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# Alkaloid quantitation and chemical fingerprinting of Icelandic *Huperzia selago* genotypes using HPLC-UV and UPLC-QToF-MS

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# ABSTRACT


## Alkaloid quantitation and fingerprinting of Icelandic *Huperzia selago* genotypes using HPLC-UV and UPLC-QToF-MS

Plants belonging to the family Lycopodiaceae (clubmosses) produce bioactive lycopodium alkaloids. The clubmoss *Huperzia serrata* has been used as herbal medicine for centuries in China for numerous of ailments, e.g., fever, swelling, and to improve memory. The alkaloid, huperzine A (hupA), was first isolated from the whole plant of *H. serrata* and has been shown to be a potent acetylcholinesterase inhibitor and of interest for development of drugs for Alzheimer's. A related species, *Huperzia selago*, which grows in Iceland, produces considerable amounts of hupA amongst eleven other lycopodium alkaloids reported so far.

The aim of this study was to characterize alkaloid variations of the three Icelandic *H. selago* genotypes. The specific objectives were to determine the hupA contents using HPLC-UV and assess its variation in genotypes and to explore the use of UPLC-MS alkaloid fingerprinting in the recognition of genotypes.

The Icelandic *H. selago* was found to have a wide range of hupA contents from 41 µg/g d.w. to 649 µg/g d.w. in the three genotypes, determined by a HPLC-UV method. Genotype 3 contains significantly higher hupA than genotype 1 and the highest amount of hupA was determined in one specimen of genotype 3 (649 µg/g d.w.). Alkaloid fingerprinting was performed using a UPLC-MS method. Principal component analysis (PCA) fingerprinting data reveals that alkaloid profile tends to be genotype specific. From the PCA loading plot, hupA and hupB are driving the separation of genotype 3 from the other two. It is suggested that Icelandic *H. selago* of genotype 3 is a good alternative source for natural hupA. This study highlights the importance of chemical fingerprinting and thorough plant identification in the selection of medicinal plant raw material with highest pharmaceutical interest.

# ÁGRIP


## Greining á alkalóíðainnihaldi og efnafræðilegu fingrafari í arfgerðum íslensks skollafingurs með HPLC-UV og UPLC-QToF-MS aðferðum

Jurtir af jafnaætt (Lycopodiceae) framleiða meðal annars lífvirka lýkópódíum alkalóíða. *Huperzia serrata* er jafni sem hefur verið notaður í Kína til náttúrulækninga svo öldum skiptir, meðal annars sem hitalækkandi, gegn bólgum og sem minnisaukandi. Alkalóíðinn, huperzín A (hupA), var fyrst einangraður úr heilli jurt af jafnanum *H. serrata*. HupA er mikilvirkur asetýlkólínesterasahindri og hefur vakið athygli sem mögulegur lyfjasproti við þróun lyfja gegn Alzheimerssjúkdómi. *Huperzia selago* eða skollafingur, er náskyldur jafni af sömu ættkvísl sem vex víða á Íslandi. Alls hafa verið einangraðir tólf lýkópódíum alkalóíðar úr íslenskum skollafingri þar á meðal lyfjavirki alkalóíðinn hupA.

Markmið rannsóknarinnar var að greina alkalóíðamynstur íslensks skollafingurs í þremur arfgerðum (genotypes) hans. Sértæk markmið voru magngreining á hupA með HPLC-UV til að meta breytileika magns hupA milli arfgerða og að kanna notkun á UPLC-MS til greiningar á efnafræðilegum fingraförum alkalóíða sem og greining gena í arfgerðir.

Magngreining á hupA í íslenskum skollafingri fór fram með HPLC-UV og leiddi í ljós að magn þess í þurrvigt spannar yfir mjög breitt bil í arfgerðunum þremur eða frá 41 µg/g til 649 µg/g. Arfgerð 3 innihélt umtalsvert meira magn af hupA samanborið við arfgerð 1 og mesta magn af hupA var að finna í einu sýni af arfgerð 3 (649 µg/g þ.v.). UPLC-MS var notað til að greina efnafræðileg fingraför alkalóíðanna, niðurstöður voru settar upp í meginþáttagreiningu (principal component analysis: PCA) sem sýndi að hver arfgerð hafði ákveðið alkalóíðamynstur. Einnig voru gögnin sett upp í PCA hleðslurit (loading plot) og sú greining sýndi að hupA og hupB aðgreinir arfgerð 3 frá hinum tveimur.

Niðurstöður rannsóknarinnar sýna að íslenskur skollafingur af arfgerð 3 er góður valkostur sem náttúruleg uppspretta fyrir hupA. Rannsóknin undirstrikar mikilvægi efnafræðilegra fingrafara og nákvæmrar auðkenningar þegar velja skal plöntuefni til frekari rannsókna.

# LIST OF ABBREVIATIONS

| | |
|---|---|
| AChEI | Acetylcholinesterase inhibitor |
| AD | Alzheimer's diseases |
| DC | Dendritic cell |
| ESI | Electrospray ionization |
| HupA | Huperzine A |
| HupB | Huperzine B |
| LA | Lycopodium alkaloid |
| LC | Liquid chromatography |
| M/Z | Mass-to-charge ratio |
| MS | Mass spectrometry |
| PCA | Principal component analysis |
| $T_R$ | Retention time |
| UPLC | Ultra-performance liquid chromatography |
| UV | Ultraviolet |
| 6-OH Hup A | 6-hydroxyl hupA |

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## Declaration of Contribution

This Master thesis project was conducted using research facilities in the Faculty of Pharmaceutical Sciences, University of Iceland. The project was designed and supervised by Elín Soffía Ólafsdóttir and Maonian Xu. Plant specimens were collected by Elín Soffía Ólafsdóttir, Elvar Örn Viktorsson, Maonian Xu and Sebastian Oddur Björnsson. Magnea Guðmundsdóttir carried out sample preparation, chemical data analysis and thesis writing. Thesis was commented and reviewed by Elín Soffía Ólafsdóttir and Maonian Xu. Plant genotypes were identified using DNA sequencing by Maonian Xu. UPLC-QToF-MS measurements were performed by Finnur Freyr Eiríksson from ArcticMass ehf.

# 1. INTRODUCTION

## 1.1 The club moss genus *Huperzia*

### 1.1.1 Taxonomy

The genus *Huperzia* belongs to the seedless vascular plant family Lycopodiaceae. Taxa in this family are also called club mosses, which reflects their common morphological features resembling mosses but distinct in scale-like or cone-like clusters of spore-bearing leaves (i.e. strobilum) (Kristinsson, Hlíðberg, & Þórhallsdóttir, 2018). There are roughly 388 species in the family, where the genus *Huperzia* consists of 25 species (PPGI, 2016). Palaeontological studies reveal that club mosses were the dominant plant group in the Carboniferous period (360-286 million years ago), and now they only account for 0,5-1% of the worlds flora (Kenrick & Davis, 2004; White & Frazier, 1986).

Based on morphological and genetic analyses, the genus *Huperzia* and its two closely related genera, *Phlegmariurus* and *Phylloglossum*, constitute the subfamily Huperzioideae (Field, Testo, Bostock, Holtum, & Waycott, 2016). Taxonomic ranking of the type species *Huperzia selago* is shown in Table 1.

**Table 1.** Classification of Huperzia selago, from Kingdom to subspecies.

| Kingdom | Plantae – plants |
|---|---|
| **Sub-kingdom (phylum)** | Tracheobionta – Vascular plants (Phylum: Tracheophyta) |
| **Division** | Lycopodiophyta – Lycopods |
| **Class** | Lycopodiopsida |
| **Order** | Lycopodiales |
| **Family** | Lycopodiaceae – Club moss family |
| **Subfamily** | Huperzioideae |
| **Genus** | *Huperzia* Bernh. |
| **Species** | *Huperzia selago* (L.) Bernh. ex Schrank and Mart. |
| **Subspecies** | *H. selago* ssp. *appressa,* ssp. *arctica and* ssp. *selago* |

Compared to closely related genera in other subfamilies (i.e., *Lycopodium* and *Diphasiastrum*), the genus *Huperzia* is distinct in its absence of creeping stems and

strobili. Strobilus is a cone-like reproductive structure on erect branches, bearing spores (Jonsell & Karlsson, 2000). These morphological differences are illustrated in Figure 1.

