Chapter 1

Introduction and scope of this thesis

Cancer is one of the leading causes of premature deaths worldwide[1]. In 2018, this led to over 9.5 million deaths and the discovery of 18 million new cancer cases. The most common cancer types were lung, breast, colorectum, prostate, skin and stomach cancer[2]. For the treatment of patients, we largely depend on natural products and the knowledge of traditional medicine. Between 1981 and 2019, around 30 % of the approved anticancer drugs were natural products or their derivatives and another 50 % contained structures inspired by nature[3]. On the WHO list of essential anticancer drugs there are several natural products or their derivatives, such as bleomycin, cytarabine, doxorubicin, etoposide, paclitaxel, vinblastine and vincristine[4]. The dependence on natural products and their derivatives can lead to shortages. For example, high demand and production issues have led to a shortage of etoposide in 2018[5,6].

Etoposide is an important anticancer drug that is listed for the treatment of various cancer types, such as lung, testicular, ovarian, lymphoma cancer and leukemia[4]. Etoposide is obtained via semisynthesis from the lignan podophyllotoxin[7], which has been reported to occur in various plants, such as the *Callitris, Dysosma, Hernandia, Hyptis, Juniperus, Linum, Nepeta, Podophyllum, Teucrium* and *Thuja* species[8]. Most of these species produce low amounts of podophyllotoxin. One of the exceptions is *Podophyllum hexandrum* (Himalayan mayapple) that produces the highest podophyllotoxin levels reported to date with yields up to 6 – 7 % (dry weight) in the roots[9,10]. The excessive harvesting has resulted in the inclusion of *P. hexandrum* in the Convention of International Trade in Endangered Species[11]. Furthermore, chemical synthesis of podophyllotoxin is difficult due to the presence of four contiguous chiral centers and the presence of a base sensitive trans-lactone moiety[12]. Therefore, we decided to explore alternative production routes toward podophyllotoxin, such as *in vitro* or *in vivo* production hosts that produce podophyllotoxin or related lignans.

For *in vitro* production, knowledge on the biosynthetic pathway in plants is required. Podophyllotoxin is produced in *P. hexandrum* via the lignan pathway[13]. In short, matairesinol is converted into deoxypodophyllotoxin by 5 consecutive enzymatic steps; before further conversion into podophyllotoxin by a hitherto unidentified enzyme. Deoxypodophyllotoxin can be converted to epipodophyllotoxin, the C-7 epimer of podophyllotoxin, by a human liver cytochrome P450[14]. Therefore, we assume that the enzyme responsible for this conversion in *P. hexandrum* is also a cytochrome P450. For catalytic activity, cytochrome P450 requires a NADPH-cytochrome P450 reductase as redox partner[15]. A transcriptome database of *P. hexandrum* is publicly available; therefore, searching for both enzymes should be possible followed by recombinant conversion of deoxypodophyllotoxin in an *in vitro* system, such as *Eschericia coli* or transgenic plant cultures. For plant cultures, first deoxypodophyllotoxin generating cultures need to be regenerated. Complete regeneration of *Anthriscus sylvestris* has been reported[16]; therefore, regenerating root cultures should

be possible. When *in vitro* conversion is possible, large quantities of deoxypodophyllotoxin are required, which can be obtained from the roots of the weed *A. sylvestris* (wild chervil) that is very common in the Netherlands[17,18]. A large-scale and environmentally friendly extraction method should be designed for the extraction of deoxypodophyllotoxin from *A. sylvestris* roots.

Instead of focusing on the *in vitro* conversion of deoxypodophyllotoxin into podophyllotoxin, research into the controlled cultivation of *P. hexandrum* is an option. For example, investigating if large-scale cultivation of *P. hexandrum* is possible in glasshouses in the temperate latitudes, like the Netherlands.

Scope of the thesis

The research presented in this thesis focuses on production routes toward podophyllotoxin. In **chapter 2**, we first elaborate further on the importance of etoposide and other podophyllotoxin derivatives for chemotherapy. This is followed by a systemic literature review describing the lignan biosynthetic pathway toward podophyllotoxin in plants. In addition, we discuss the engineering possibilities for recombinant production of podophyllotoxin, such as conversion of deoxypodophyllotoxin into podophyllotoxin by a plant cytochrome P450 in a heterologous host.

For the *in vitro* production of podophyllotoxin, we first need to identify the cytochrome P450 responsible for the conversion of deoxypodophyllotoxin into podophyllotoxin in *P. hexandrum*. In **chapter 3**, we searched for this cytochrome P450 in the publicly available *P. hexandrum* transcriptome database. To this end, we combined knowledge on cytochrome P450 transcript expression under stress conditions and sequence characteristics, such as highly conserved domain sequences in plant cytochrome P450s. In addition, we searched for the endogenous NADPH-cytochrome P450 reductase by searching for proteins containing the highly conserved domain sequences for NADPH-cytochrome P450 reductases in the predicted products dataset of the *P. hexandrum* transcriptome database. Subsequently, we demonstrated the expression of one *P. hexandrum* cytochrome P450 and NADPH-cytochrome P450 reductase in our *E. coli* expression system.

When the *in vitro* production of podophyllotoxin is possible, large quantities of deoxypodophyllotoxin are required. Deoxypodophyllotoxin can be obtained from the roots of the very common weed *A. sylvestris*. In **chapter 4**, we assessed the extraction of deoxypodophyllotoxin by the environmentally friendly supercritical carbon dioxide extraction

method and compared this to the traditional solvent-based extraction method. Not only the extraction of deoxypodophyllotoxin, but also the improvement of deoxypodophyllotoxin production by *A. sylvestris* is of interest. For this, a small scale extraction method with high throughput is necessary; therefore, we designed a quick method vortex method for this (**chapter 4**).

Another source for deoxypodophyllotoxin could be *in vitro* cultures of *A. sylvestris*. In **chapter 5**, we regenerated root cultures of *A. sylvestris* and determined if they produced deoxypodophyllotoxin. For the large-scale cultivation of these roots, a disposable bioreactor system with integrated oxygen sensors was designed. An alternative route toward podophyllotoxin production would be controlled large-scale cultivation of *P. hexandrum*. In **chapter 6**, we report the cultivation of *P. hexandrum* in a glasshouse in the Netherlands under various conditions. We investigated the influence of soil type, temperature and hormone treatment on the biomass formation and podophyllotoxin production.

Finally, a summary of all study results described in this thesis is presented in **chapter 7** (English) and the **Appendix** (Dutch).

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