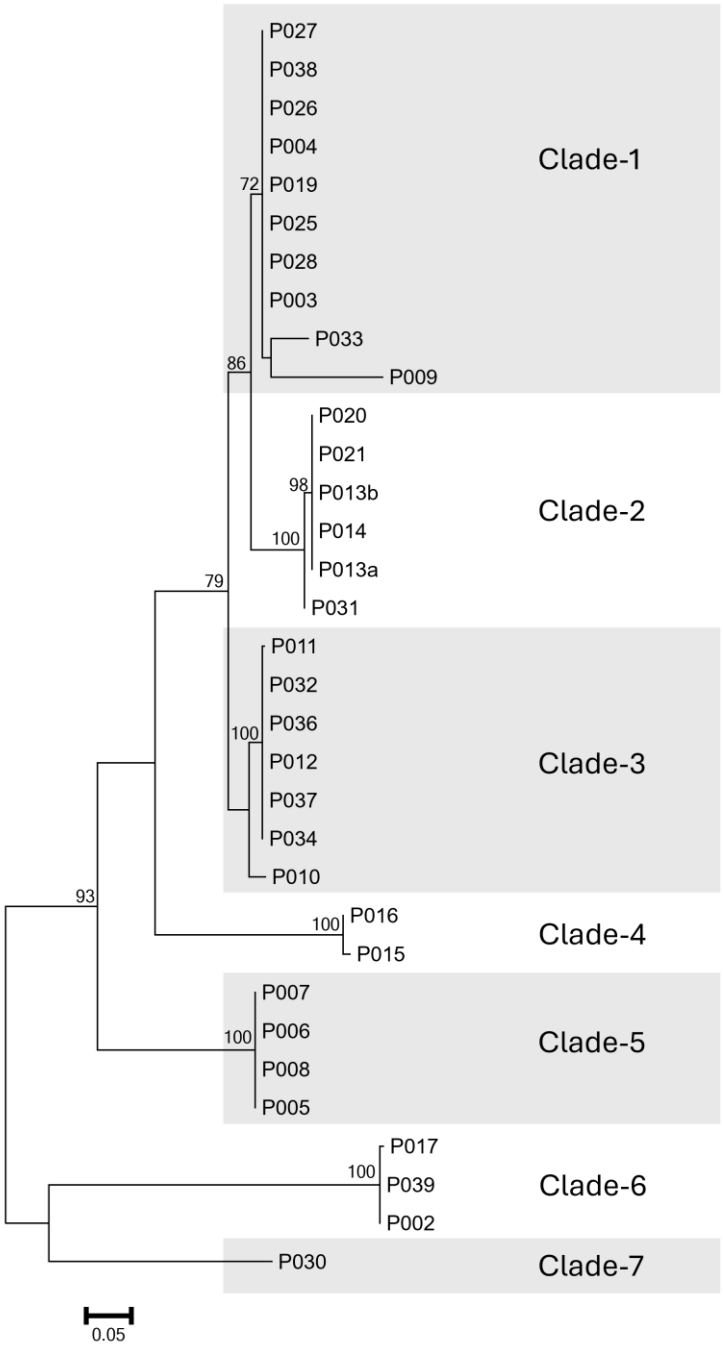

Figure 22. Maximum Likelihood tree of the ITS sequences of the isolates, grouped in seven clades. ML bootstrap support values (≥70) are shown. The scale bar indicates expected changes per site per branch.

6. Conclusions

The results of thesis work illustrate that the root of *Anthriscus sylvestris* produces significant amount of lignan derivatives, so it may be suitable for isolating some members of this group of compounds.

During the examination of the plant samples, at certain retention times, ions with given masses were detected by positive ionization, on the total ion chromatograms. Based on these masses, the possible presence of each of the four investigated lignans was checked in the endophytes. During the analysis, however, the presence of the desired *Anthriscus*-type lignans could not have been detected in the isolated fungi.

Similarly to other studies isolated endophytes produce many other physiologically and medicinally important compounds. One such example is brefeldin A, expressed by several isolates.

7. Summary

In the Diploma Work, the model plant used for the investigations was the cow parsley (*Anthriscus sylvestris*) belonging to the family Apiaceae, subtribe Scandicinae. The so-called 'crown' clade includes *Anthriscus* and some of the closest related taxa, among which the morphology of the fruits is the basis of differentiation.

Plants can live in a symbiotic relationship with various organisms, including endophytic fungi. Although the definition of endophytes has changed several times over the decades, one of the most accepted definitions is that endophytes live within the plant tissue (without causing any harmful symptoms), at least in a certain period of their life cycle.

The secondary metabolic products can be classified into different larger groups, these are terpenoids, phenolics, polyketides (including fatty acid derivatives), and N-containing compounds (including alkaloids). The families of compounds belonging to these groups are extremely diverse and their many medicinal uses are known, such as certain compounds used in cancer research (e.g. PTOX from *A. sylvestris* or vermelhotin from the endophyte *F. fulophazii*). In addition to the anti-tumor effect of terpenoids, it is worth mentioning additional physiological effects, including antibacterial, antiviral, or antimalarial properties. Another important group of compounds are the phenolics containing at least one hydroxylated aromatic ring. Phenolics can be divided into several subgroups, among which lignans and flavonoids should be mentioned. Lignans are phenylpropane dimers and have been shown to have many

important physiological effects, such as the cytotoxic (anti-cancer) podophyllotoxin and its derivatives.

To determine the metabolic composition, the lyophilized and grinded samples were examined using HPLC-UV-MS methods.

Determining the plant metabolites, four lignans characteristic for the genus *Anthriscus* were detected. They were the widely researched deoxypodophyllotoxin, yatein, anhydropodorhizol and angeloylpodophyllotoxin. The detected lignans were present in both the leaf and root samples, with a higher concentration in the latter one.

The most abundant genera of isolated fungi were *Pyrenochaeta*, *Ilyonectria*, *Dactylonectria*. Several compounds were identified in the different groups, with interesting and significant physiological effects in many cases (see Chapter 5.8). In some samples of genus *Pyrenochaeta*, monodictyochromone A and B, phomopsidin or nigrosporapyrone and fusarpyrone B were detected. In connection with genus *Ilyonectria*, it is worth mentioning the radicicol derivatives and the 8,9-dihydrogreensporone C compound. Brefeldin A, which has already been isolated from several groups of fungi, was presented in genus *Dactylonectria*. In case of order Diaporthales, a probable new metabolite was detected during the examination.

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STATEMENT

Name: Péter Mészáros
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ELTE Faculty of Science: Biology MSc

specialization: Plant Biology and Mycology specialization

Title of diploma work:

Secondary metabolites of the endophytic fungi isolated from the root of *Anthriscus sylvestris*

As the author of the diploma work I declare, with disciplinary responsibility that my thesis is my own intellectual product and the result of my own work. Furthermore I declare that I have consistently applied the standard rules of references and citations.

I acknowledge that the following cases are considered plagiarism:
– using a literal quotation without quotation mark and adding citation;
– referencing content without citing the source;
– representing another person's published thoughts as my own thoughts.

Furthermore, I declare that the printed and electronical versions of the submitted diploma work are textually and contextually identical.

Budapest, 6 May, 2024

Mészáros Péter
Signature of Student