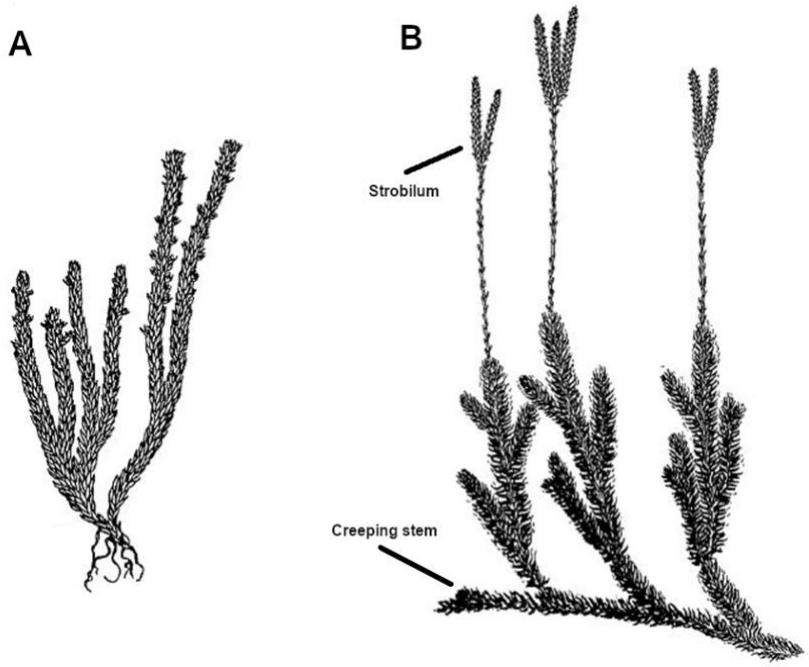



**Figure 1.** Morphology of representative club mosses in Nordic countries. Huperzia selago (A) does not have strobili and creeping stems as other related taxa, such as Lycopodium clavatum (B).

*Huperzia selago*, *Diphasiastrum alpinum*, *Lycopodium clavatum*, and *Spinulum annotinum* are the four club mosses that grow in Iceland (Kristinsson et al., 2018). The species *L. clavatum* is a protected species only found in eastern Iceland.

### 1.1.2 Classification of Icelandic *Huperzia selago*

Due to large morphological variations, species boundaries of *H. selago* are still under debate. Different morphotypes were treated as unresolved taxa in "*H. selago* group" (Ollgaard, 1987) due to a lack of macroscopically discernible morphological differences. Some taxonomists take a more extreme opinion regarding morphotypes as different species (e.g., "*H.* arctica," "*H.* appressa" and "*H.* selago") (Gilman & Testo, 2015). However, morphological intermediates are quite common between morphotypes, and morphological species boundaries are still not sure.

*H. selago* grows around Iceland (Figure 2) and includes all three described morphotypes; appressa, arctica, and selago (Xu. et al., 2019a) These morphotypes are challenging to separate just by looking at them, the difference between them is

mainly found in different shades of the green color and the length of the shoots, in which direction the leaves grow and where sporophylls are located on the shoots (Kristinsson et al., 2018). Appressa has the widest distribution around Iceland and can be found in the mountains, and on the lowlands as well, the stems are thin. Their length is usually 5-8mm (Kristinsson et al., 2018), the leaves point upwards, and their color is yellow to yellowish-green (Kristinsson et al., 2018; Xu et al., 2019a), the sporophylls are located on the entire stem (Kristinsson et al., 2018). The distribution and the quantity of arctica are limited in Iceland, and it grows in high hills of mountains. The stems are thin and quite short or about 4-6mm commonly with sporophylls, although they don't always have sporangium (Kristinsson et al., 2018; Xu et al., 2019a). Last but not least is selago; it grows, especially in western Iceland (Xu et al., 2019a), although it can be found in several places around the country. The selago morphotype has the tallest shoots, around 10-15mm and the leaves are more spread out (Kristinsson et al., 2018) than the other types and the color of it is much greener sometimes even dark green (Xu et al., 2019a), the sporophylls are usually at the top of the sprouts, and sometimes they are inconspicuous (Kristinsson et al., 2018). Genetic analysis suggested that different morphotypes should be regarded as subspecies under *Huperzia selago*, three genotypes have been found in Icelandic taxa (Xu et al., 2019a). Therefore, the results support the classification (Table 1) made by Kristinsson (2010) and Jonsell and Karlsson (2000). Alkaloid fingerprinting also reveals that each genotype tends to have its own alkaloid fingerprint (Xu et al., 2019b).

**Figure 2.** Geographic distribution of Huperzia selago in Iceland (Kristinsson et al., 2018).

**Figure 3.** Photograph of *Huperzia selago* representing the appressa morphotype (picture taken on September 5th, 2018 in Helgafellssveit, Snæfellsnesi by Maonian Xu).

### 1.1.3 Ethnic use

Humans have recognized and exploited herbal plants as folk medicines for thousands of years. Plants were the first medicine used by a human being. They played and still play an essential role in curing or relieve human illnesses and are also very important for new drug discovery for many diseases that threaten human health (Fatemeh Jamshidi-Kia, 2017).

*Huperzia* species that belong to the family of Lycopodiaceae are apart from being explored as drug candidates, used in folk medicines and cultural ceremonies (Armijos et al., 2016).

Examples of traditional medicine using the genus *Huperzia* and its two closely related genera, *Phlegmariurus* and *Phylloglossum* in different cultures are listed below.

**Argentina:** In Argentina, it is common to use herbal medicines by the Sierra de Comechingones commune; they have been using leaves and stems from *Phlegmariurus saururus* to increase sexual potency, as antialopecic medicine (Goleniowski, Bongiovanni, Palacio, Nunez, & Cantero, 2006) as well in folk medicine to improve memory (Ortega et al., 2006).

**Canada:** Canadian natives used *H. selago* for external use to alleviate the suffering caused by pain, they laid hot compress on the person's head. The natives also used *H. selago* orally to induce vomiting and diarrhea, likewise to produce toxic effects, which results in unconsciousness (Róbertsdóttir, 2011).

**China:** The *Huperzia* and *Phlegmariurus* species have been used in folk medicines for centuries in China. There is a long history of using the whole plant of *H. serrata* as a

component in Chinese herbal medicine, “Qian Cen Ta”. “Qian Cen Ta” is used to treat several kinds of disorder and has a history of use as a memory improver, for strains, rheumatism, fever (Patocka, 1998), myasthenia gravis, schizophrenia, organophosphate poisoning, swelling, and bruises (Ma, Tan, Zhu, & Gang, 2005; Orhan, Kupeli, Sener, & Yesilada, 2007). The first registration usage of “Qian Cen Ta” is found in the Chinese pharmacopeia “Ben Cao Shi Yi” that was written by Zangqi Chen in the year 739. In the pharmacopeia the herb is called “Shi Song” and its activity listed to treat arthritis, colds, to relax muscles and tendons, and to increase blood flow (Ma, Tan, Zhu, Gang, & Xiao, 2007).

**Ecuador:** In Southern Andes of Ecuador, there is a rich tradition of using herbs as folk medicines, including species of *Phlegmariurus*. Saraguro visionary healers use *Phlegmariurus* species like *P. compacta*, *P. espinosana*, *P. brevifolia*, *P. crassa*, *P. weberbaueri*, *P. columnaris*, *P. tetragona* in folk medicine and for cultural ceremonies. The Saraguro community regards these plants as sacred, psychoactive potent, and they are believed to have magical power. The plants are either given to people and animals in mixture with other plants or just alone. The Saraguro healers make mixtures with alcohol, and the herbal blend can be administered in three different ways, depending on what mixture it is. It can be drunk, given by nose inhalation or blown over the patient (Armijos et al., 2016).

**Iceland and other Nordic countries:** *H. selago* is considered to be toxic when used internally, but the sources simulate that *H. selago* was used by the Scandinavians to induce vomiting and to treat constipation. *H. selago* was also used as a developer for yarn coloring (Róbertsdóttir, 2011).

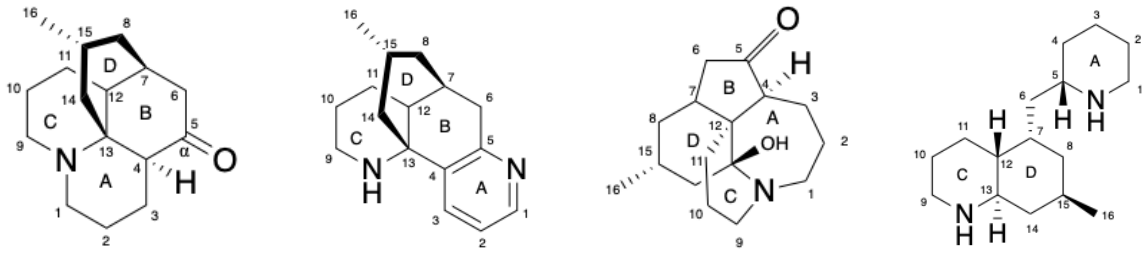
**North Sumatra, Indonesia:** Kabanjahe traditional market is a market in the Kabupaten district in Karo. This market has existed for over 40 years; approximately 350 plant species from around 90 families can be found there. These plants are used as ingredients for traditional medicines. Lycopodiaceae is one of them, and they have four *Phlegmariurus* species from that family. *P. carinatum* Desv. Ex Poir (leaves) and *P. nummularofolium* Blume (leaves) are believed to affect bone fractures and cancer. *P. proliferum* Blume (whole) is also used for bone fractures and to increase sexual potency. *P. phlegmaria* L (leaves) is used for kidney disease and also to treat cancer (Silalahi, Nisyawati, Walujo, Supriatna, & Mangunwardoyo, 2015).

