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Antiproliferative Effect of Common *Nepenthes* Species on Adenocarcinoma cells

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Declaration

I hereby declare that I have done this thesis entitled “Antiproliferative Effect of Common *Nepenthes* Species on Adenocarcinoma Cells” independently, all texts in this thesis are original, and all the sources have been quoted and acknowledged by means of complete references and according to Citation rules of the FTA.

Prague, 18th April

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Nicola Dřímalová

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Abstract

Pitcher plants, renowned for their intriguing pitcher-shaped leaves, have long fascinated botanists and researchers alike. These remarkable plants have been the subject of many studies due to their diverse array of bioactive compounds with potential antiproliferative properties, including plumbagin. Despite the growing interest in pitcher plants, their efficacy against colorectal adenocarcinoma, a prevalent and one of the most occurred cancers worldwide, has remained unexplored.

In this study, various *Nepenthes* species and their parts were evaluated in the activity against colorectal adenocarcinoma cell lines, along with their cytotoxicity on healthy human intestine cells. Additionally, the antioxidant activity was assessed using the DPPH assay. Furthermore, chemical analyses were conducted to characterize the bioactive compounds present and investigate differences between parent and hybrid plants.

The results unveil the considerable potency of *Nepenthes* × *hookeriana* roots, which exhibit robust antiproliferative activity against both HT29 and Caco-2 colon adenocarcinoma cell lines, with half maximal inhibitory concentration values (IC_{50}) of 10.59 μg/mL and 2.68 μg/mL, respectively. Notably, these roots demonstrated minimal cytotoxicity to healthy human cells while exhibiting significant inhibition of DPPH (IC_{50} 15.36 μg/mL), underscoring their potential as promising therapeutic agents. Besides that, leaves of *N. ampullaria* emerged with IC_{50} value of 13.61 μg/mL, indicated its potential in mitigating oxidative stress-related pathologies.

LC-MS/MS analysis revealed a rich phytochemical profile characterized by the abundance of various compounds in *Nepenthes species* and discovered distinct differences between parents and hybrids. This analysis with using of UV indicated a low level of plumbagin, implying the involvement of other compound(s) with anticancer properties. This discovery makes these species unique plants which could be potentially considered as a future crucial chemotherapeutics against colorectal cancer, as this is the first study mentioned *N.* × *hookeriana* for its antiproliferative activity against colorectal cancer cell lines. Moreover, the results are very promising showing low IC_{50} in comparison with other *Nepenthes* species.

Key words: anticancer properties, carnivorous plants, colorimetric assay, Hooker's pitcher plant

Abstrakt

Láčkovky, známé svými zajímavými listy ve tvaru džbánků, již dlouho fascinují botaniky a vědce. Tyto pozoruhodné rostliny se staly předmětem mnoha studií díky své rozmanité škále bioaktivních látek s potenciálními protinádorovými vlastnostmi, např. plumbagin. Navzdory rostoucímu zájmu o láčkovky zůstává jejich účinnost proti adenokarcinomu tlustého střeva a konečníku, který je rozšířeným a celosvětově jedním z nejčastěji se vyskytujících nádorových onemocnění, dosud neprozkoumána.

V této komplexní studii byla hodnocena aktivita různých druhů rodu *Nepenthes* a jejich částí vůči buněčným liniím kolorektálního karcinomu a jejich cytotoxicita na buňky zdravého lidského střeva. Kromě toho byla hodnocena antioxidační aktivita extraktů z láčkovek pomocí DPPH testu. Dále byly provedeny chemické analýzy, které charakterizovaly bioaktivní sloučeniny přítomné v láčkovkách a také zkoumaly rozdíly mezi mateřskými a hybridními rostlinami.

Výsledky odhalily značnou účinnost kořenů *N.* × *hookeriana*, které vykazovaly silnou protinádorovou aktivitu vůči buněčným liniím HT29 a Caco-2 s hodnotami poloviční maximální inhibiční koncentrace (IC_{50}) 10,69 μg/mL, resp. 2,68 μg/mL. Navíc tyto kořeny vykazovaly minimální cytotoxicitu vůči zdravým lidským buňkám a zároveň vykazovaly významnou inhibici DPPH radikálu (IC_{50} 15,36 μg/mL), což potvrzuje jejich potenciál jakožto slibných léčivých látek. Kromě toho *N. ampullaria*, zejména její listy s hodnotou IC_{50} 13,61 μg/mL, vykazuje určitý potenciál pro zmírňování patologických stavů souvisejících s oxidačním stresem.

LC-MS/MS analýza odhalila bohatý fytochemický profil charakterizovaný množstvím různých sloučenin a odhalila výrazné rozdíly mezi rodiči a hybridy. Tato analýza s využitím UV záření ukázala nízký obsah plumbaginu, což naznačuje výskyt dalších látek, které mají protinádorové vlastnosti. Tento objev činí z těchto druhů unikátní rostliny, které by mohly být potenciálně považovány za budoucí zásadní léčiva proti kolorektálnímu karcinomu, protože *N.* × *hookeriana* je prvním zmíněným druhem rodu *Nepenthes* s tak vysokou protinádorovou aktivitou proti buněčným liniím tohoto typu.

Klíčová slova: kolorimetrický test, masožravky, protinádorové vlastnosti, Hookerova láčkovka

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List of the abbreviations used in the thesis

Caco-2	colorectal human adenocarcinoma cell line
CCD 841 CoN	healthy human intestine cell line
CRC	colorectal cancer
DMSO	dimethyl sulfoxide
DPPH	2,2-diphenyl-1-picrylhydrazyl
EGFR	epidermal growth factor receptor
EMEM	eagle's minimum essential medium
EVO	evolution
FBS	fetal bovine serum
GFL	Gesellschaft für Labortechnik
HDI	human development index
H-ESI	heated electrospray ionization
HT29	colorectal human adenocarcinoma cell line
IC_{50}	half maximal inhibitory concentration
LC-MS/MS	liquid chromatography tandem mass spectrometry
m/z	mass-to-charge ratio
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
NCDs	non-communicable diseases
SD	standard deviation
UV	ultraviolet light

1. Introduction

Plants are in many ways irreplaceable source of bioactive compounds widely used in pharmaceuticals. Nature still hides its full potential, and our purpose is to explore these possibilities. Many plants have the ability to interrupt the cell proliferation of various types of cancer (Zhang et al. 2017). One of them is the genus *Nepenthes.* These species are rich in the context of phytochemical profile and have recently caught the eye of many scientists in the field (Wójciak et al 2023). Various hybrids and species in general have been positively tested against several types of cancer, such as breast and lung cancer or melanoma. Their extracts have been able to slow or completely stop the proliferation of cancer cells (Yang et al. 2021; Liu et al. 2021; Lin et al. 2022; Lin et al. 2023). In addition, these plants have also recently been found to have a great ability to inhibit free radicals, making them versatile tool for use as medicinal agents (Naheeda et al. 2023)

Although pitcher plants have always been known for their ethnobotanical purposes, these plants as a subject of research have spread in recent years, thus, their abilities towards colon adenocarcinoma have not been investigated by anybody before. This is the main reason for this thesis – to fill the gap regarding other possibilities of pitcher plants. It is important to actively seek answers because these plants have them.

This study attempts to approach the general knowledge about these interesting plants, plant-based bioactive compounds and summarize findings known so far. The main objectives of the research were evaluating the antiproliferative effects of different parts of *Nepenthes* species and hybrids, assessing their ability to inhibit DPPH free radical in comparison to parent and hybrid plants, and evaluating its potential cytotoxicity to healthy human cells. As the pitcher plants are known for their high content of plumbagin (strongly bioactive), the next direction was to investigate the presence of this compound with a total mass-spectrometric analysis to determine the general content of chemical features of all plants, focusing on the differences in compound content hybrid plants (Rahman-Soad et al. 2021). Hybrids should have better results in both the antioxidant assay and colorimetric assay targeting the proliferation of colon adenocarcinoma cell lines (Zhu et al. 2016).

2. Literature Review

2.1. Colon Adenocarcinoma

Non-communicable diseases (NCDs) have risen in recent years. Based on the prediction by Wang & Wang (2020), 76.2 % of all deaths worldwide will be caused by NCDs. Unfortunately, cancer is one of the main NCDs besides cardiovascular and chronic respiratory diseases or diabetes (World Health Organization 2020). In 2020, cancer emerged as a prominent contributor to global mortality, resulting in approximately 10 million fatalities. This staggering figure signifies that cancer claimed the lives of almost one-sixth of the total deaths recorded during that year (Ferlay et al. 2020). The three most frequently encountered types of cancer are lung, breast, and colorectal (Sung et al. 2021). Many of these can be successfully treated in their preliminary stages, but in 2020, colorectal cancer (CRC) stood out as one of the leading causes, accounting for almost 1 million deaths (Morgan et al. 2020). These numbers are magnified by oxidative stress which increases degradation of healthy cells and promotes rapid carcinogenesis (Carini et al. 2017). Free radicals are capable of distinct damages of DNA, proteins and all cell components including membrane. Ionization can be the source of free radicals producing in human body. Moreover, environmental toxins can significantly contribute to abnormal production of free radicals causing faster progression of colon adenocarcinoma (National Cancer Institute 2017). Although free radicals can be responsible for adenocarcinoma development, antioxidants can make a significant contribution to slow or prevent this type of cancer (Järvinen et al. 2000). It is well estimated that a diet rich in antioxidants can prevent cancer occurrence and that antioxidants work as synergetic compounds to other medicinal agents (Collins 2005).

Colorectal adenocarcinoma is a type of cancer that mainly arises from adenomatous polyps. These are clearly demarcated masses of epithelial dysplasia characterized by uncontrolled division of crypt cells (Fig. 1). An adenoma is deemed malignant when neoplastic cells break through the muscularis mucosae and invade the submucosa (Ponz de Leon & Di Gregorio 2001). The progression of colorectal cancer is gradual, commencing with a mild inflammation, followed by the formation of adenomatous polyps in the epithelium, and ultimately culminating in the emergence of adenocarcinoma.

Furthermore, this process is propelled by the accumulation of mutations and genetic alternations, spanning a duration of 10-15 years (Sawicki et al. 2021).

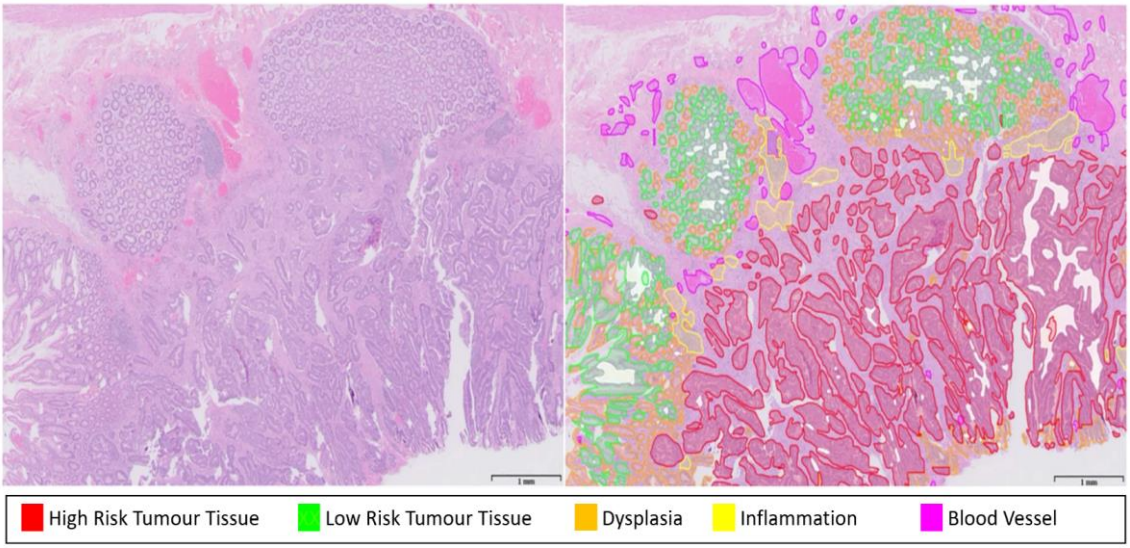


Figure 1 Histopathological Screening of CRC (Ho et al. 2021)

2.1.1. Epidemiology

Typically, CRC affects men around age 50, but the occurrence is also reported in women around 75 years of age (Maclnnis et al. 2006). For men, this type of cancer is the third most commonly occurring, whereas for women is the second after reproductive organs (Torre et al. 2017). The majority of patients aged 70 and above tend to have early-stage disease, whereas younger patients, typically in their 40s, are more likely to have a more aggressive form of this cancer at the same stage of presentation (Amersi et al. 2005).