## 1.2 Lycopodium alkaloids

It was in the year 1881 that the first lycopodium alkaloid (LA) was isolated, lycopodine, from club mosses (i.e., *Lycopodium complanatum*) (Bödeker, 1881). The lycopodine structure was not fully elucidated by then (Olafsdottir, Halldorsdottir, Pich, & Omarsdottir, 2013). Studies on LAs were progressing slowly at first, but the number of discovered LAs slowly expands as more club moss are chemically investigated. Due to the structural uniqueness of alkaloids isolated from Lycopodiaceae, they are collectively called lycopodium alkaloids (Ma et al., 2005). A milestone study was the discovery of huperzine A (hupA) as a potent acetylcholinesterase inhibitor (AChEI), which is an important drug target in Alzheimer's diseases (AD) (Liu et al., 1986). Since then, there has been an increasing interest in LAs; in 2004 were registered and reported numbers of discovered LAs 282 (Ma & Gang, 2004) since then the number must have exceeded. LAs bioactivities were gradually revealed and shown to involve multiple mechanisms apart from AChEI (Ayer & Trifonov, 1994; Bai, Tang, & He, 2000; Olafsdóttir, Halldorsdottir, Pich, & Omarsdottir, 2013). Examinations on Lycopodiaceae herbals show that the main bioactivity comes from the LAs, both *in vivo* and *in vitro* (Borloz, Marston, & Hostettmann, 2006). These are further supported from aforementioned ethnic uses of club mosses as herbal medicines. For example, *H. serrata* has been used for centuries in Chinese herbal medicines for numerous ailments, e.g., swelling, fever, to improve memory and more symptoms. Therefore bioactive LAs, particularly hupA, were suggested for the treatment of one of the significant diseases affecting seniors, Alzheimer's disease, and also myasthenia gravis (Nilsu et al., 2018; Shan, Luo, Pan, Zou, & Kong, 2016).

### 1.2.1 Structural groups

Lycopodium alkaloids are polycyclic alkaloids with a unique skeleton (Ma & Gang, 2004; Wang et al., 2018). Lycopodium alkaloids are classified into four different structural groups (Figure 4), due to their differences in biosynthesis and physio-chemical characteristics. The four groups are lycodane, lycopodane, fawcettimine, and miscellaneous. The largest one is lycopodane-group, and it is the most widely distributed (Aver & Trifonov, 1994; Ma & Gang, 2004).



**a** **b** **c** **d**

**Figure 4.** Representative compounds for each lycopodium alkaloid structural group. a) lycopodine, from the lycopodane group; b) lycodine, from the lycodane group; c) fawcettimine from the fawcettimine group and d) phlegmarine from the miscellaneous group.

### 1.2.1.1 Lycopodane class

Compounds in this class have four connected hexagons (e.g. lycopodine; Figure 4a), where ring A and C make a quinolizidine ring system with the nitrogen. It is common to have carbonyl group in these compounds; generally, it is at C5 in ring B, but sometimes it is at C6 like in huperzine E (Figure 5a). In this class oxygenation is usually at C4, C5 at α-position, C6, C7, and the C12. Rings A, B, and C are stable, but ring D has the most diversity, like in annopodine (Figure 5b), there is the ring open at C8 to C15 (Ma & Gang, 2004).

**a** **b**

**Figure 5.** Structure of a) huperzine E and b) annopodine.

### 1.2.1.2 Lycodane class

Compounds in this class consist of four rings (e.g lycodine; Figure 4b), like the compounds in the lycopodane class. The difference between the rings here from the one in lycopodane is found in ring A, the A ring is either pyridone or pyridine ring. The different variation of compounds in this class is located in the C ring. The C ring opens after a cleavage then C9 eliminates with a result of the $C_{15}N_2$ skeleton, e.g. hupA

(Figure 6). Most of the lycopodium alkaloids with a potent anticholinesterase activity are compounds belonging to this class, e.g. hupA (Ma & Gang, 2004).

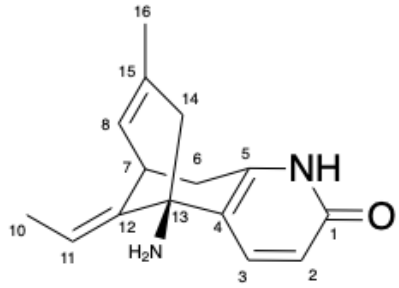



**Figure 6.** Structure of huperzine A.

### 1.2.1.3 Fawcettimine class

Compounds in the fawcettimine group (e.g fawcettimine; Figure 4c) are mainly tetracyclic compounds like the other two classes above, three of the rings are six-membered A, C, D, and the B ring is five-membered with a carbonyl group at C5. The B ring is the one that alters from the lycopodine group, C4 bonds to C13 in the lycopodane group, but in fawcettimine, it bonds from C4 to C12 (Ma & Gang, 2004).

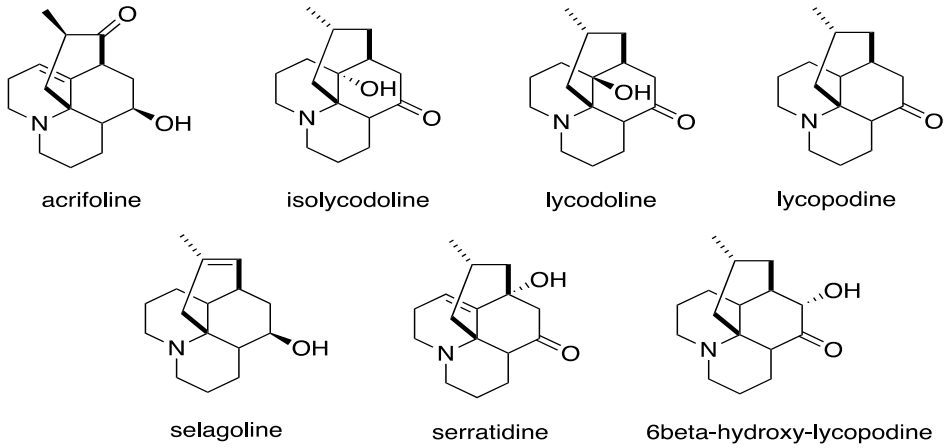
### 1.2.1.4 Miscellaneous class

The representative compound for this group is phlegmarine (Figure 4d) since all of the compounds belonging to this group can be induced from phlegmarine. There is a wide range of structures in this class, e.g., some have 12 carbons, and some have three nitrogen's (Aver & Trifonov, 1994). Compounds in the other three types have phlegmarine as an intermediate in their biosynthesis, and LAs that doesn't fit in the other classes are grouped in this class. This class is characterized by three rings A, C, and D, where C4 is not connected to C12 or C13, like in the other types. There is an exception from the three rings system. Some LAs in this group have six rings, and there are also known LAs with only two rings in their skeleton. The compounds with only two rings are supposed to be the chemically simplest ones (Aver & Trifonov, 1994; Ma & Gang, 2004).

### 1.2.2 Lycopodium alkaloids in Icelandic *Huperzia selago*

Twelve lycopodium alkaloids have been reported in *H. selago* so far. Seven of them belong to the lycopodane class (Figure 7), including acrifoline, isolycodoline, lycodoline, lycopodine, selagoline, serratidine, and 6α-hydroxylycopodine. Five of them belong to the lycodane class (Figure 7), including α- and β- obscurine, huperzine A and B, and 6β-hydroxyhuperzine A (Achmatowicz, 1956; Ayer, Browne, Elgersma, & Singer, 1990; Ayer, Browne, Orszanska, Valenta, & Liu, 1989; Grynkiewicz, 1968; Valenta, Yoshimura, Rogers, Ternbah, & Wiesner, 1960).

**Lycopodane - type**




**Lycodane - type**

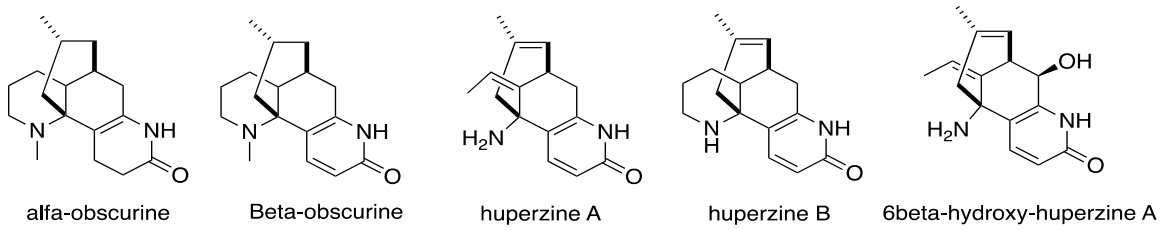



**Figure 7.** Structures of the twelve reported lycopodium alkaloids in *Huperzia selago*.

Three LAs were isolated from the Icelandic *H. selago*, where selagoline was discovered as a new natural product from this plant along with hupA and serratidine (Staerk et al., 2004). Ten alkaloids have been annotated in Icelandic taxa using alkaloid fingerprinting except for α- and β- obscurine, and two unannotated peaks might be new (Xu et al., 2019).

Quantitate variation of huperzine A and huperzine B (hupB) contents has been described among three genotypes of Icelandic *H. selago* (Xu et al., 2019). Genotype

3 (264.39 – 679.82 mg/g d.w) contains significantly higher amount of hupA than genotype 1 (20.63 – 193.84 mg/g d.w), while genotype 2 has a rather broad content (113.13 – 599.63 mg/g d.w). HupB was only detected in genotype 3 and 2 (Xu et al., 2019).

### 1.2.3 Bioactivity

Pharmalogical and bioactivity studies, *in vivo* and *in vitro*, on LAs have reflected potent biological activity, such as anti-inflammation, anticancer, AChEI activity and effectiveness against neurodegenerative diseases such as AD (Hardardottir, Olafsdottir, & Freysdottir, 2015; Mandal et al., 2010; Olafsdóttir et al., 2013). It has also been discovered that hupA has a positive effect on memory and thus increased study abilities (Ma et al., 2007).

**Anti-inflammation:** Immuno-modulatory effects of annotine from *Lycopodium annotinum* was investigated in an Icelandic study. Annotine was examined for effects on maturation of dendritic cells (DCs) and its capability to activate allogeneic CD4+ T helper cells. The results demonstrate that annotine can induce an inflammatory response by DCs, leading to activation of T cells and differentiation to the Th2/regulatory T-cells (Hardardottir et al., 2015). According to this study, annotine might be beneficial as a drug candidate for the treatment of Th1-and/or Th17-mediated inflammatory diseases, (Hardardottir et al., 2015) like the autoimmune diseases, multiple sclerosis and rheumatoid arthritis (Hardardottir et al., 2015; Leung et al., 2010).

**Anticancer:** *In vivo* study on HeLa cells derived from cervical cancer were investigated for anticancer effects of lycopodine from *Lycopodium clavatum* extract (Mandal et al., 2010). The results showed multiple effects lycopodine on the HeLa cells by increasing cell population in the sub-G1 region, fragmentation of inter-nucleosomal DNA, chromatin condensation induction, and caspase-3 activation leading to involvement in apoptosis of the HeLa cells (Mandal et al., 2010). This study revealed that lycopodine might be interesting as a drug candidate in the treatment of cervical cancer.

**Acetylcholinesterase inhibitory:** HupA has exhibited a variety of biological actions, and today's researches focus mainly on its effect on memory and its neuroprotective effects (Olafsdóttir et al., 2013; Skolnick, 1997). Several activity studies have been done and studies on safe usage of hupA, such as the study performed on adult rats and rats with cognitive impairment where it was concluded that hupA improved the

rats' memory (Lu, Shou, & Tang, 1988). HupA resulted in enhanced mind and behavior within AD patients with mild to moderate symptoms in placebo-controlled, double-blind, and randomized trials that were performed on clinical action and safety of hupA (Zhang et al., 2002). HupA AChEIs activity had longer half-life, more affect, and more specific inhibition on AChE compared to synthetic AChEIs tacrine and donepezil in the comparative study that was performed on rats (Heydorn, 1997; Wang & Tang, 1998).

### 1.2.4 Liquid chromatography-mass spectrometry analysis of lycopodium alkaloids

Liquid chromatography (LC) - mass spectrometry (MS) is a powerful approach in phytochemical analyses, which combines separation efficiency from LC and sensitive molecular mass detection from MS (Allwood & Goodacre, 2010). Nowadays, bioactive substances like alkaloids (Zhu et al., 2015) in medicinal plants are usually subjected to chemical profiling/fingerprinting using LC-MS methods (Allwood & Goodacre, 2010). With smaller particle size (ca. 1.7 µm) and versatile column-packing chemistry, the chromatographic peak resolution was enhanced considerably which offers a variety of selectivity to different chemical structures (Liang et al., 2010). To identify analytes, it is necessary to combine the UPLC instrument with different detectors which provides spectroscopic and/or spectrometric data for separated peaks (Konishi et al., 2007). As a sensitive detector, MS captures not only ionized compounds with their possible fragment ions, but also impurities at low levels. Single-charged molecular ions and their fragments contribute to compound identification (Shan et al., 2016) by providing data in molecular formula and structure predictions (Konishi et al., 2007). Annotation and identification of compounds could also be aided by comparing in-house data with a curated MS database of natural products (Konishi et al., 2007).

In lycopodium alkaloid research, LC-MS has been mostly applied to chemical fingerprinting of plants in the subfamily Huperzioideae, while taxa in other subfamilies were less studied. LC-MS methods have been developed and used to analyze several clubmoss species, e.g. *Diphasiastrum alpinum* (Bai et al., 2000) *H. selago* (Borloz et al., 2006; Xu et al., 2019b) *H. serrata* (Shan et al., 2016; Wu & Gu, 2006; Zhao, Wang, Luo, & Yang, 2015), *Phlegmariurus squarrosa* (Cuthbertson, Piljac-Zegarac, & Lange, 2012), *Spinulum annotinum* (Borloz et al., 2006), *Lycopodiastrum casuarinoides* (Shan et al., 2016), *Lycopodium clavatum* (Borloz et al., 2006) and *Phlegmarirus carinatus*

(Shan et al., 2016). From these studies, the composition of LAs in respective plants was characterized, and major alkaloids were annotated (Shan et al., 2016).

Quantification of lycopodium alkaloids using mass spectrometry is highly restricted to lycodane-type alkaloids, particularly hupA (Cuthbertson et al., 2012; Shan et al., 2016). These alkaloids are readily fragmented under low collision energy, and this allows the generation of multiple fragment ions and multiple reaction monitoring. Other structural groups (e.g. lycopodane-group) are more resistant to MS fragmentation, and usually limited structural information could be obtained apart from molecular mass (Shan et al., 2016). High collision energy is expected to bring about sufficient fragmentation (Shan et al., 2016; Xu et al., 2019b). Mass spectrometry has shown improved sensitivity compared to ultraviolet (UV) based quantification, but more efforts should be made to method validation (e.g. analyzing matrix effects) (Cuthbertson et al., 2012). The latter is still the preferred method for rapid quantification of hupA analogues, since these compounds have a 2-pyridone chromophore (Sangster & Stuart, 1965). Therefore, simultaneous quantification of LAs from different structural groups is apparently a problem.

Chromatographic separation of lycopodium alkaloids is also a challenge. Alkaloids are basic compounds, and they are usually analyzed in their protonated form under buffered acidic conditions, such as ammonium acetate buffer around pH 5.5 or even formic acid. Positively charged alkaloids are poorly retained on reversed phase columns and may co-elute quickly.

The following research was based on this literature.

# 2. AIMS OF THE STUDY

In this study, the aim was to characterize alkaloid variations of Icelandic *Huperzia selago* genotypes.

Specific objectives are:

1. To determine the hupA contents using HPLC-UV and assess its variation in genotypes.

2. To explore the use of UPLC-MS alkaloid fingerprinting in the recognition of genotypes.

# 3. EQUIPMENTS, MATERIALS AND METHODS

## 3.1 Equipments

**Table 2.** Equipments.

| **Equipment** | **Manufacturer** |
|---|---|
| Acuity UPLC™ system | Waters |
| Dionex UltiMate 3.0 HPLC system | Thermo Fisher Scientific |
| Eclipse XDB-C18 column (HPLC) | Agilent |
| Eppendorf tubes (polypropylene) | SARSTEDT AG & Co. KG |
| Finnpipette | Thermo Scientific |
| Glass tubes | DWK Life Sciences, GmbH |
| Glass vials | Waters |
| Luna Omega Polar C-18 column (UPLC) | Phenomenex |
| Milli-Q water purification system | Millipore GmbH |
| MS Semi-Micro Balance | Mettler-Toledo |
| Pasteur pipette | Brand GmbH |
| pH paper | Sigma-Aldrich |
| Pico 17. Thermo Centrifuger | Thermo Electron Corporation |
| Sonicator bath | Fisher Scientific |
| Syringe | B. Braun Melsungen AG |
| Syringe filter (0.45mm) | Phenomenex |
| Vortex (stirrer) | Scientific Industries, Inc. |

## 3.2 Materials

**Table 3.** Materials.

| Material | Manufacturer |
|---|---|
| Acetic acid | Sigma-Aldrich |
| Ammonium acetate ($NH_4OAc$) | Sigma-Aldrich |
| Ammonium hydroxide | Sigma-Aldrich |
| Dichloromethane | Sigma-Aldrich |
| Huperzine A (98% purity) | PhytoLab GmbH & Co. KG |
| Methanol | Sigma-Aldrich |
| MilliQ water | Millipore GmbH |
| Tartaric acid | Sigma-Aldrich |

## 3.3 Methods

### 3.3.1 Plant material

In total 49 *H. selago* specimens were sampled for this study, including three from Reykjanesfólkvangur, six from Aðalvík and 40 from Snæfellsnes. The whole plant of *H.selago* was collected by Elín Soffía Ólafsdóttir, Elvar Viktorsson, Maonian Xu, and Sebastian Oddur Björnsson from June 23, 2018 to September 5, 2018.

Plants were morphologically identified by the collectors following identification keys specified in Flora Nordica (Jonsell & Karlsson, 2000). After collection, the whole plant materials were air-dried for two weeks. For each plant specimen, a voucher was spared and stored in the herbarium of Icelandic Institute of Natural History, Akureyri Division. Genotypes of collected *H. selago* were determined by sequencing chloroplastic markers, as described before (Xu et al., 2019a). In the 49 *H.selago* specimens were three genotypes detected which supports previous published study (Xu. et al., 2019a). Haplotype network of relationship between the three genotypes are shown in Appendix A.