Generally, this type of cancer started to affect people below age 55. The incidence has been annually increasing by 1 % - 2 % since 1995 worldwide. Subsequently, there was a substantial growth in the percentage of cases reported among those under age 55, with an increase from 11 % in 1955 to 20 % in 2019 (Siegel et al. 2024). In the coming decade, there will be a notable surge in the global burden of CRC, with an anticipated increase of 60 %. This surge will lead to the emergence of over 2.2 million new cases and 1.1 million deaths associated with this disease (Sawicki et al. 2021). The rise of colorectal cancer cases and deaths is predominantly observed in countries with medium and high human development index (HDI) that are embracing a “western” lifestyle. Among these nations, developed countries face the greatest susceptibility to colon cancer (Arnold et al. 2017, Wong et al. 2021).

2.1.2. Risk Factors

Family or Personal History – In the case where a first-degree relative has been diagnosed with CRC or colon polyps before the age of 60, or if two or more first-degree relatives have been diagnosed with CRC or polyps at any age, a patient is identified as having a significant family history of colorectal cancer (Amersi et al. 2005). Besides directly inherited anamnesis, other significant effects on colon cancer development have inflammatory bowel diseases such as Crohn's disease or ulcerative colitis, which lead to negative affection of the gastrointestinal immune system (Hnatyszyn et al. 2019). People with diabetes also tend to be at higher risk. An increase in insulin and inflammatory condition associated with the disease are considered to be factors in the development of colorectal cancer. The direct influence of excessive insulin production on stimulating colonic cell proliferation indicates its potential role in the process of colorectal carcinogenesis (Pang et al. 2018).

Age and Sex – The underlying reasons for gender variations in health outcomes are not fully comprehended, but it is theorized in the scientific paper by Keum & Giovannucci (2019) that differences in exposure to risk factors such as alcohol drinking or smoking and sex hormones may be contributing factors. The risk of developing colorectal cancer is approximately 30 % higher in men than in women. Men diagnosed with this form of cancer have a worse prognosis, with mortality rates approximately 40 % higher than those of women (Sawicki et al. 2019). In addition to the fact that men around age 55 are the most susceptible to colon cancer, people under age 50 years of age have predominated since 2005 (Wong et al. 2021).

Ethnicity and Race – The survival rates for colorectal cancer exhibit notable differences that can be dependent on race and ethnicity. In the United States, Native Americans and African Americans are at higher risk of CRC development and have lower survival rates across all stages of this type of cancer as opposed to Hispanic and white Americans (Rawla et al. 2019).

Lifestyle – A sufficient number of recent scientific papers recorded that the primary cause of colon cancer development is due to consumption of red and processed meat (Chapelle et al. 2020; Abu-Ghazaleh et al. 2021; Kossenas & Constantinou 2021; Farvid et al. 2021; Vernia et al. 2021). As a potential second cause of colon cancer development are considered sedentary jobs and lack of exercise with general movement. Compared to

individuals with a normal weight, overweight or obese men and women have a 50 % and 20 % higher risk of developing colorectal cancer (Rawla et al. 2019). Contribution to the active expansion of CRC is also strongly influenced by smoking and alcohol intake. It is estimated that consuming 2-3 drinks daily can increase the risk of colorectal cancer by approximately 20 %, whereas consuming more than three alcoholic beverages can further heighten this risk by about 40 % (Sawicki et al. 2019). Amersi et al. (2005) recorded that diet low in fibre increased the risk of CRC by up to 50 %. Thus, high consumption of fruits and vegetables may prevent the development of this disease (Gandomani et al. 2017).

2.1.3. Treatment

In the management of colorectal cancer, patients with node-positive disease are typically advised to undergo adjuvant chemotherapy as part of the standard treatment approach. Conversely, for individuals diagnosed with stage II or III rectal cancer, the current standard of care entails the administration of adjuvant chemoradiotherapy in combination with high-dose radiotherapy. It is estimated that these well-tolerated measures reduce the recurrence rate by over 30 %. These types of treatment may cause harmful side effects, with the exception of plant-based targeted therapy, which uses drugs that directly target cancer cells and usually cause less damage to normal cells [Colon Cancer Treatment (PDQ®)–Patient Version 2022]. In the USA, the total annual medical cost of treating colorectal cancer in 2020 is $24.3 billion, with an average cost per patient of $2,000 for oral prescription drugs in the last year of life.

Increasing the screening prevalence and the use of new natural agents can rapidly reduce the number of deaths and economic impact and increase the detection of early-stage cancers (Health and Economic Benefits of Colorectal Cancer Interventions 2022). The treatment of colorectal cancer has advanced in the last 20 years, but it is important to note that the optimal treatment approach for this type of disease has not yet been definitively established. New synthetic drugs are constantly being developed, although their abilities are limited, or lose their benefits over time (Florescu-Tenea 2019). Plant-based compounds are less toxic to human body and have certain advantages over the synthetic ones (Mohan 2021).

2.2. Antiproliferative Effect of Plants and Plant-Derived Compounds

Plants are generally the most studied object for potential use against various types of cancer. Considering the fact that lung cancer, breast cancer, and colorectal cancer are the most common cancers in the world, plants, and their derivates, are also commonly used as chemotherapeutics or supported therapy (Sung et al. 2021). One of the most-known plant chemotherapeutics are **vinca alkaloids** from the plant *Catharanthus roseus*, known as the Madagascar periwinkle. These compounds, such as **vinblastine**, **vincristine** or **vinorelbine** are part of chemotherapy for some types of lung cancer and breast cancer (Adenis et al. 1995; Johnson et al. 1996; Zhang et al. 2017). These stood out as second in the isolation of the first plant-based drugs ever (El-Sayed 2021). They are irreplaceable in the context of chemotherapy. In addition, taxanes, such as **paclitaxel** or **docetaxel**, are compounds isolated from the bark of trees in the *Taxus* genus (yews) and have their purpose in chemotherapy (Francis et al. 1995; Crown et al. 2004). Some studies indicated that the use of **curcumin** or **ginseng** may also help to stop cell proliferation (Lee et al. 2016; Ashrafizadeh et al. 2020).

In the treatment of colorectal cancer are widely used **camptothecin** derivates, fluoropyrimidines, and epidermal growth factor receptor (EGFR) inhibitors. Camptothecin is a natural product found in the bark and stem of the *Camptotheca acuminata* tree (Thomas et al. 2004). Derivates, such as **irinotecan** and **topotecan** are used in chemotherapy regimens for CRC (Wang et al. 2013). While not directly derived from plants, fluoropyrimidines like **5-fluorouracil** (5-FU) and **capecitabine** are synthetic analogues of the natural pyrimidine nucleoside uracil, which are widely used in treatment of colorectal cancer (Malet-Martino & Martino 2002). Some plant-derived compounds, such as monoclonal antibodies like **cetuximab** and **panitumumab**, target EGFR and are used as chemotherapeutics (García-Fonsillas et al. 2019). Besides that, certain flavonoids or polyphenols are positively tested against CRC. **Quercetin** and **kaempferol** have demonstrated anticancer properties in preclinical studies against this type of cancer along with **resveratrol** and **plumbagin** (Shaafsma & Hsieh 2016; Rahman-Soad et al. 2021; Neamtu et al. 2022; Nejabati & Roshangar 2022). All these compounds and many more are reported to be present in *Nepenthes* species making them interesting topic for scientists (Wójciak et al. 2023).

2.3. Nepenthes Species

Nepenthes species are commonly known as pitcher plants, according to their pitcher-shaped traps used for catching prey from insects to small vertebrates (Cooley et al. 2023), or simply gathering organic material such as leaf litter (Moran et al. 2003). Evolution of *Nepenthes* spp. reach into Northern Tethys from the European Eocene and into Gondwana with the separation of the Indian plate from Madagascar. Diversification of the genus from others was done in the Early Eocene. The botanical carnivory of *Nepenthes* species had a monophyletic origin from the genus *Drosera* (Biswal et al. 2018). The range of species in the genus *Nepenthes* is incredibly large, occurring from eastern India to northern Australia, including New Caledonia, Madagascar, Southeast Asia - Indonesia, Malaysia, the Philippines, or Sumatra, and especially Borneo, with the highest diversity of *Nepenthes* species in the world (Nishida & Takashi 2001).

2.3.1. Taxonomy

The first record of pitcher plants was made in 1658 by the French colonial governor of Madagascar, Étienne de Flacourt (1607-1660), in his seminal work (Phillipps & Lamb 1996). The scientific name was later introduced in 1737 by the Swedish scientist Carl Linnaeus (1707-1778) in relation to Homer´s Odyssey and the drug nepenthe mentioned therein ("Pitcher Plant" 2023). These plants belong to the family Nepenthaceae, which is close to families Droseraceae and Drosophyllaceae (Ellisson & Gotelli 2009). The genus *Nepenthes* comprises mostly liana-forming plants with approximately 160 species and includes numerous natural and cultivated hybrids, making it one of the most comprehensive genera of all carnivorous plants in the world (Ellison & Adamec 2017).

The genus can be divided into two groups – lowland species (below 1300 meters above sea level) and highland species (1500-10 000 meters above sea level and more). The difference in their habitats can have an impact on their anatomical structures such as root growth, leaf and trap formation (Arimy et al. 2017). Examples of differences possibly depending on habitat include *N. aristolochioides*, which has a vertical trap entrance to attract specific insects which occurs at higher altitudes about 2000 meters

above sea level (Fig. 2), while *N. pudica*, recently discovered by Czech scientists Dančák et al. (2022), forms unique achlorophyllous traps underground (Fig. 3). This species seems to be negatively phototrophic with occurrence at 1100-1300 meters above sea level (Nerz 1998).

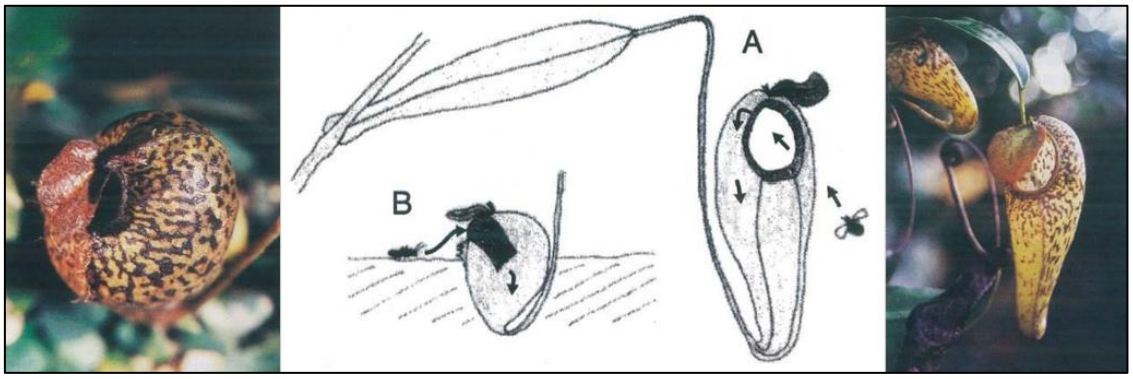


Figure 2 *Nepenthes aristolochioides*; A) proposed trapping mechanism of upper pitcher, B) proposed trapping mechanism of lower pitcher (Nerz 1989)

Figure 3 *Nepenthes pudica*; A) detail of lower pitchers excavated from the soil, B) pitchers in a cavity under tree roots, C) pitchers revealed under a moss mat, D) pitchers extracted from a cavity-note achlorophyllous shoot and reduced phyllodia formed in total darkness (Dančák et al. 2022)

2.3.2. Morphology

Pitcher plants are dioecious, prostrate, climbing, epiphytic shrubs or half shrubs. All species of the genus *Nepenthes* have 2*n*=80 (Devi et al. 2012). The inflorescence type is thyrse or botryoids, the flowers are unisexual without petals, sepals are 3(4), imbricate or basally connate. The stamens are (4-6)8-24, gynoecium is fused of (3)4(6) carpels forming a compound ovary with the same number of locules as carpels. The fruit is

a loculicidal capsule containing numerous filiform seeds with a small straight embryo surrounded by a starchy, proteinaceous, and oily endosperm (Kubitzki & Bayer 2003).

Stem is climbing, often woody. Leaves are alternate, sword-shaped with elongated tendrils ending in metamorphosed leaves – traps (Nepenthes species 2016). Traps can be divided into lower pitchers and upper pitchers. Lowers are smaller, variable, facing tendril attachment, while uppers are bigger, constant, with often prehensile tendril and facing away from tendril attachment (Ngai Lam & T. W. Tan 2020). *Nepenthes* is characteristic by ontogeny dimorphism, traps are forming and changing through its growth (Gaume & Di Giusto 2009). On the upper part is a lid (Fig. 4), covering the entry of the trap often seen with glandular crest in the shape of a tooth producing pheromones. Around the entry is a wettable peristome, the very first step where the prey can be caught (Bohn and Federle 2004). The inner edge of the peristome contains secretive glands producing nectar, remaining flower scent (Di Giusto et al. 2010). When the prey is captured, there is no escape due to waxy slippery walls of the pitcher plant (Gaume & Di Giusto 2009). On the sides of a pitcher are digestive glands (Fig. 5) producing enzymes which are inducing decomposition of the prey. Insect falls into liquid consisting of enzymes and water (Gorb et al. 2004).

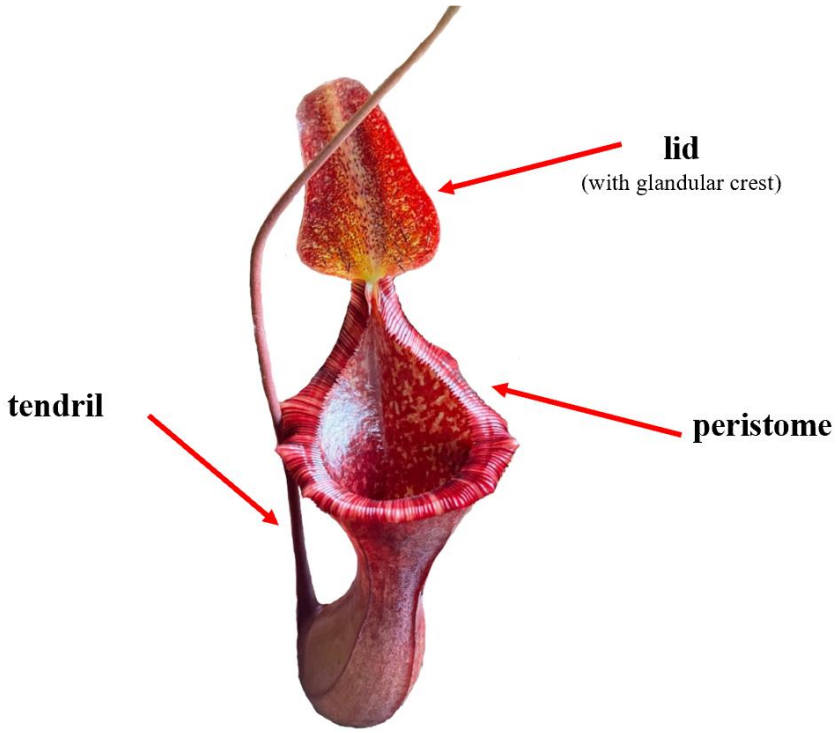


Figure 4 Pitcher Morphology (photo author)

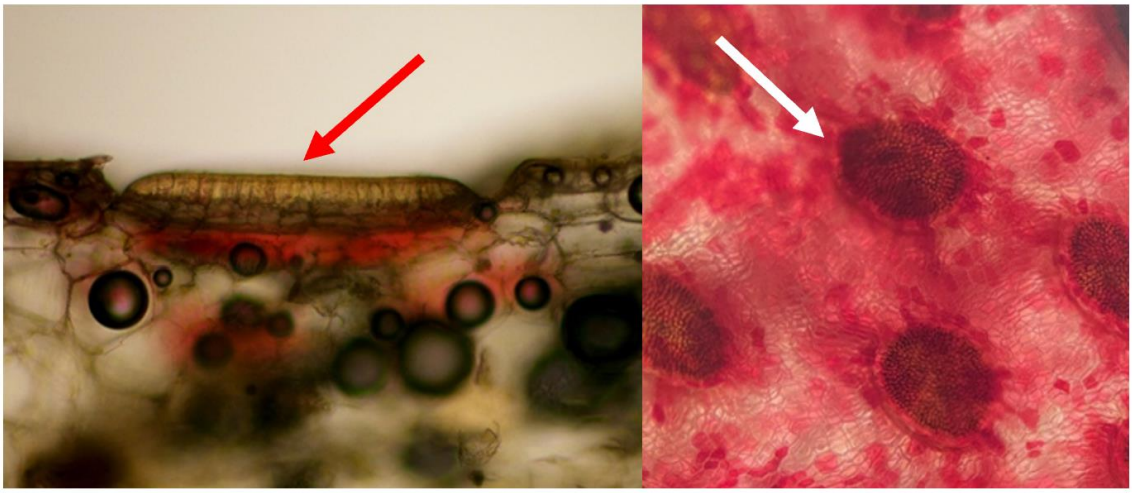


Figure 5 Digestive Glands (photo author)

2.3.3. Botanical Description of Selected *Nepenthes* species

Nepenthes ampullaria Jack is a lowland pitcher plant with occurrence in Southeast Asia mainly in Borneo (Moran et al. 2003). It can be often found near the ground beneath the forest canopy of tropical forest with its traps laying on the ground and waiting for the first leaves to fall into them. *N. ampullaria* is a detritivore, which means that it primarily consumes fallen leaves from plants above (Setiawan et al. 2018; Pavlovič et al. 2018). The traps are small and ovoid. In lighter habitats can be green to light greenish (Fig. 6) on the other hand in dark, shady areas are darker with reddish spots (Harapan et al. 2022).

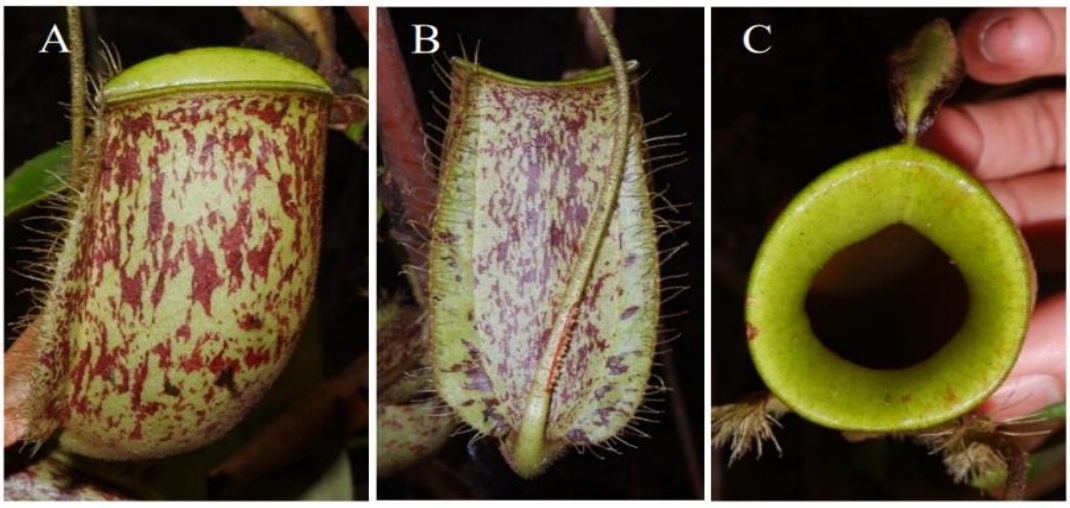


Figure 6 Body parts of *Nepenthes ampullaria*; (A) longitudinal look, (B) front, (C) peristome (Harapan et al. 2022)

Nepenthes rafflesiana Jack occurs mostly in Peninsular Malaysia, Brunei, Sumatra or Singapore (Moran 1996). Its traps are long with reddish to dark brown spots and marble peristome. Trapping can be divided into two forms – typical form has highly viscoelastic fluid but lacks wax crystals on the insides of the pitcher (Fig. 7A) in contrast elongated form (Fig. 7B) is characterized by a thick wax layer inside the pitcher, but its fluid consistency is watery (Bauer et al. 2011).

Figure 7 Two forms of *Nepenthes rafflesiana*; (A) typical form, (B) elongate form (Bauer et al. 2011)

Nepenthes × *hookeriana* H. Low has a synonym Hooker's pitcher plant after Sir Joseph Dalton Hooker. It is natural hybrid between *N. ampullaria* and *N. rafflesiana* with the same occurrence as its parent plants (Yulita & Mansur 2012). The shape of the pitchers is inherited from *N. ampullaria* also with its typical "marbly" pattern, but the large peristome is from *N. rafflesiana* (Mansur 2016). As is usual for *Nepenthes*, also this hybrid displays ontogeny dimorphism with lower traps (Fig. 8A) resembling *N. ampullaria* and upper traps (Fig. 8B) looking-like *N. rafflesiana* (Adam & Hamid 2006). Although it seems to be more similar to *N. ampullaria*, the opposite is true, because this hybrid takes more characteristics from *N. rafflesiana* then from *N. ampullaria* from the genetics' point of view (Yulita & Mansur 2012).

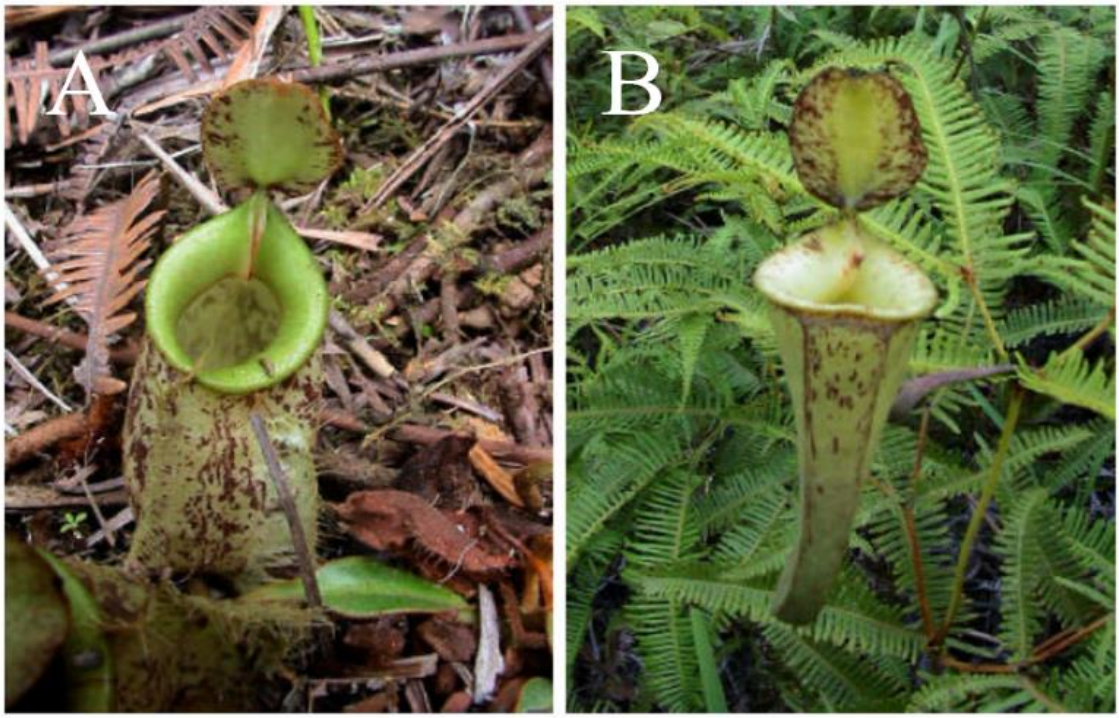


Figure 8 Pitcher shapes of *Nepenthes* × *hookeriana*; (A) urceolate shape of lower pitcher, (B) infundibulate shape of upper pitcher (Adam & Hamid 2006)

Nepenthes ventricosa Blanco is one of the most well-known representatives of the family Nepenthaceae. Its main habitat is the Philippines generally in highland areas (Gronemeyer et al. 2016). The identification is uncomplicated due to its characteristic green bulbous pitchers and tightly ridged, pink-to-red peristome (Fig. 9) (*Nepenthes ventricosa* 2024A). *Nepenthes ventricosa* is highly grown as a house plant, its maintenance is not hard so it can be frequently found in flower shops and hobby markets (*Nepenthes ventricosa* 2024B).

Figure 9 *Nepenthes ventricosa* from private collection in Denmark (Nepenthes ventricosa Blanco 2021)

Nepenthes graciliflora Elmer has been previously confused with another species – *N. alata* Blanco, but according to the only publication of Cheek & Jebb (2013), differences between these species have been clarified. Moreover, due to its high world distribution as it is the most widespread *Nepenthes* species in the Philippines, the majority of available hybrids of this species named as "hybrids of *N. alata*" are probably relatives of *N. graciliflora*. Base of the pitcher is broadest, with sudden narrowing to the long upper part. Typical ribbed peristome is reduced (Fig. 10) together with glandular crest (Cheek & Jebb 2013).

Figure 10 *Nepenthes graciliflora* (photo author)

Nepenthes × *ventrata* R. Fleming is one of the most popular hybrids of *Nepenthes* species due to its low maintenance requirements. This species is a result of natural crossbreeding of *N. ventricosa* and *N. graciliflora* (former *N. alata*). Pitchers are lightly green, with bright sunlight, can be red blushed (Nepenthes × ventrata 2023). The higher the traps are, the lighter their colouration is. Older traps tend to be coloured more (Fig. 11). This hybrid is also one of the most cultivated pitcher plants in *in vitro* conditions (Nepenthes × ventrata 2024).