For each plant specimen, the rest plant material after taking a voucher part was used for chemical analysis. Plants with entire shoots were submerged in liquid nitrogen and pulverized with pestle and mortar. Then frozen pulverized materials were lyophilized overnight to produce dried plant materials ready for alkaloid extraction. Small plant specimens containing single shoots are not included in chemical analysis. Treatment of plant materials and the entire workflow are summarized in Figure 8.

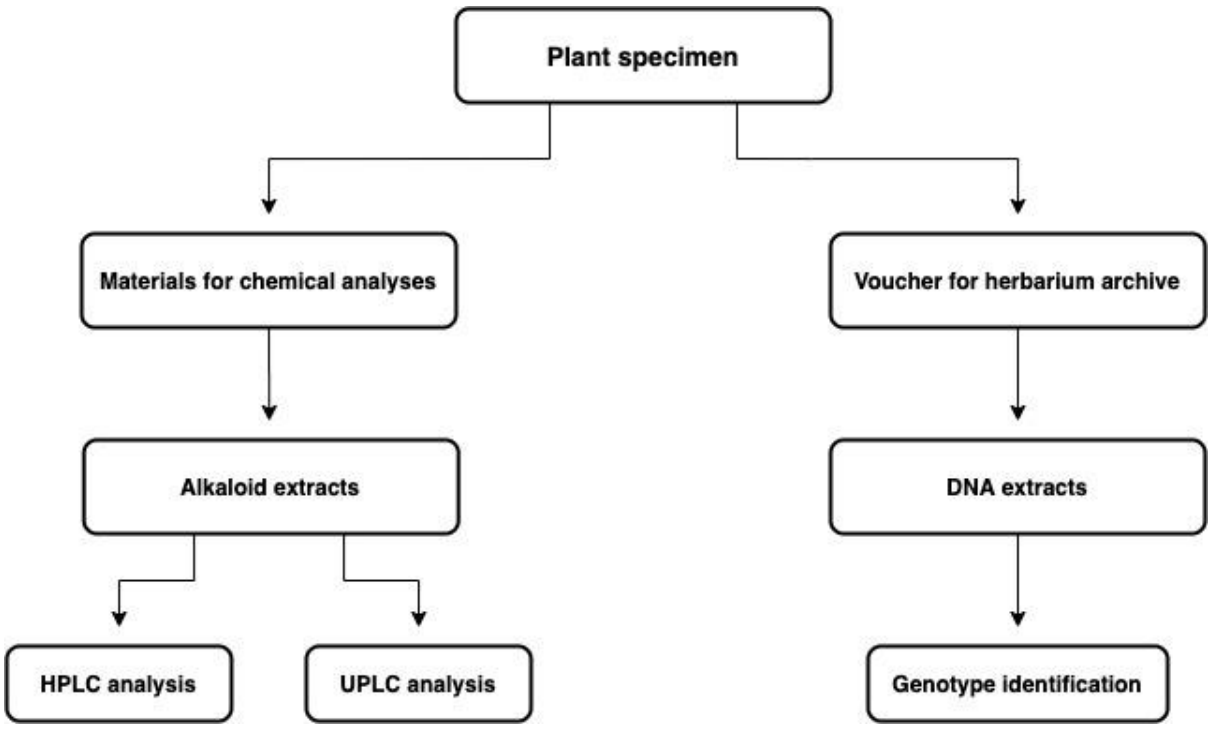



**Figure 8.** Treatment of plant materials and workflow of the study. The current study focused on the chemical analysis, and the genotype identification was carried out by another study.

## 3.3.2 Sample preparation

### 3.3.2.1 Preparation of sample solution

For each specimen in chemical analysis, lyophilized plant materials ca. 40 mg (two to three replicates for each specimen, depending on the amount of lyophilized plant materials) were weighed. The plant materials were extracted with 1 mL of 3% acetic acid in a sonicator for 30 min, and then centrifuged at 8.000 rpm for 6 min. The extraction was further repeated twice, and all supernatants combined. Combined solutions were defatted with 3 mL of dichloromethane and vortexed; the aqueous layer was transferred to a new glass tube and, its pH was adjusted to 10 by adding 30%

ammonium hydroxide drop by drop. The alkaloids were extracted twice from the aqueous layer by adding 3mL of dichloromethane and mixed on vortex, the organic layer was transferred into a new glass tube. Combined organic extracts were evaporated overnight under continuous air flow in a fume hood. Dried residues were dissolved in 50% aqueous methanol solution, sonicated for 1 min, and filtered through 0.45 mm regenerated cellulose filter into 2.0 mL HPLC vials. These HPLC test solutions were stored in the refrigerator at 6°C until HPLC and UPLC analyses were performed. An alternative extraction solution, 3% tartaric acid (Cuthbertson et al., 2012) was compared with 3% acetic acid. Higher peak areas from HPLC-UV analyzes were found using 3% acetic acid as extraction solution, and there for the 3% tartaric acid was not selected, the HPLC-UV chromatographs of the two acid extraction methods are shown in Appendix B.

### 3.3.3 Alkaloid analysis

#### 3.3.3.1 Determination of huperzine A using HPLC-UV

HupA contents in plant specimens were quantified using a published HPLC-UV method (Xu et al., 2019a) with minor modifications in gradient elution. The analysis was performed on a Dionex UltiMate 3.0 HPLC system, equipped with a column oven compartment kept at 30°C, an autosampler with temperature control kept at 10°C, an UltiMate 3000 pump, and an UltiMate 3000 photodiode array detector. The system was controlled with Dionex Chromeleon software v7.2. The chromatographic column Eclipse XDB-C18 (4.6 x 150mm, 5 µm; Agilent) was used for the alkaloids separation and eluted with gradient of A (10mM ammonium acetate buffer, pH 5.5) and B (acetonitrile) at a flowrate of 0.8 mL/min: 0-8 min, 25% B; 8-10.5 min, linear gradient 25%-90% B; 10.5-11 min, 90% B; 11-12.5 min, linear gradient 90%-25% B; 12.5-16 min, 25% B. The flow rate of the mobile phase was set at 0.8 mL/min and the injection volume of each sample was 20 µL. UV detection wavelength was set at 310 nm.

Standard solution was prepared at a concentration of 50 µg/mL of hupA in methanol then serially dilution of the standard was performed in methanol to get an appropriate concentration of hupA, 0.5, 1, 2.5, 5, 10 and 25 µg/mL.

### 3.3.3.2 Alkaloid fingerprinting

UPLC analysis was performed on an Acuity UPLC™ system (Waters corp., Milford, USA) coupled to a QToF SYNAPT G1 mass spectrometer equipped with electrospray ionization (ESI) interface (Waters MS Technologies, Manchester, UK). Luna Omega Polar C-18 column (2.1 mm x 100 mm, 1.6 µm, Phenomenex, UK) at ambient temperature at 22°C was used for LA separation. The mobile phase was composed of 10 mM ammonium acetate buffer pH 5.5 (solvent A) and methanol (solvent B). The flow rate of the mobile phase was set at 0.4 mL/min and the injection volume was 2 µL. The following gradient program was used: 5% B, 0-0.5 min; linear gradient 5% B-80% B, 0.5-9 min; 80% B, 9-10 min; linear gradient 80% B-5%B, 10-10.1 min; 5% B, 10.1-12 min. Quality control samples were prepared by pooling all alkaloid samples. MS analysis was performed on a SYNAPT G1 mass spectrometer and spectra were acquired in positive ionization mode. Mass spectrometer settings were as follows: cone voltage 42 V, cone gas flow 50 L/h at source temperature 120°C, desolvation temperature 400°C, desolvation gas flow 800 L/h and capillary voltage 3.2 kV. Collision energy ramped was set from 10.0 – 50.0 eV, and ion scan was set from mass to charge ratio (m/z) 100 to 1550.

### 3.3.3.3 Principal component analysis

MassLynx v4.1 (Waters corp., Milford, USA) was used for acquisition and data processing to create a data matrix for principal component analysis (PCA), following a reported procedure (Xu et al., 2019b). Following parameters were chosen; retention time ($t_R$) was set from 2 to 8 min, $t_R$ window 0.2 min, mass range 100 to 700 Da, mass tolerance 0.05 Da and marker intensity threshold 250 counts. In order to perform PCA loading plot, processed MS data was imported to the software SIMCA v14.1 (Sartorius Stedim Data Analytics, Umea, Sweden). PCA was used to reveal the relationship between variables of the alkaloid fingerprints by identifying chromatograms of each sample who are highly correlated to each other (Figure 14A).

# 4. RESULTS

## 4.1 Quantification of the huperzine A in Icelandic *Huperzia selago* using HPLC-UV

HupA was identified in alkaloid extracts by comparing the retention time with authentic standard. It eluted out at 9.558 min, well separated from other peaks with a good peak shape (Figure 9). From the HPLC chromatograph, hupA is detected as the major component at the wavelength of 310 nm, and differences in minor components (i.e. small peak eluting earlier than hupA) can also be found between genotype. A calibration curve with a good linearity ($R_2$ = 0.9997) was made for the calculation of hupA contents in alkaloid extracts: Y = 8028X – 621.72, where Y is peak area of hupA, and X is the concentration of hupA in ug/mL.