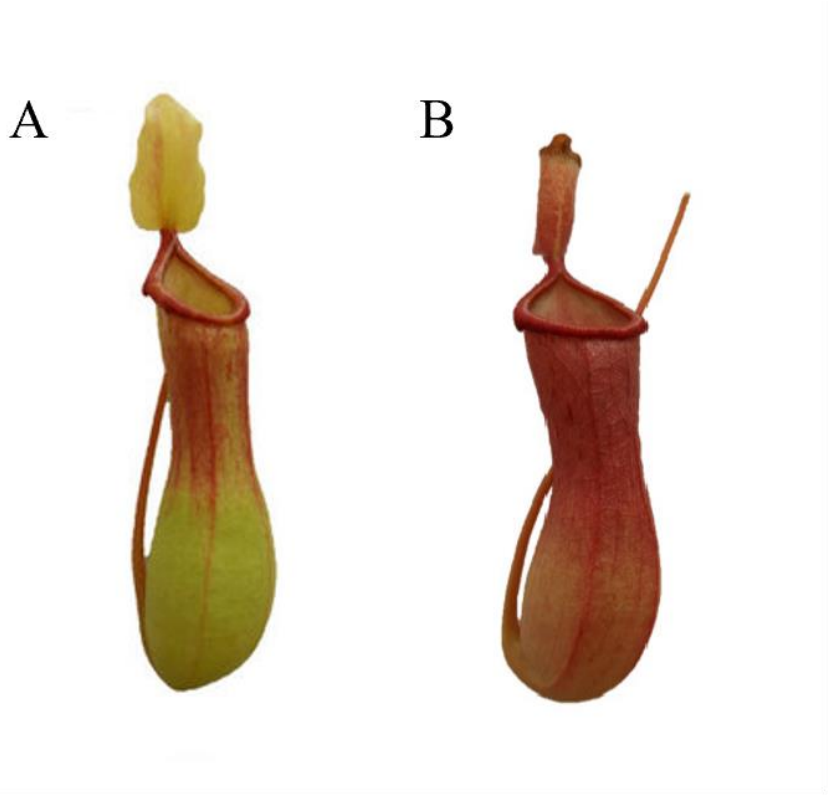


Figure 11 Traps of *Nepenthes* × *ventrata*; (A) younger trap, (B) older trap (Wal et al. 2022)

2.3.4. Ethnobotany and Bioactive Compounds in *Nepenthes*

As mentioned, pitcher plants are mainly found in Borneo and tribe people here use them almost daily. They prepare rice inside pitchers believing it adds better taste and texture (Schwallier et al. 2015). Furthermore, the utilization of pitcher plants extends beyond mere sustenance, as highlighted by the research of Platt et al. (2018). Their study sheds light on the diverse culinary practices surrounding pitcher plants, with the plants being widely embraced and consumed as vegetables in the Bornean diet. This revelation underscores the versatility of pitcher plants in local cuisine, where they are valued not only for their nutritional benefits but also for their unique taste and culinary appeal.

Through these culinary practices, pitcher plants not only serve as a source of nourishment but also contribute to the cultural identity and culinary heritage of the region.

Over the course of history, the genus *Nepenthes* has played a prominent role in traditional medicinal practices. Pitcher plants have been used for treatment of numerous illnesses since ancient times (Sanusi et al. 2017). In Ancient Greece, imported powder from Egypt made of *Nepenthes* used to be added to wine with other substances. This beverage used to bring joy and happiness, make people to forget painful memories, and had sedative effects (A Brief History of the Drugs: From the Stone Age to the Stoned Age 1999). Ancient Greek and Egyptian pharmacopoeias called this drink *nepenthes*, which means in translation "no grief", "no sorrow" or "no pain". This plant had been officially grown in gardens of Egypt (Rosso 2010).

Picher plants have their place in traditional tribes. The people from Dayak Serebuang use unopened traps with liquid to treat stomach ache and cough and use pitcher plants to cure gastroenteritis and diarrhoea (Setiawan 2015; Idamansyah 2020). Garo people apply fine paste from pitchers to treat leprosy, whereas Khasi tribe uses pitcher fluid as a digestive (Uriah et al. 2016). Beside frequent use of pitchers, consuming other parts of *Nepenthes* spp. is also reported. As the most consumed and used species is considered *N. ampullaria.* Drinking of a stem decoction of this species is an abundant step in the treatment of malaria for people in Malaysia hand in hand with root decoction applied in curing of asthma and tuberculosis (Sabran et al. 2016; Saidon et al. 2023).

The diverse effects of these plants can be attributed to the presence of natural bioactive compounds. Compounds such as kaempferol, cyanidin, quercetin, plumbagin, derivates of shinanolone etc., are reported to be presented in leaves of *Nepenthes* species (Aung et al. 2002). Beside enzymes, traps also contain droserone and 5-*O*-methyldroserone so far confirmed by Eilenberg et al. (2010). For roots is typical content of novel naphthoquinones; nepenthones – A, B, C, D, E, F, G, nepenthosides A, B and plumbagin (Cannon et al. 1980; Thanh et al. 2015; Thao et al. 2015). Overall, Ismail et al. 2015 reported presence of flavonoids, alkaloids, tannins, terpenoids and phlobatannins steroids. Most of these compounds for instance naphtoquinones are confirmed as potent antioxidants and are reported to have cytotoxicity to several cancer cell lines. Due to such wide spectrum of bioactive compounds in *Nepenthes*, it is possible that these plants will be cultivated as medicinal crops in the future (Thanh et al. 2015; Wójciak et al. 2023).

3. Aims of the Thesis

Pitcher plants are widely used in traditional medicine against stomach issues. Moreover, many studies confirmed its ability to inhibit breast cancer cells, melanoma, lung, or oral cancer cell lines. However, research about these plants in the context of the third most common cancer, colon adenocarcinoma, has not yet been assessed. *Nepenthes* species frequently contain a significant amount of plumbagin, which has anticancer properties. We hypothesize that pitcher plants can also contribute to the inhibition of colon adenocarcinoma cell lines. Moreover, the hybrids should provide better results than parents.

The main aim of this study was to determine the antiproliferative effect of various *Nepenthes* species against colon adenocarcinoma cell lines HT29 and Caco-2 and assess the cytotoxicity to healthy human intestine cell line CCD 841 CoN. Given the fact, that free radicals may play a role in the development of cancer, the ability of extracts to inhibit these radicals was evaluated by DPPH antioxidant assay. In addition, the chemical analysis was also performed to discover differences between parent and hybrid plants and to clarify the presence of plumbagin, which is often mentioned as the principal active compound in *Nepenthes* species.

The specific aims are:

a) Preparation of ethanolic extracts from various plant parts of each *Nepenthes* species.
b) Determination of the cytotoxicity of obtained extracts species using MTT assay against two cancer cell lines and one healthy cell line.
c) Assession of the antioxidant activity of selected *Nepenthes* species using DPPH assay.
d) Providing chemical analyses with a focus on plumbagin.

4. Materials and Methods

4.1. Plant Material

In total, six *Nepenthes* species have been analysed. *N. ampullaria* Jack, *N. rafflesiana* Jack, *N. ventricosa* Blanco, *N.* × *ventrata* R. Fleming (*N. ventricosa* × *N. graciliflora*) (Cheek & Jebb 2013) were collected at the Botanical Garden in Troja, *N.* × *hookeriana* H. Low (*N. ampullaria* × *N. rafflesiana*) was obtained from private collection and *N. graciliflora* Elmer was acquired from the Botanical Garden of Charles University in Prague, Czech Republic. The plants were cultivated under controlled and stable conditions in local greenhouses (temperature approximately 30°C, humidity 80-90 %). Fresh plant material of each species was divided into four parts (if possible) – trap, leaf, stem, root (Table 1), then air-dried for several days in a dryer at 40°C to full dryness, and afterward stored in paper packs until further use.

4.2. Extracts Preparation

Air-dried plant material was finely grinded using Grindomix apparatus (GM100 Retsch, Haan, Germany) and subsequently 5 g of each plant powder was extracted in 150 ml of 80 % ethanol for 24 h using a laboratory orbital shaker (GFL3005, GFL, Burgwedel, Germany). A total of twenty ethanolic extracts were produced, with each extract being filtered and then concentrated until dryness using a rotary evaporator R-200 (Buchi, Switzerland) under vacuum at 40°C. The dry residues were dissolved in 100 % dimethyl sulfoxide (DMSO) to a stock concentration of 51.2 mg/mL and stored at -80°C until further evaluation. The weight of the herbs utilized and the weight of the dry residue after extraction were used to compute the % yield of extracts.

4.3. Cell Cultures

All cell cultures were purchased from the American Type Culture Collection (Rockville, MD, USA). Caco-2 cells were cultured in Eagle's Minimum Essential Medium (EMEM) and HT29 cell lines were nurtured in Dulbecco's Modified Eagle Medium, enriched with 20 % (Caco-2) and 10 % (HT29) fetal bovine serum (FBS), 1 mM sodium

bicarbonate, 1 mM sodium pyruvate, 1 % non-essential amino acids, and a 1 % solution of penicillin-streptomycin (containing 10,000 units/mL of penicillin and 10 mg/mL of streptomycin from Sigma-Aldrich (St. Louis, MO, USA). As for the normal cells, CCD 841 CoN cells were sustained in EMEM, complemented with 10 % FBS, 2.5 mM sodium bicarbonate, 0.5 mM sodium pyruvate, a 1 % solution of penicillin-streptomycin, and 10 mM glutamine. The cultures were maintained at 37°C with 5 % CO_2, and the culture media were refreshed every 2–3 days, with passage occurring every 7 days.

4.4. Cytotoxicity Assay

Cell viability was assessed through the colorimetric MTT assay, originally devised by Mosmann in 1983, with certain modifications. Caco-2, HT29 and CCD 841 CoN cells were seeded in 96-well plates at a density of 2.5×10^3 cells per well. Following a 24 h incubation period, the cells were exposed to two-fold serially diluted samples ranging from 0.24 to 500 μg/mL for 72 h. Subsequently, the previous medium in each well was replaced with fresh EMEM or Hybri-Care medium containing MTT reagent (1 mg/mL). The plates were then incubated for an additional 2 h at 37°C and after that, formazan crystals were dissolved in 100 μL of DMSO. The absorbance was measured at 555 nm using a Tecan Infinite M200 spectrometer (Tecan Group, Mannedorf, Switzerland). The % mortality for each concentration of genotype was plotted and utilized to determine the 50 % inhibitory concentration (IC_{50} value) with standard deviation (±SD) in μg/mL. All samples were tested as duplicates in three independent tests.

4.5. Antioxidant Assay

The assessment of antioxidant activity in the extracts was conducted following the approach outlined by Sharma and Bhat (2009), which measures their ability to inhibit DPPH radicals. Trolox (control) and sample working solutions were prepared at 512 μg/mL concentration in methanol. Employing the Freedom EVO 100 pipetting platform (Tecan, Mannedorf, Switzerland), each sample underwent a two-fold serial dilution in 96-well microtiter plates. Subsequently, 75 μL of methanol and 25 μL of 1 mM DPPH in methanol were dispensed into each well. The final concentration range for both

samples and Trolox in the microtiter plate spanned from 0.125 to 256 μg/mL. Incubation of the plates occurred in the dark at room temperature (25°C) for 30 min. Subsequent spectrophotometric measurement of absorbance at 517 nm was conducted using a Multimode Reader Cytation 3 (BioTek Instruments, Winooski, VT, USA). The experiments were conducted in triplicate as three independent tests, and the results were expressed as the mean of IC_{50} ±SD in μg/mL.

4.6. LC-MS/MS Analysis with UV

Each DMSO extract was diluted in 80 % ethanol to a final concentration of 51.2 μg/mL and subsequently filtered through a 0.22 μm polytetrafluoroethylene membrane filter. Liquid chromatography tandem mass spectrometry analysis with ultraviolet light (UV LC-MS/MS) was performed using a Vanquish™ Flex UHPLC System coupled to an Orbitrap ID-X Tribrid mass spectrometer, equipped with heated electrospray ionization (H-ESI). UV absorbance was measured at 254 and 390 nm. The LC column used was Waters ACQUITY BEH C18 (2.1 x 150 mm, 1.7 μm) with a flow gradient of 0.350 mL/min, column oven temperature of 40°C, and an injection volume of 1 μL. The solvents used were water with 0.1 % formic acid (A) and acetonitrile with 0.1 % formic acid (B). The following method was used for the analysis of all the samples: linear gradient of 5 to 100 % B over 15.5 min, isocratic at 100 % B for 2 min, and 2 min at 5 % B. H-ESI was achieved in positive mode and with following mass spectrometer parameters: ion transfer tube temperature set to 325°C, auxiliary gas flow rate at 10 L/min, vaporizer temperature set to 350°C, sheath gas flow rate set to 50 L/min, capillary voltage set to 3000 V, MS resolution at 60 000, quadrupole isolation, scan range from m/z 100-1000, RF Lens set to 45 %, and a maximum injection time of 118 ms.

The standard of plumbagin was prepared as a 2 % solution, transferred into vial, and subsequently detected by using UV LC-MS/MS analysis. The samples, where the plumbagin peak was detected based on the mass-to-charge ratio (m/z), were run again with the use of UV.

Data processing

1) Raw data were converted through MZConvert programme, as an output directory was selected *mzML* format. Further processing was done in program MZmine developed by Pluskal et al. (2010).

2) The converted *.mzML* files were used as input in MZmine processing. The output consisted of a *.cdv* file containing information about all detected features (intensity, retention time) and *.mgf* text file that contains information about precursor mass, charge, m/z and abundance of peaks.

3) The *.mzML* files were imported through *Raw data methods → Raw data import → .mzML.*, then saved.

4) To create a file for further use in SIRIUS-GUI (Fig. 12), it was important to load the previously downloaded *.xml* file and in the *Batch queue* provide the *Export/Submit to SIRIUS* location where the output files were stored.

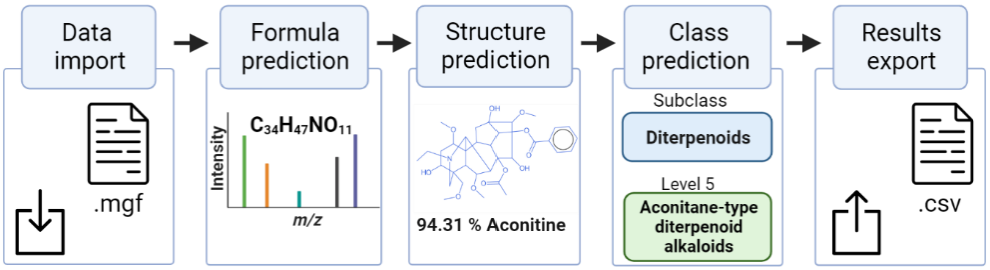


Figure 12 Schematic workflow of the main steps of SIRIUS analysis (photo Lana Mutabdžija)

5) The *.mgf* output file from MZmine was imported to SIRIUS-GUI and to analyse all imported features was done by clicking *Compute all*.

Figure 13 Parameter settings for SIRIUS-GUI run. Each panel (**a**–**d**) refers to different modules within the SIRIUS suite (SIRIUS, ZODIAC, CSI:FingerID and CANOPUS). (**a**) The SIRIUS module generates molecular formula candidates for each feature and is database-independent. (**b**) ZODIAC ranks the best formula for each feature. (**c**) CSI:FingerID provides each feature's structural information (i.e. fingerprint), which is database-independent, and searches structure databases for a structure which best matches the feature's predicted fingerprint. (**d**) The fingerprint information is used by CANOPUS to predict the compound class (photo author)

6) New pop-up window with parameters opened, where could be parameters for each stage of the run adjusted (Fig. 13). Then select Compute.

7) For our analyses general parameters were selected as: *Instrument:* Orbitrap, *Use DD formulas:* all, *Possible Ionization:* all, *Predicted FBs:* all, *CSI:FingerID:* all.

8) In each analysed feature can be seen SIRIUS module prediction, ZODIAC module prediction, CSI:FingerID fingerprint prediction in databases and CANOPUS class assignments of the compounds. For our purposes, two modules and class assignments were used.

5. Results

5.1. Antiproliferation Against Cancer Cell Lines and Cytotoxicity

In this study, 20 extracts derived from different parts of six *Nepenthes* species were obtained in yields ranging from 0.28 % to 3.93 % (*v*/*w*). The results of the MTT assay performed with colorectal cancer cell lines and healthy cells, including extract yields, are listed in Table 1. The strongest antiproliferative activity in both cancer cell lines showed the root extract of *Nepenthes* × *hookeriana* (IC_{50} 10.59 μg/mL for HT29 and 2.68 μg/mL for Caco-2). Other potent species with IC_{50} values ranging from 22.91 μg/mL to 32.74 μg/mL were *N. ventricosa* leaf and trap, *N.* × *ventrata* stem, and root of *N. graciliflora*. As for the moderate IC_{50} values, ranging from 45.79 μg/mL to 86.83 μg/mL were *N. ampullaria* (trap, leaf, stem, root), *N. ventricosa* (trap, leaf) and trap from *N.* × *hookeriana*. The rest of the results were considered weak (IC_{50} >90 μg/mL). Almost all samples did not affect healthy human intestinal cells (IC_{50} >512 μg/mL), except stem extract from *N.* × *ventrata* with IC_{50} 21.81 μg/mL. Thus, the rest of the samples could be interpreted as non-harmful (root of *N.* × *hookeriana*, stem and root of *N. graciliflora*, trap and leaf of *N. ventricosa*, trap, leaf and root of *N.* × *ventrata*).

5.2. Antioxidant Activity of Extracts

The ability of the radical inhibition of each *Nepenthes* extracts is shown in Table 1. Although almost all the samples exhibited antioxidant activity, the most active ones were leaf extracts of *Nepenthes ampullaria* with IC_{50} 13.61 μg/mL and of *Nepenthes* × *hookeriana* with a 15.36 μg/mL value of IC_{50}. Moreover, other plant parts of *Nepenthes ampullaria* showed remarkable antioxidant activity with IC_{50} ranging from 22.24 to 49.07 μg/mL. Similarly, strong activity could be noted for *N. graciliflora* (root, stem, trap), *N. ventrata* (stem, root), *N.* × *hookeriana* (trap), and *N. rafflesiana* (leaves and root) with IC_{50} values from 21.16 to 34.06 μg/mL. Other samples, including *N. ventricosa* (leaves), *N.* × *hookeriana* (root), and of *N. ventrata* (leaves) provided moderate antioxidant effect with IC_{50} from 46.82 to 52.71 μg/mL. The rest of the samples exhibited only a weak ability to inhibit DPPH (IC_{50} 99.63 – 216.33 μg/mL)

Table 1 Antiproliferation, cytotoxicity and antioxidant activity of *Nepenthes* species

Species	Part tested	Yield (%)	MTT assay IC_{50} ± SD µg/ml			DPPH assay IC_{50} ± SD µg/ml
			HT29	**Caco-2**	**CCD 841 CoN**	
N. ampullaria	trap	15.64	86.83 ± 5.26	148.66 ± 14.64	>512	49.07 ± 15.57
	leaf	39.28	45.79 ± 4.28	145.20 ± 14.54	>512	13.61 ± 1.41
	stem	11.34	68.51 ± 6.58	187.00 ± 1.15	>512	25.57 ± 1.86
	root	34.45	62.39 ± 1.66	49.33 ± 0.04	>512	22.24 ± 4.37
N. rafflesiana	trap	5.76	138.71 ± 4.39	249.68 ± 22.54	>512	204.19 ± 21.28
	leaf	18.88	146.80 ± 7.26	>512	>512	33.86 ± 8.92
	stem	9.29	135.92 ± 9.29	>512	>512	33.03 ± 10.40
N.* × *hookeriana	trap	13.96	74.40 ± 2.37	63.53 ± 1.55	>512	21.97 ± 2.89
	leaf	25.63	179.40 ± 15.85	>512	>512	15.36 ± 2.00
	root	9.30	10.59 ± 0.46	2.68 ± 0.65	172.38 ± 3.86	52.60 ± 16.54
N. graciliflora	trap	15.35	120.38 ± 2.81	239.84 ± 46.24	>512	34.06 ± 13.32
	leaf	25.67	138.01 ± 7.61	>512	>512	101.15 ± 41.36
	stem	9.53	119.37 ± 59.02	241.42 ± 4.88	165.26 ± 14.68	30.77 ± 9.79
	root	18.36	32.10 ± 4.62	29.79 ± 0.14	40.93 ± 18.37	21.16 ± 2.04
N. ventricosa	trap	11.26	24.15 ± 4.10	53.36 ± 0.99	52.44 ± 10.50	99.63 ± 20.01
	leaf	20.5	22.91 ± 0.63	73.04 ± 2.22	73.65 ± 23.00	46.82 ± 10.38
N.* × *ventrata	trap	6.00	97.86 ± 7.11	168.89 ± 53.24	118.98 ± 17.96	216.33 ± 19.66
	leaf	9.57	274.93 ± 4.91	340.59 ± 35.47	289.01 ± 36.30	52.71 ± 13.11
	stem	15.62	27.11 ± 5.89	92.73 ± 6.56	21.81 ± 9.77	22.75 ± 4.49
	root	22.28	32.74 ± 4.14	31.54 ± 0.63	84.51 ± 13.94	22.26 ± 4.02
Vinorelbine			NT	22.67 ± 4.75	43.75 ± 3.03	
5-fluorouracil			6.29 ± 3.04	179.81 ± 23.50	NT	
Trolox						9.30 ± 0.96

Footnotes: *N.* stands for *Nepenthes*; MTT: 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; IC_{50}: half maximal inhibitory concentration; HT29: colorectal carcinoma human cell line; Caco-2: colorectal carcinoma human cell line; CCD 841 CoN: healthy intestinal cells; DPPH: 2,2-diphenyl-1-picrylhydrazyl; NT stands for "not tested"

5.3. Chemical Analyses

According to LC-MS/MS analysis in all extracts were detected 4,561 features, and the major classes of bioactive compounds in all extracts were flavonoids, phenolic compounds or alkaloids. Despite the similarities in feature content with its parents, *N.* × *hookeriana* contained 45 compounds which were not presented in either parent, and *N.* × *ventrata* had 31 more compounds than its parents (see Table 2). The presence of plumbagin was evaluated in roots of *N. graciliflora*, *N. ventricosa* and of *N.* × *hookeriana*, however there was a lower intensity compared to the 2 % standard. The retention time was shifted in the root sample of *N.* × *hookeriana* which suggested the presence of some plumbagin isomer (Figure 14). Overall, there was a low abundance of this compound.