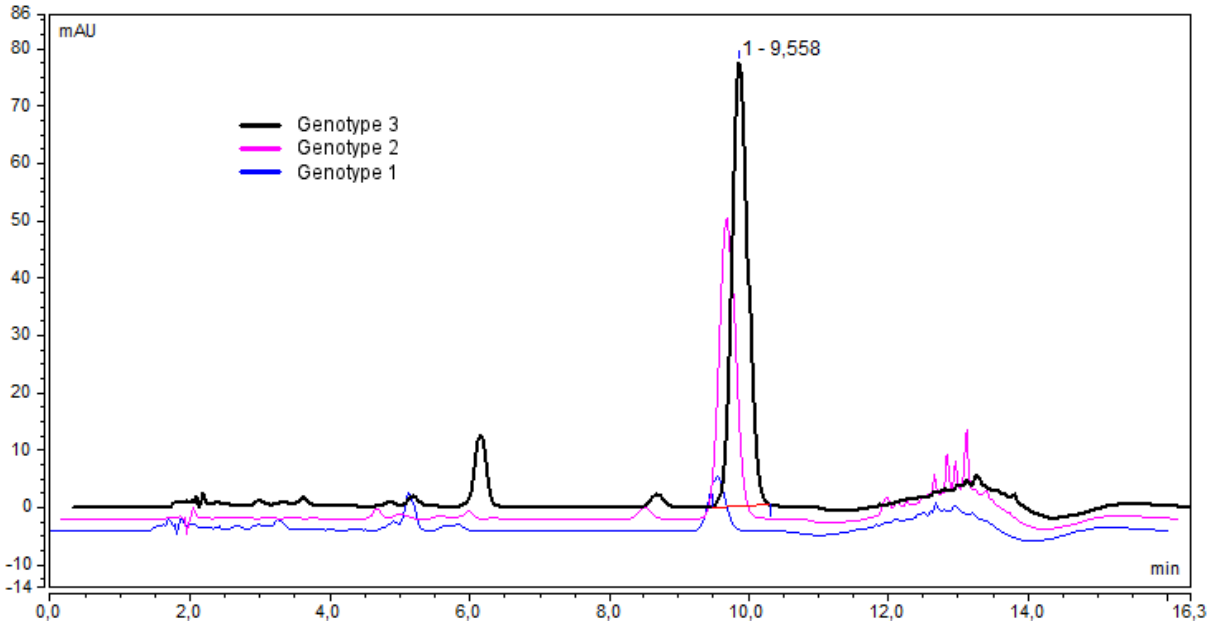



**Figure 9:** HPLC-UV chromatographs of alkaloid extracts from three Icelandic *Huperzia selago* genotype, showing variation of huperzine A contents between genotypes. Detection wavelength was at 310 nm.

Icelandic *H. selago* displays a high variation of hupA contents, from 41 µg/g to 649 µg/g. The highest hupA content (649 µg/g) was found in genotype 3, and the lowest (41 µg/g) in one specimen of genotype 2. A wide range was found in genotype 2, from 41 to 486 µg/g. HupA contents in all investigated *H. selago* were listed in Figure 10, where genotypes were marked in different colors. HupA contents in genotype 3 are

significantly higher than those in genotype 1. The determined values of hupA in all specimens are provided in the table in Appendix C.

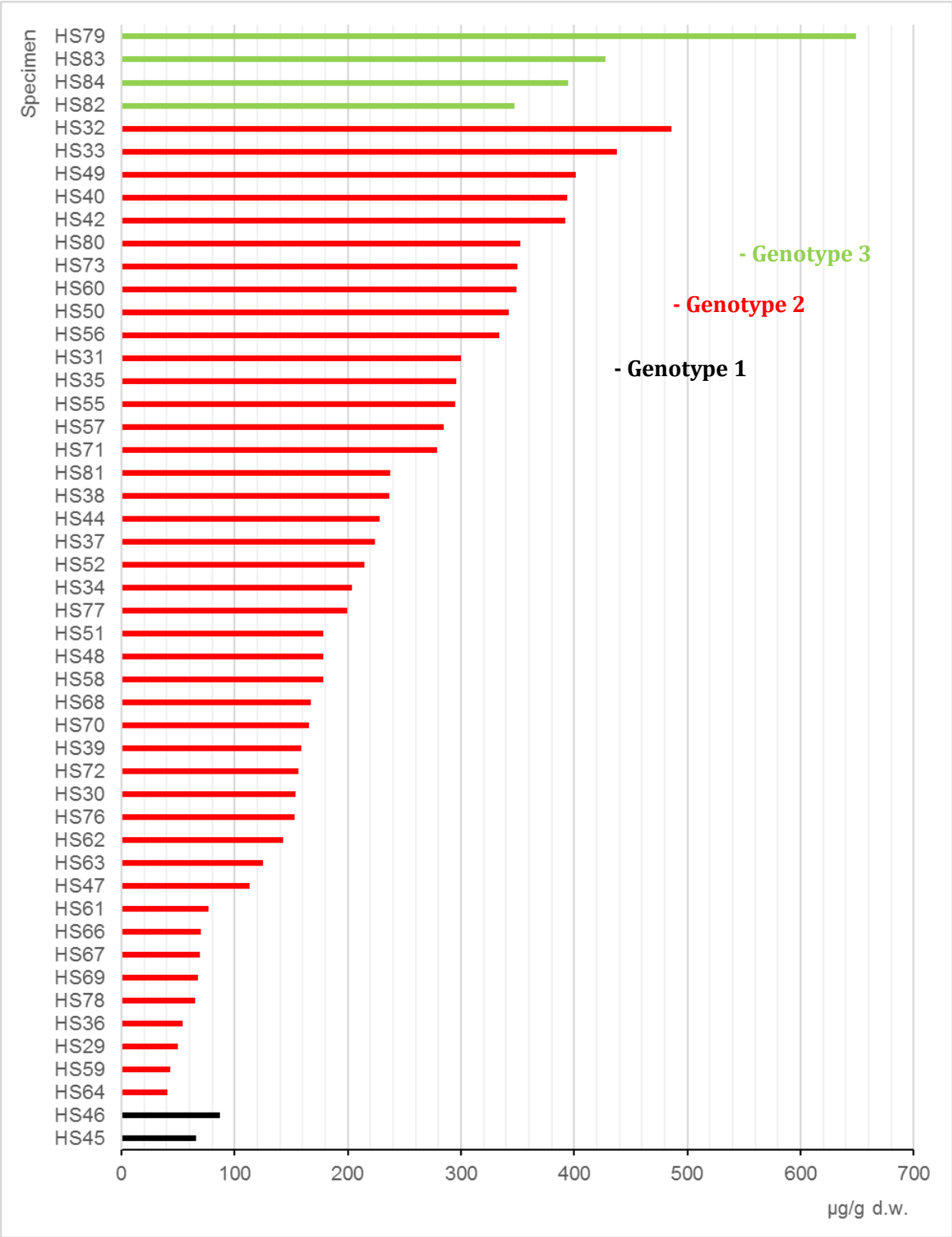



**Figure 10:** Huperzine A contents (µg/g) in Icelandic *Huperzia selago* specimens, genotypes are marked with different colors

## 4.2 Alkaloid fingerprinting of Icelandic *Huperzia selago* genotypes using LC-MS

With a gradient elution, more alkaloids eluted out before hupA ($t_R$ = 5.59 min) using the UPLC-MS method, and the alkaloid fingerprints of three genotypes are shown in Figure 11.

Genotypes displayed similar alkaloid fingerprints at first glance, with the majority of low-molecular-weight alkaloids (e.g. m/z 248 and 264) eluting before hupA. However, a few discernible peaks/alkaloids are much more intense in genotype 3 than other two genotypes, such as the peak eluting at $t_R$ 5.26 min with m/z 378 and the peak eluting at $t_R$ 7.21 and m/z 375 is only pronounced in genotype 2 and 3. On the contrary, there are also peaks more pronounced in genotype 1 and 2, such as the peak with $t_R$ 6.66 min and m/z 438.