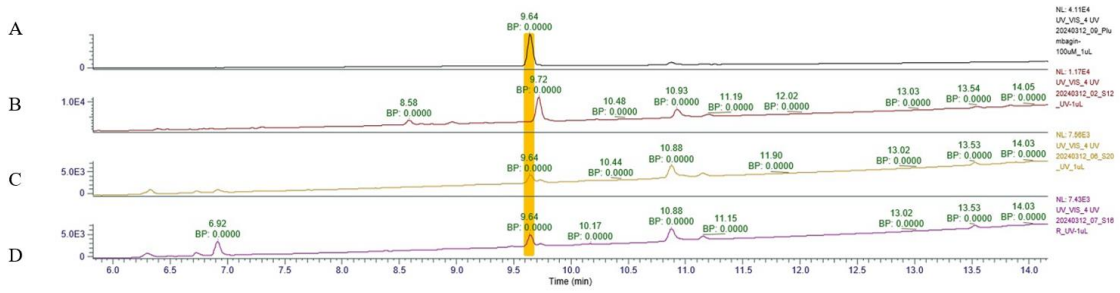
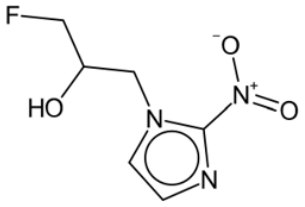
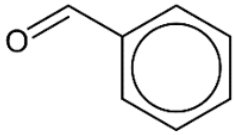
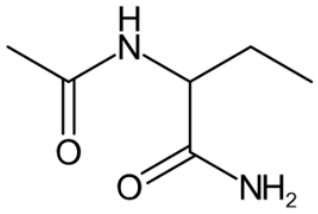
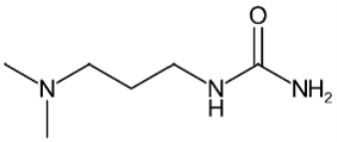
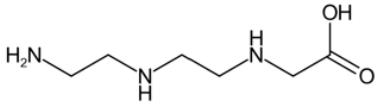
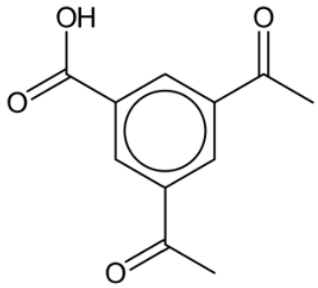
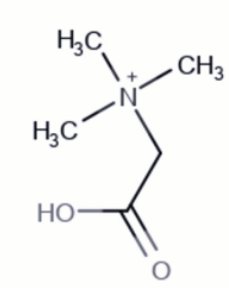
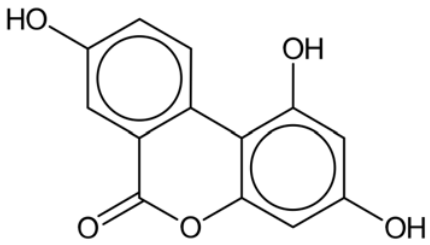
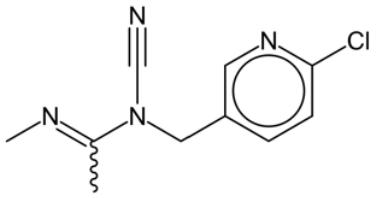
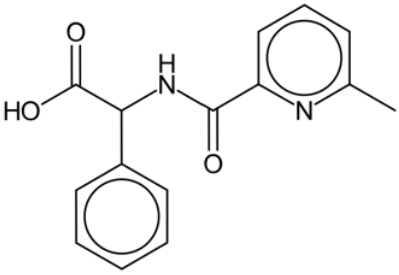
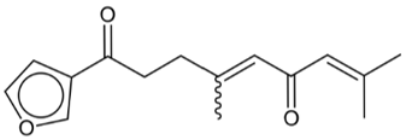
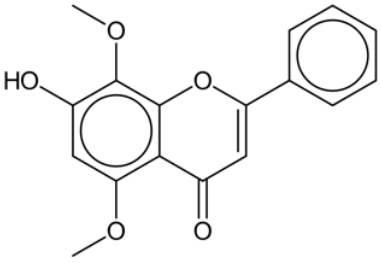
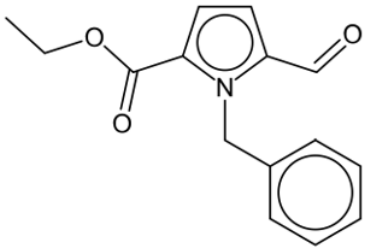
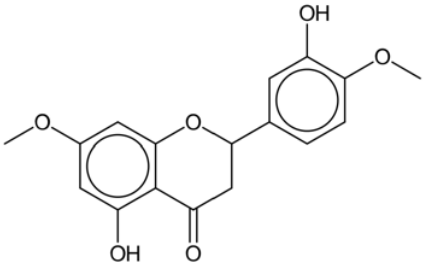
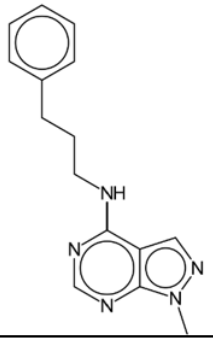
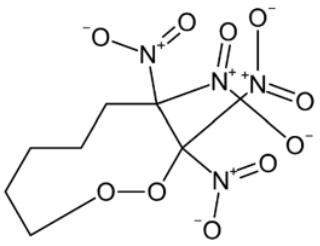
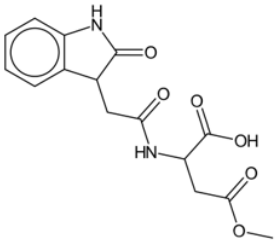
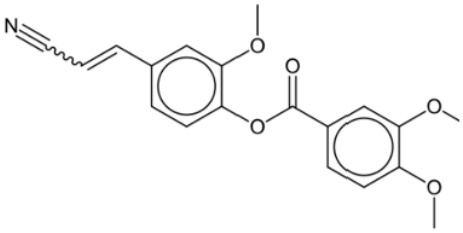
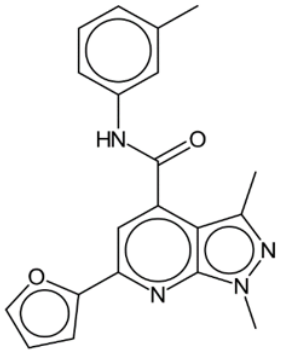
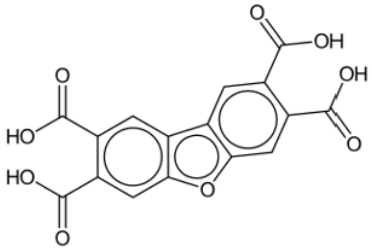
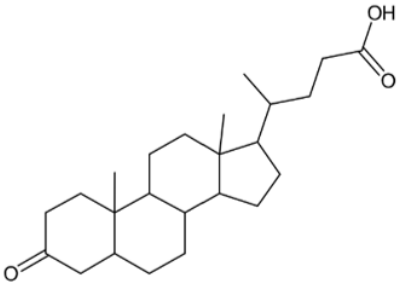
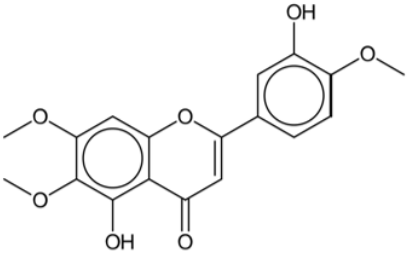
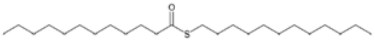


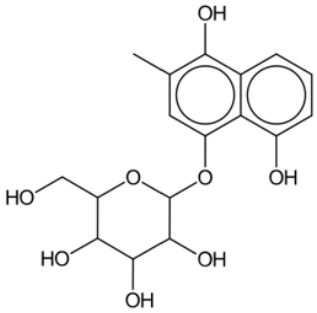
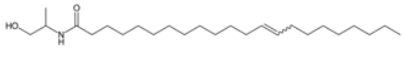
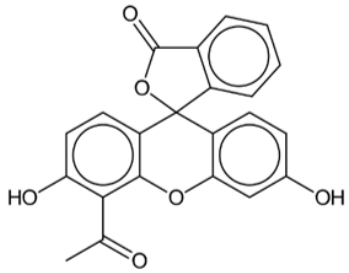
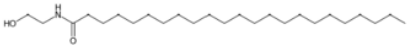
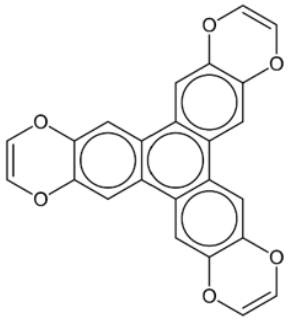
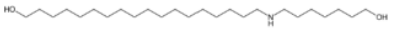
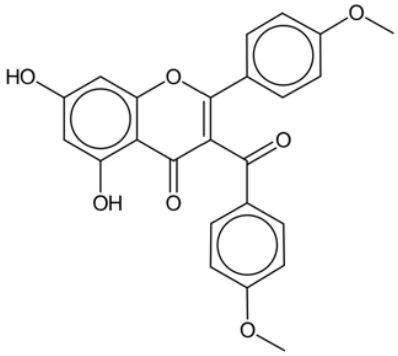
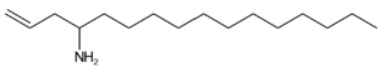
Figure 14 Plumbagin detection by UV LC-MS/MS analysis in extract of (A) plumbagin standard, (B) roots of *Nepenthes* × *hookeriana*, (C) roots of *Nepenthes* × *ventrata* (D) roots of *Nepenthes graciliflora*

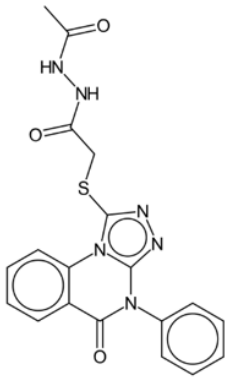
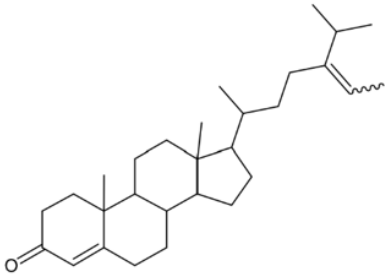
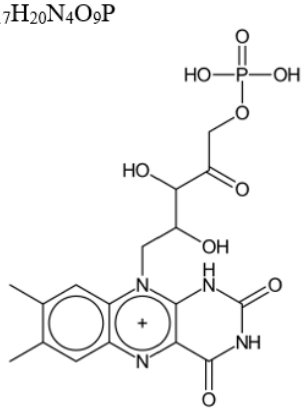
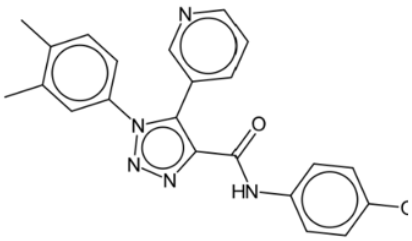
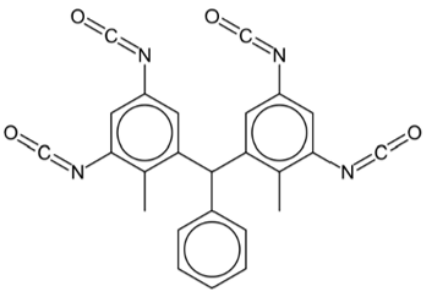
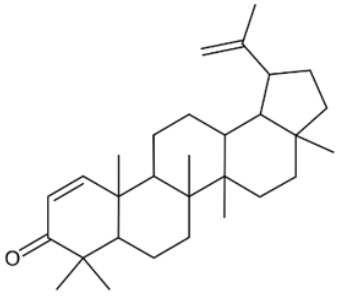
Table 2 Chemical structures, formulas, and names of extra compounds in hybrid plants

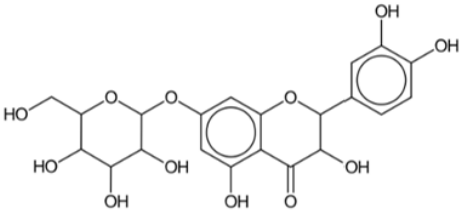
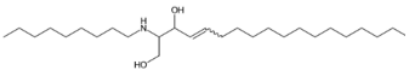
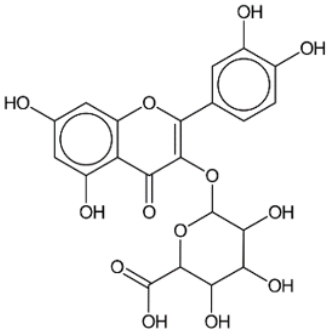
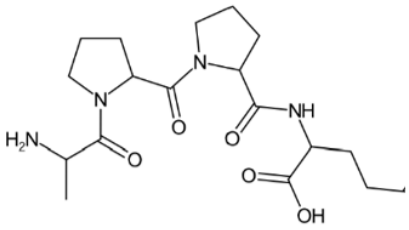
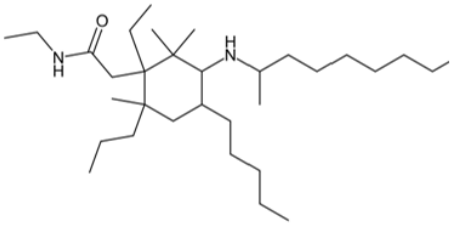
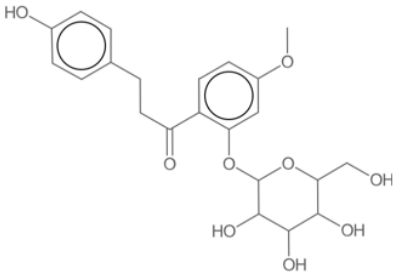
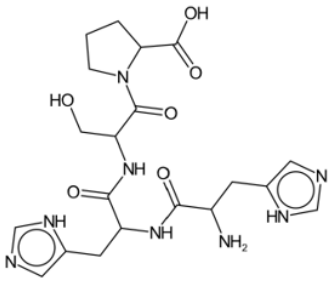
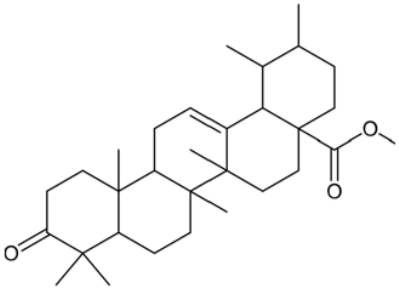
Compound Order	Species	
	N.* × *hookeriana	***N.* × *ventrata***
1	Fluoromisonidazole $C_6H_8FN_3O_3$ 	Benzaldehyde C_7H_6O 
2	2-acetamidobutanamid $C_6H_{12}N_2O_2$ 	
3	3-dimethylaminopropylurea $C_6H_{15}N_3O$ 	
4	Glycine, N-[2-[(2-aminoethyl)amino]ethyl]- $C_6H_{15}N_3O_2$ 	
5	3,5-diacetylbenzoic acid $C_{11}H_{10}O_2$ 	Betaine monohydrate $C_5H_{13}NO_3$ 

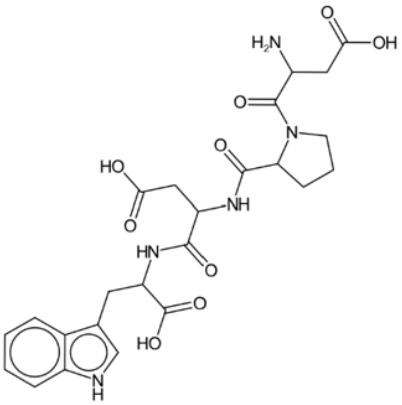
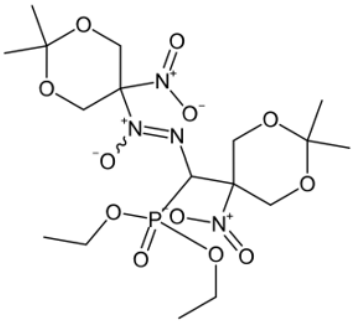
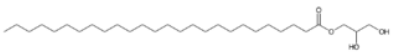
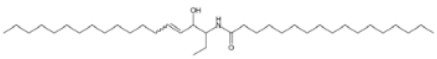
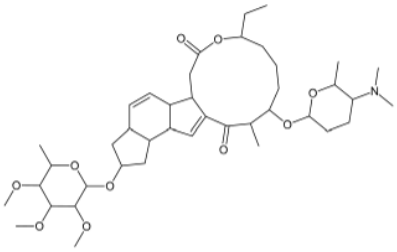
6	1,3,8-trihydroxybenzo[c]chromen-6-one $C_{13}H_8O_5$ 	N-[(6-chloropyridin-3-yl)methyl]-N-cyano-N”-methylethanimidamide $C_{10}H_{11}ClN_4$ 
7	2-[(6-methylpyridine-carbonyl)amino]-2-phenylacetic acid $C_{15}H_{14}N_2O_3$ 	4,7-Nonadiene-1,6-dione $C_{15}H_{18}O_3$ 
8	Norwogonin 5,8-dimethyl ether $C_{17}H_{14}O_5$ 	Ethyl 1-benzyl-5-formylpyrrole-2-carboxylate $C_{15}H_{15}NO_3$ 
9	Persicogenin $C_{17}H_{16}O_6$ 	1-methyl-N-(3-phenylpropyl)pyrazolo[3,4-d]pyrimidin-4-amine $C_{15}H_{17}N_5$ 

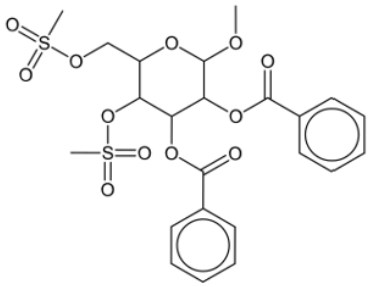
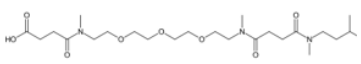
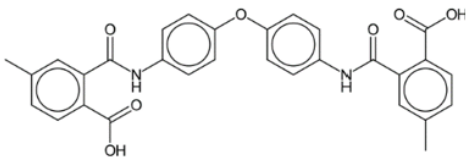
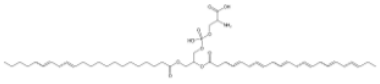
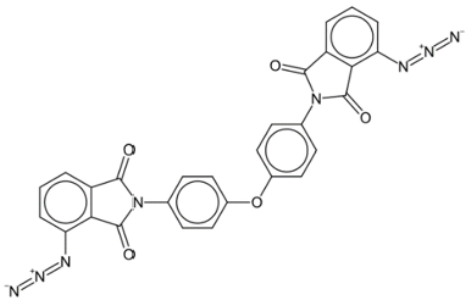
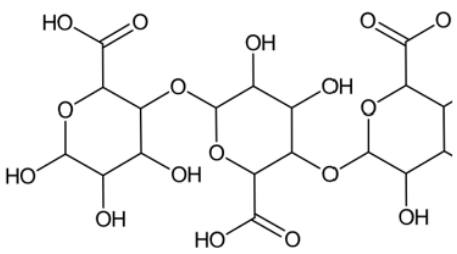
10	3,3,4,4-tetranitrodioxonane $C_7H_{10}N_4O_{10}$ 	4-methoxy-4-oxo-2-[[2-(2-oxo-1,3-dihydroindol-3-yl)acetyl]amino]butanoic acid $C_{15}H_{16}N_2O_6$ 
11	[4-[(Z)-2-cyanoethenyl]-2-methoxyphenyl]3,4-dimethoxybenzoate $C_{19}H_{17}NO_5$ 	6-(furan-2-yl)-1,3-dimethyl-N-(3-methylphenyl)pyrazolo[3,4-b]pyridine-4-carboxamide $C_{20}H_{18}N_4O_2$ 
12	2,3,7,8-Dibenzofurantetracarboxylic acid $C_{16}H_8O_9$ 	3-keto-LCA $C_{24}H_{38}O_3$ 
13	Eupatorin $C_{18}H_{16}O_7$ 	Dodecanethioic acid $C_{24}H_{48}OS$ 

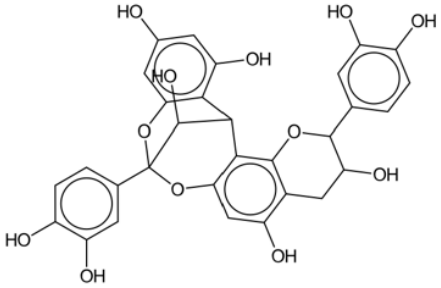
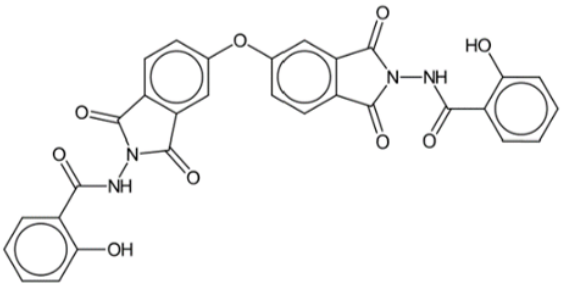
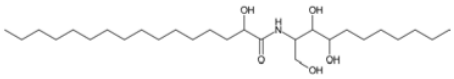
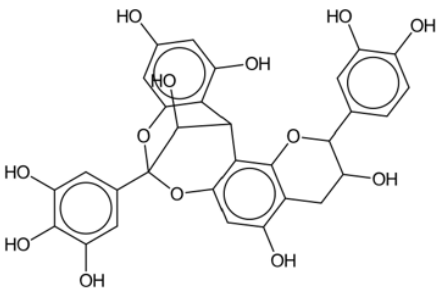
14	(2S,3R,4S,5S,6R)-2-(4,8-dihydroxy-3-methylnaphtalen-1-yl)oxy-6-(hydroxymethyl)oxane-3,4,5-triol $C_{17}H_{20}O_8$ 	(Z)-N-((2R)-1-hydroxypropan-2-yl)docos-13-enamide $C_{25}H_{49}NO_2$ 
15	4”-acetyl-3”,6”-dihydroxispiro[benzofuran-3,9”-xanthene]-1-one $C_{22}H_{14}O_6$ 	Tricosanoyl-EA $C_{25}H_{51}NO_2$ 
16	Triphenyleno[2,3-b:6,7-b”:10,11-b””]tris[1,4]dioxin $C_{24}H_{12}O_6$ 	18-(7-hydroxyheptylamino)octadecane-1-ol $C_{25}H_{53}NO_2$ 
17	3-p-Anisoyl Acacetin $C_{24}H_{18}O_7$ 	hexadec-1-en-4-amine $C_{16}H_{33}N$ 

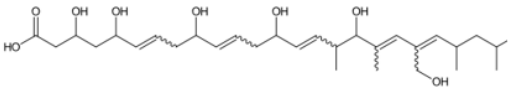
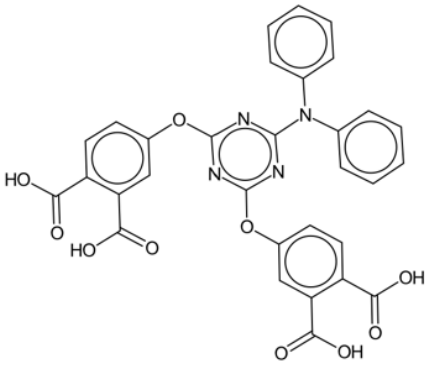
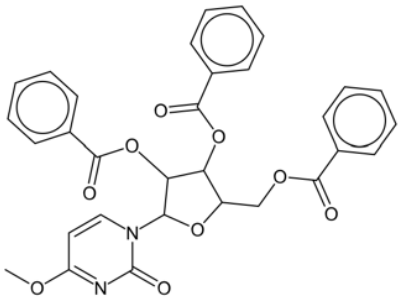
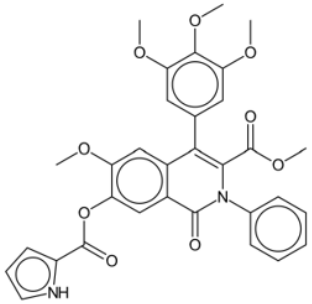
18	N”-acetyl-2-[(5-oxo-4-phenyl-[1,2,4]triazolo[4,3-a]quinazolin-1-yl)sulfanyl]acetohydrazide $C_{19}H_{16}N_6O_3S$ 	Fucostenone $C_{29}H_{46}O$ 
19	[(3S,4S)-5-(7,8-dimethyl-2,4-dioxo-1H-benzo[g]pteridine-10-ium-10-yl)-3,4-dihydroxy-2-oxopentyl]dihydrogen phosphate $C_{17}H_{20}N_4O_9P$ 	1-(3,4-dimethylphenyl)-N-(4-ethoxyphenyl)-5-(pyridin-3-yl)-1H-1,2,3-triazole-4-carboxamide $C_{24}H_{23}N_5O_2$ 
20	Benzene 1,1” -(phenylmethylene)bis[3,5-diisocyanato-2-methyl]- $C_{25}H_{16}N_4O_4$ 	Glochidone $C_{30}H_{46}O$ 

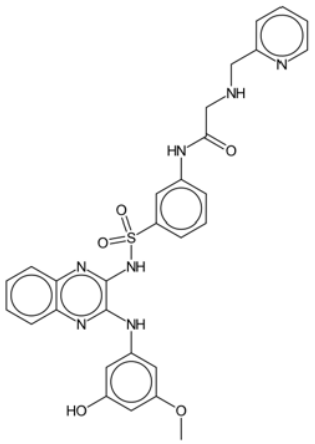
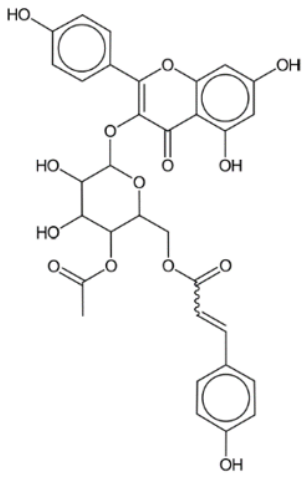
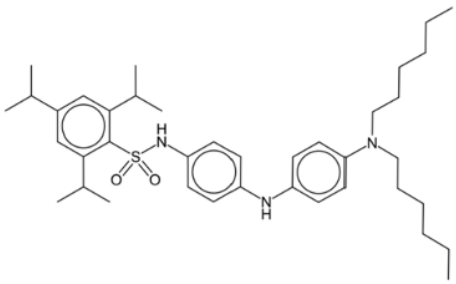
21	Quercetin-7-glucoside $C_{21}H_{22}O_{12}$ 	C-8 Ceramine $C_{27}H_{55}NO_2$ 
22	Mikwelianin $C_{21}H_{18}O_{13}$ 	5-amino-2-[[1-[1-(2-aminopropanoyl)pyrrolidine-2-carbonyl]pyrrolidine-2-carbonyl]amino]-5-oxopentanoic acid $C_{18}H_{29}N_5O_6$ 
23	2-[3-(decan-2-ylamino)-1-ethyl-2,2,6-trimethyl-4-pentyl-6-prpylcyclohexyl]-N-ethylacetamide $C_{33}H_{66}N_2O$ 	4”-O-Methyldavidioside $C_{22}H_{26}O_9$ 
24	1-[2[[2-[[2-amino-3-(1H-imidazol-5-yl)propanoyl]amino]-3-hydroxypropanoyl]pyrrolidine-2-carboxylic acid $C_{20}H_{28}N_8O_6$ 	Methyl 3-oxours-12-en-28-oate $C_{31}H_{48}O_3$ 