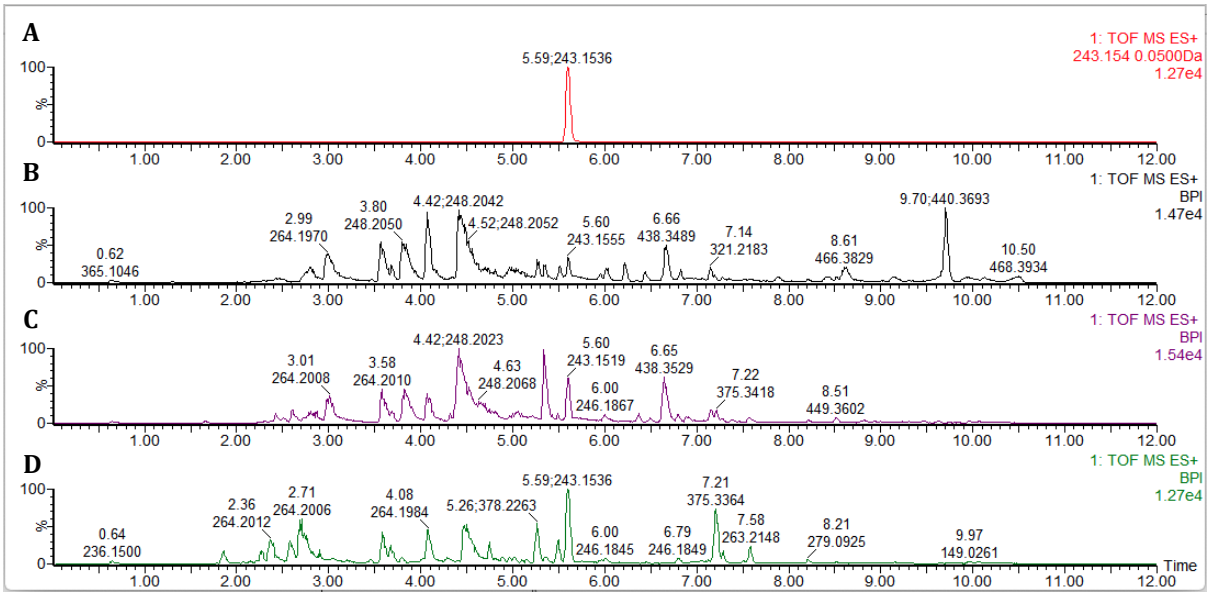



**Figure 11.** UPLC-QToF-MS chromatograms of A) standard compound, huperzine A (tR = 5.59 min), B) Icelandic Huperzia selago genotype 1, C) Icelandic Huperzia selago genotype 2 and D) Icelandic Huperzia selago genotype 3.

The peak of hupA standard (Figure 11A) shows an ion at mass to charge ratio (m/z) of 243.1536. Element composition analysis embedded in MassLynx predicted the formula as $C_{15}H_{19}N_2O$, which corresponds to protonated molecular ion of hupA.

MS/MS spectrum provides more structural information of hupA by showing fragment ions (Figure 12), the following fragments were identified in the spectra: 265.1281 ($[M+Na]_+$), 243.1303 ($[M+H]_+$), 226.1179 (loss of $NH_3$), 210.0912 (loss of $NH_3$ and $CH_4$) and 196.0764 (loss of $NH_3$ and $C_2H_6$).

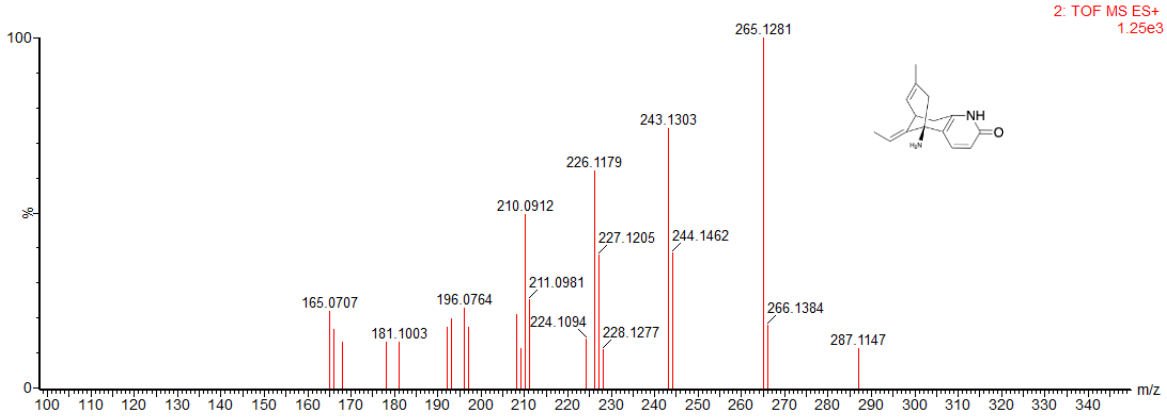



**Figure 12.** MS/MS spectra of the [M+H]+ ions for huperzine A.

The other two lycodane-type alkaloids, namely hupB (Figure 13A) and 6-hydroxy huperzine A (Figure 13B) were annotated by selected ion monitoring, shown in their extracted ion chromatograms. However, lycopodane-type alkaloids did not generate fragments at a high energy ramp up to 50 eV.

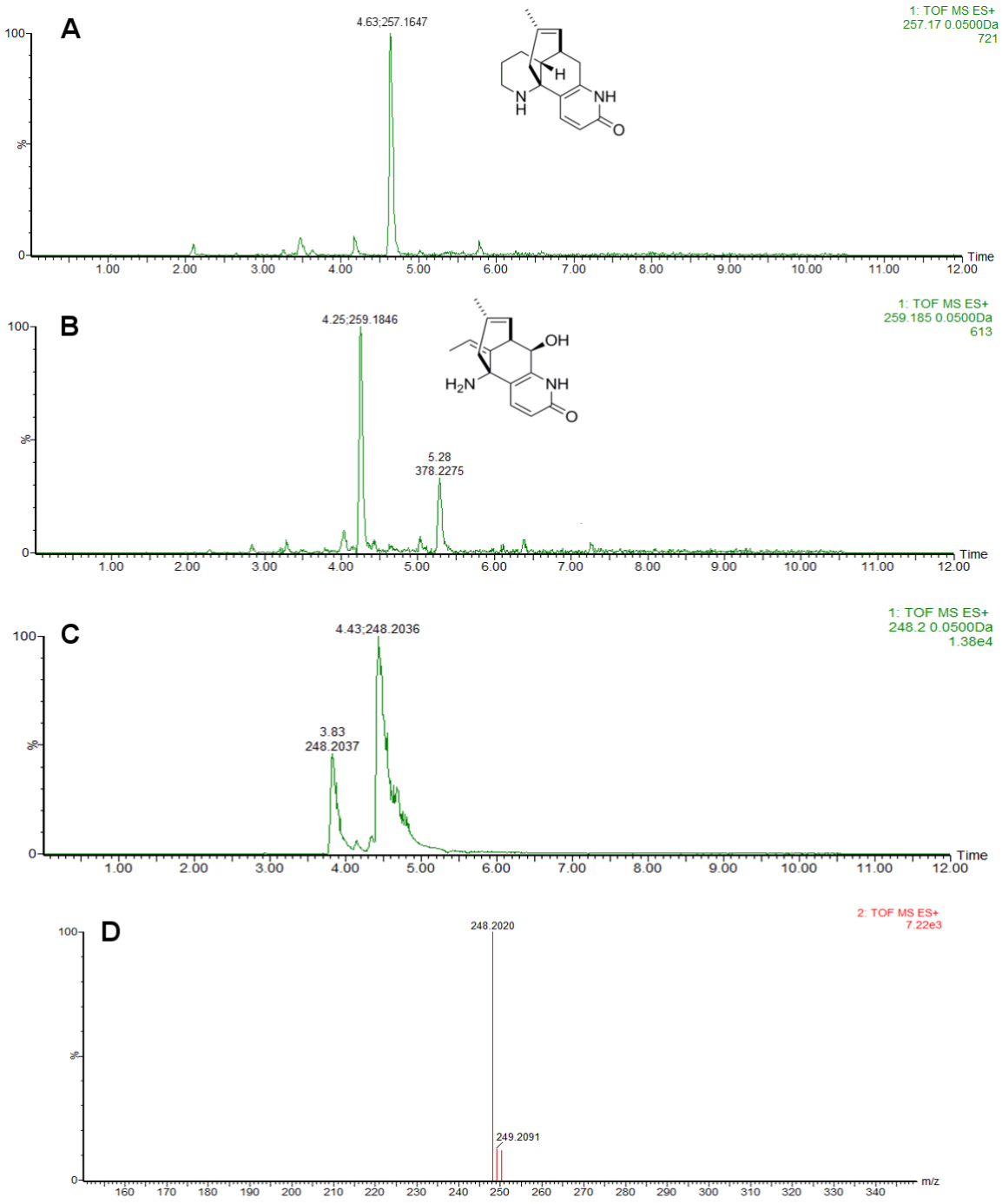



**Figure 13.** The extracted ion chromatography of A) huperzine B, B) 6-hydroxy huperzine A, C) lycopodine-like alkaloid at m/z 248 and D) MS/MS spectra for lycopodine-like alkaloids at 50 eV energy.

## 4.3 Principal component analysis of alkaloid fingerprints

Raw MS data were processed with the software MassLynx for peak alignment and normalization. Processed raw data were sent to SIMCA for principal component analysis (PCA). In the PCA plot (Figure 14A), plant genotypes are well separated using their alkaloid fingerprints, which was based on a total of 301 variables/ions. It indicated

that each genotype has his own alkaloid fingerprints. The first component can explain 16.84% variation and the second 13% variation. PCA loading plot shows the contribution of individual variables to the separation of genotypes (Figure 14B). HupA and hupB contribute to the separation of genotype 3 from the other two, and 6-hydroxyl huperzine A is driving the separation of genotype 2 and 3 from genotype 1.