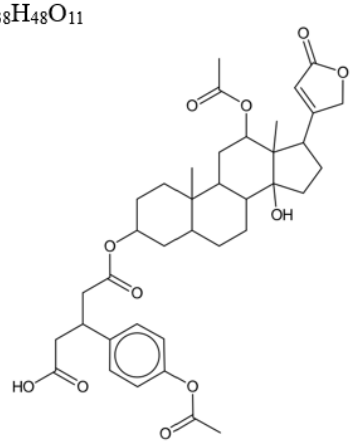
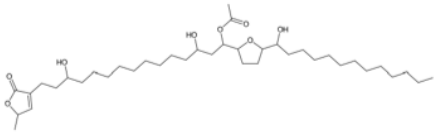
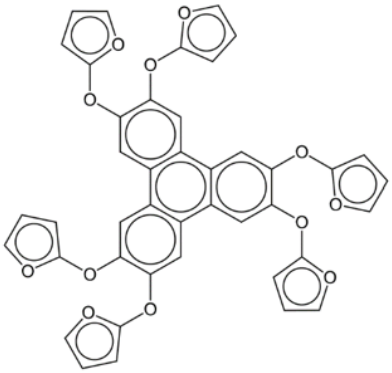
25	3-amino-4-[2-[[3-carboxy-1[[1-carboxy-2-(1H-indol-3-yl)ethyl]amino]-1-oxopropan-2-yl]carbamoyl]pyrrolidine-1-yl]-4-oxobutanoic acid $C_{24}H_{29}N_5O_9$ 	
26	[diethoxyphospforyl-(2,2-dimethyl-5-nitro-1,3-dioxan-5-yl)methyl]imino-(2,2-dimethyl-5-nitro-1,3-dioxan-5-yl)-oxidoazanium $C_{17}H_{31}N_4O_{12}P$ 	2,3-dihydroxypropyl hexacosanoate $C_{29}H_{58}O_4$ 
27	N-[(E)-hydroxynonadec-5-en-3-yl]heptadecanamide $C_{36}H_{71}NO_2$ 	Spinosyn A $C_{41}H_{65}NO_{10}$ 

28	Methyl 2,3-di-O-benzoyl-4,6-bis-O-(methylsulfonyl) hexopyranoside $C_{23}H_{26}O_{12}S_2$ 	4-[methyl-[2-[2-[2-[2-[methyl-[3,7,11,15-tetramethylhexadecyl)amino]-4-oxobutanoyl]amino]ethoxy]ethyl]amino]-4-oxobutanoic acid $C_{39}H_{75}N_3O_8$ 
29	2-[[4-[4-[(2-carboxy-5-methylbenzoyl)amino]phenoxy]phenyl]carbamoyl]-4-methylbenzoic acid $C_{30}H_{24}N_2O_8$ 	1-(13Z,16Z-docosadienoyl)-2-(4Z,7Z,10Z,13Z,16Z,19Z-docosahexaenoyl)-glycero-3-phosphoserine $C_{50}H_{82}NO_{10}P$ 
30	4-azido-2-[4-[4-(4-azido-1,3-dioxoisoindol-2-yl)phenoxy]phenyl]isoindole-1,3-dione $C_{28}H_{14}N_8O_5$ 	
31	Trigalacturonate $C_{18}H_{26}O_{19}$ 	✕

32	Procyanidin A2 $C_{30}H_{24}O_{12}$ 	×
33	2-hydroxy-N-[5-[2-[(2-hydroxybenzoyl)amino]-1,3-dioxoisoindol-5-yl]oxy-1,3-dioxoisoindol-2-yl]benzamide $C_{30}H_{18}N_4O_9$ 	
34	(2R)-2-hydroxy-N-()3S,4R)-1,3,4-trihydroxy-16-methyloctadecan-2-yl)hexadecanamide $C_{35}H_{71}NO_5$ 	×
35	Epigallocatechin-(2beta->7,4beta->8)-epicatechin $C_{30}H_{24}O_{13}$ 	×

36	Phomenoic acid $C_{34}H_{58}O_8$ 	×
37	4-[[4-(3,4-dicarboxyphenoxy)-6-(N-phenylanilino)-1,3,5-triazin-2 yl] oxy] phthalic acid $C_{31}H_{20}N_4O_{10}$ 	×
38	4-methoxy-1-(2,3,5-tri-o-benzoyl]pentofuranosyl)pyrimidin-2(1h)-one $C_{31}H_{26}N_2O_9$ 	×
39	Methyl 6-methoxy-1-oxo-2-phenyl-7-(1H-pyrrole-2-carbonyloxy)-4-(3,4,5-trimethoxyphenyl)isoquinoline-3-carboxylate $C_{32}H_{28}N_2O_9$ 	×

40	4-[3-[[3-(-hydroxy-5-methoxyanilino)quinoxaline-2-yl]sulfamoyl]phenyl]-2-(pyridine-2-ylmethylamino)acetamide $C_{29}H_{27}N_7O_5S$ 	×
41	Kaempferol 3-(4”-acetyl-6”-p-coumarylglucoside) $C_{32}H_{28}O_{14}$ 	×
42	N-(4-(4-(dihexylamino)anilino)phenyl)-2,4,6-tri(propan-2-yl)benzenesulfonamide $C_{38}H_{59}N_3O_2S$ 	×

43	5-[[(3S,5R,8R,9S,10S,12R,13S,17R)-12-acetyloxy-14-14-hydroxy-10,13-dimethyl-17-(5-oxo-2H-furan-3-yl)-1,2?3,4,5,6,7,8,9,10,11,12,15,16,17-tetradecahydrocyclopenta[a]phenathren-3-yl]oxy]-3-(4-acetyloxyphenyl)5-oxopentanoic acid $C_{38}H_{48}O_{11}$ 	×
44	Uvaribonin $C_{39}H_{70}O_8$ 	×
45	2-[3,6,7,10,11-pentakis(furan-2-yloxy)triphenylene-2-yl]oxyfuran $C_{42}H_{24}O_{12}$ 	×

Footnotes: *N.* stands for *Nepenthes*

6. Discussion

Nepenthes spp. are currently one of the most investigated plants for their content of various compounds with bioactive activities (Devi et al. 2016). Previous studies have raised an interest in the use of *Nepenthes* species as a new source of antitumor agents. The anticancer activity of these plants has been reported in several cancer cell lines, but the evaluation of activity against colorectal cell lines has never been mentioned before. Despite the abundance of wild species, the selection of the specific species used in this study was based on local availability, focusing on the activity of *Nepenthes* spp. observed so far (Yang et al. 2021; Liu et al. 2021; Lin et al. 2023) and potential differences between parent plants and their hybrids. Studies reported cytotoxicity of aerial plant parts as whole extract (Ou-Yang et al. 2021), thus focusing on dividing of the plant parts, including roots, was crucial for strong valid results. Although none of the species/hybrids evaluated in our study have ever been tested for anticancer activity, some of them have been reported as useful remedies for stomach pain or gastroenteritis (Miguel et al. 2018; Idamansyah 2020) which influenced the aim of this study to test these species against colorectal cancer. However, the incorporation of pitcher plant leaves or roots in traditional medicine has not been acknowledged in any scientific publication. Thus, ethnobotanical studies are recommended to facilitate additional research on these captivating plants and enhance our understanding of their potential medicinal properties.

Our results discovered that the most active extract against cancer cell lines was the one from the roots of *Nepenthes* × *hookeriana*. In many studies, IC_{50} values of pitcher plants ranged from 8 μg/mL to 32 μg/mL (Tang et al. 2019; Ou-Yang et al. 2019; Peng et al. 2020). Our results (IC_{50} 2.68 μg/mL) reported for the first time such high activity in *Nepenthes* species. This discovery naturally rised the question of the presence of plumbagin, which is frequently reported in many studies of pitcher plants and has strong antitumor activity, especially against Caco-2 cell line (Kuete et al. 2016; Sanusi et al. 2017; Devi et al. 2019).). Based on this assumption we performed a DPPH assay to investigate the connection between antiproliferative and antioxidant activity. In the study by Keute et al. (2016), the IC_{50} of plumbagin was determined as 13.17 μg/mL against Caco-2. Sundari et al. (2017) described the antioxidant activity of an extract containing 1.3 % of plumbagin with IC_{50} 3.99 μg/ml. Both of the assays should indicate

low numbers at the same time, but our results indicated low numbers in case of cancer cell lines and significantly higher in antioxidant activity and vice versa. There was no connection between these two assays performed which has clearly supported our theory. Cytotoxicity to cancer cells was remarkably distinct, although the antioxidant activity was not strong at all. This means that plumbagin was not the source of antiproliferative activity in our samples. To support our results, the UV LC-MS/MS was also performed to detect the presence of plumbagin and reveal its intensity in samples which showed m/z of plumbagin (189.04) (Rodrigues et al. 2007). Plumbagin, was found in several samples (*N.* × *ventrata* root, *N. graciliflora* root, *N.* × *hookeriana* root), but according to a study by Sanusi et al. (2017), plumbagin should be presented just in leaves, however, our study revealed the presence of this compounds in roots. Compared to our 2 % standard of plumbagin, its intensity was low, which could possibly indicate a small amount of concentration. According to Chen et al. (2016), low intensity should cause no inhibition or light cytotoxicity. Thus, this also supported our theory that the anticancer activity could be influenced by other compound(s) than plumbagin itself.

In this study, *N. ampullaria* showed the strongest antioxidant activity in leaves with IC_{50} 13.61 μg/mL. According to a study by Satrimafitrah et al. (2021), *Nepenthes* leaves possessed IC_{50} of 72.79 μg/mL. However, they tested other species and *N. ampullaria* was tested for the first time here. Our results indicated great abilities of *N. ampullaria* to inhibit free radicals, making this plant also first with such a high antioxidant ability.

Hybridization is due to hybrid vigour nowadays commonly used in crossbreeding of many medicinal plants for its ability to inherit or multiply desirable effects of its parents due to the variable number and concentrations of secondary metabolites produced by these plants (Namdeo 2018; Wang et al. 2020). In the study by Rosli et al. (2021), the content of bioactive compounds in *N. ampullaria*, *N. rafflesiana*, and their hybrid *N.* × *hookeriana* was compared. The hybrid was found to differ by 43.3 % in the content of new compounds that were not present in either parent (Rosli et al. 2021). Our data from LC-MS/MS reveals another than content of features, it also showed several compounds, unique for the species *N.* × *hookeriana* and *N.* × *ventrata*, however, these numbers are lower than what was reported in the study mentioned before. Based on the assumption of possible differences in the activity of parent plants and their hybrids, we assumed that

the hybrid vigour increased the inhibition abilities of the hybrid plant compared to the inhibitory activity of its parents, which has been confirmed (Zhu et al. 2016).

Chemical analysis of extra compounds in the hybrid plants revealed a high abundance of flavonoids, anthocyanins or amino derivates. Nevertheless, several very interesting compounds were found in *N.* × *ventrata*, such as Spinosyn A, which is well-known and used substance as insecticide. According to a study by Ou-Yang et al. (2021) the presence of quercetin was detected in another hybrid of *N. ventricosa*, although we found this compound in *N.* × *hookeriana* and not in *N.* × *ventrata*. The hybrid also contained Glochidone, a compound recognized for its anticancer effects on skin tumours, as detailed by Tanaka et al. (2004). Nonetheless, the compound spectrum of *N.* × *hookeriana* was found to be much more diverse than that of other plants examined. In addition to the presence of quercetin and kaempferol, the roots of *N.* × *hookeriana* revealed the exclusive presence of several unique compounds. Among these were fluoromisonidazole, persicogenin, and eupatorin, each demonstrating promising properties as agents for inhibiting the growth of cancer cells. This distinct array of compounds found exclusively in the roots of *N.* × *hookeriana* holds significant implications for its pharmacological potential. A study by Mottagipisheh et al. (2024) determined the synergistic effect of these compounds suggesting that these compounds along with others may contribute to the species' robust antiproliferative activity. Thus, it is likely that the roots of *N.* × *hookeriana*, with their rich and diverse chemical profile, harbour potent bioactive agents that confer them the strongest observed antiproliferative activity among the examined samples.

7. Conclusion

In this study we reported for the first time antiproliferative effects of six *Nepenthes* species (*N. ampullaria, N. rafflesiana, N. × hookeriana, N. graciliflora, N. ventricosa, N. × ventrata)* dividing into twenty ethanolic extracts based on the various plant parts (leaves, stem, traps, roots) against colorectal cancer cell line HT29 and Caco-2 as well as the cytotoxicity to healthy cell line CCD 841 CoN. Most of the samples exhibited distinct antiproliferative properties, while the cytotoxicity test confirmed the relative safety of all samples that have been evaluated. Our hypothesis was confirmed by the fact that both hybrid plants exhibited better results than their parents but as a most promising was determined root extract of *N. × hookeriana* providing the strongest cytotoxic effect to both cancer cell lines especially to Caco-2 and at the same time, being safe for healthy human intestine cells. Moreover, this hybrid also showed a strong antioxidant effect on DPPH. Thus, this plant appeared to have great potential in the development of new medicinal agents in the future. With a use of UV LC-MS/MS analysis, we detected a lower abundance of plumbagin than the plumbagin standard leading to the significant finding that has shed light on the possibility of other interesting compound(s) being present in roots of *N. × hookeriana*, causing the antiproliferative activity. It is possible that the presence of fluoromisonidazole, persicogenin, and eupatorin in combination with antioxidants and other compounds can have a synergistic effect and strengthen the effect of each compound. This study not only underscores the unique chemical composition of *N. × hookeriana* but also underscores its potential as a valuable source of bioactive compounds with therapeutic implications in cancer research. Further exploration into the compounds of *N. × hookeriana* roots is imperative for the potential use in the future development of new cytotoxins targeting colon adenocarcinoma.

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