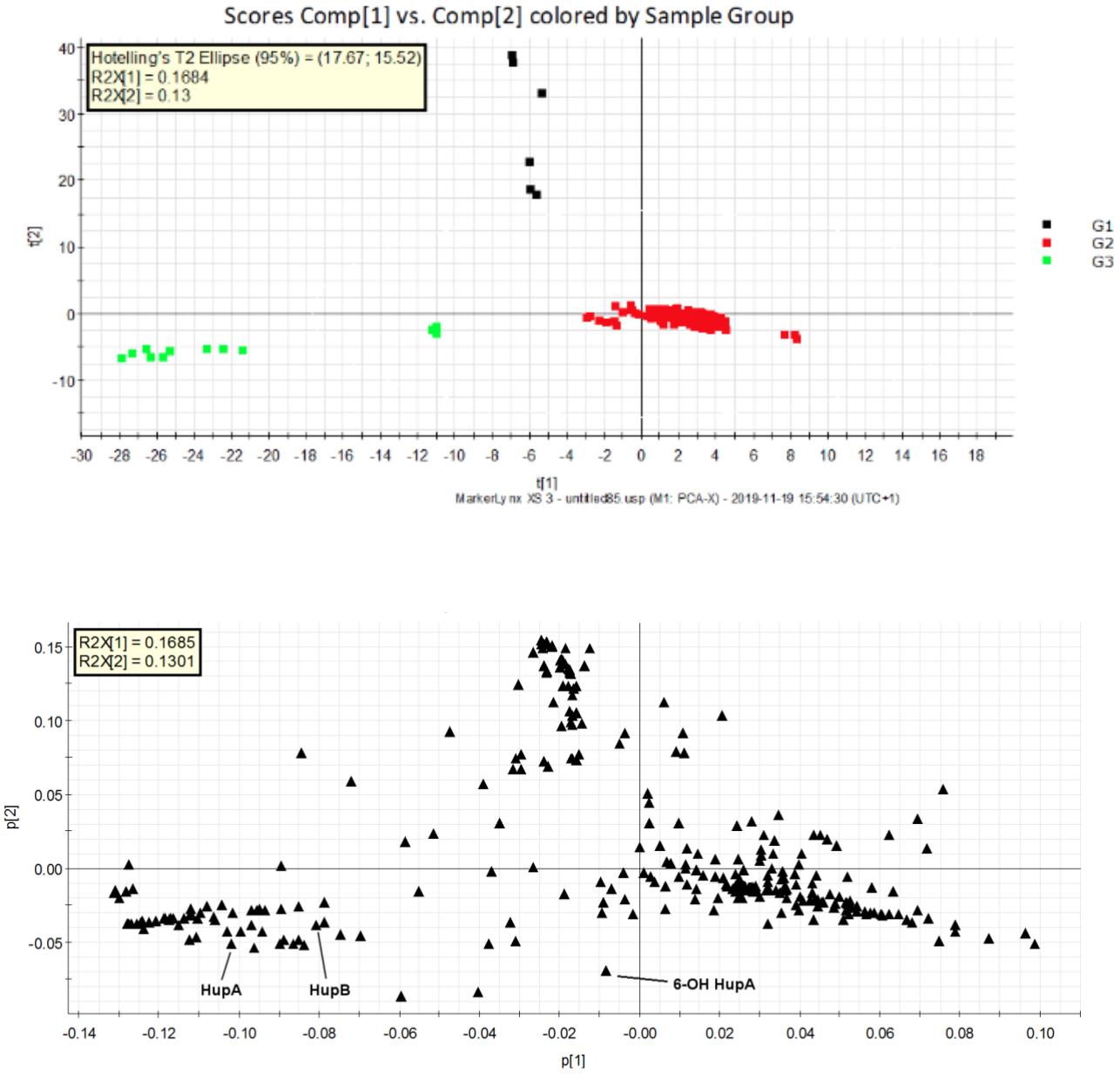



**Figure 14.** A) PCA plot of alkaloid patterns from Icelandic Huperzia selago, where each cluster indicate genotype 1-3, B) PCA loading plot shows that hupA and hupB are contributing to the separation of genotype 3 from the other two and 6-hydroxyl hupA (6-OH HupA).

# 5. DISSCUSSION

## 5.1 Variation in huperzine A contents of Icelandic *Huperzia selago*

One of the goals of this study was to determine the hupA contents in the Icelandic club moss, *Huperzia selago,* using the HPLC-UV instrument, and to assess hupA variations between genotypes. HupA contents varied from 41 to 649 µg/g d.w. in the 49 *H. selago* specimens tested. This content range is close to previous published study on hupA contents in the Icelandic *H.selago*, where the range of the hupA contents was reported to vary from 20.63 to 679.82 µg/g d.w. (Xu. et al., 2019a). HupA contents in wild grown *Huperzia* species from countries like China, Poland and Australasia have been tested and published. The *Huperzia* species in China had a variation in hupA contents from 46,85 to 254,58 µg/g d.w. (Ma et al., 2005) the once from Poland had a variation from 540 µg/g to 1270 µg/g d.w. (Szypuła et al., 2005) which is the highest amount reported yet. In wild grown population and *Huperzia* species from Australasia the variation of hupA contents was from 0 to 1030 µg/g d.w. (Goodger, Whincup, Field, Holtum, & Woodrow, 2008). The highest hupA content (3300 µg/g d.w.) was reported in cultivated *H. selago* from Poland (Szypuła et al., 2005). These considerable hupA content ranges on one hand could be attributed to natural variation between plant taxa, and on that other hand, they may be caused by differences in sample preparation, such as plant material treatment and extraction buffer. Apparently, a standardized method should be in place to make a more meaningful comparison of hupA contents. Our results indicate that genotype 3 of Icelandic *H. selago* can be a high yield natural source of hupA. Future study could explore the tissue cultivation of this genotype and optimize cultivation conditions for a higher hupA yield.

HupA content in *Huperzia* species varies between plant tissues. The leaves have been shown to contain the highest amount of hupA followed by the stems, and the lowest amount is found in the roots (Ma et al., 2005; Xu. et al., 2019a). HupA contents described from three different genotypes in this study also support the previous published study (Xu. et al., 2019a), which shows that genotype 3 has significantly higher hupA contents than genotype 1 ($p > 0.05$). In this study the highest hupA content (649 µg/g) was found in genotype 3, and the lowest (41 µg/g) in one specimen of genotype 2. Two specimens of genotype 1 have low hupA contents, 66 and 87 µg/g. A wide range of hupA contents was observed in the intermediate genotype 2, from 41

to 486 µg/g (Figure 10). Genotype 1 shows hupA content in the low end of this broad range (66-87µg/g) and genotype 3 in the high end and above (347-649 µg/g).

## 5.2 Alkaloid fingerprinting of Icelandic *Huperzia selago* genotypes

Metabolomics involves identification and quantitative understanding of all small-molecule metabolites (< 1 kDa) in biological systems (Kosmides, Kamisoglu, Calvano, Corbett, & Androulakis, 2013). There are two main approaches: profiling and fingerprinting. The former focuses on quantitation of all metabolites and therefore are more time- and labor-consuming, while the latter aims at rapid analysis to discover patterns in samples and provide classifications (Ellis, Dunn, Griffin, Allwood, & Goodacre, 2007). To apply the chemical fingerprinting approach into this study, it is important to capture as many ionized alkaloid peaks as possible. It has been shown that 3% acetic acid as LA extraction solution outperformed 3% tartaric acid in reflecting alkaloid diversity and quantity (Cuthbertson et al., 2012).

Results of genotypes separation from this study shows a better separation than that in the previous study (Xu et al., 2019b). This may be due to the small sampling size of genotype 1 and genotype 3, in comparison to the published study. Variations in these two genotypes were not fully represented in this study. It is also interesting to see that specimens of genotype 2 were very much centralized in this study, even though genotype 2 has a large hupA content variation and is well sampled. The gene expression involved in alkaloid production may differ between genotypes since each genotype has its own alkaloid fingerprints (Wink, 2003).

HupA has a good UV absorbance and therefore is well detected with HPLC-UV, but not all lycopodium alkaloids have good UV absorbance, like lycopodane-type alkaloids. There are more alkaloids detected by UPLC-MS compared to HPLC-UV, since alkaloids by nature are prone to ionization. Mass spectrometry analysis of lycopodane-type alkaloids lacks characteristic fragment ions when instruments are performed at regular energy levels (10 – 35 eV) (Shan et al., 2016; Xu et al., 2019b). A high collision energy level up to 50 eV was proposed in the previous study (Xu et al., 2019b), and this was particularly tested in this study. However, no major fragment ions were found in lycopodane-type alkaloid peaks using current electrospray ionization. Therefore, even higher collision energy should be tested for analysis of these alkaloids or another ionization technique with high collision capability could be applied, such as

atmospheric pressure chemical ionization (Csupor et al., 2009). The composition of mobile phase affects e.g. elution, shapes and separations of the peaks. In this study the mobile phase was mild acidic, the peak eluting at 4.42, m/Z 248 is quite tailing and that may be improve by changing to an alkaline mobile phase.

From the loading plot, hupA and hupB are correlated in approximately 40% of the variation reflected by the two principal components. Both alkaloids drive the separation of genotype 3 from the other two. This is in agreement with a previous study of the three genotypes (Xu. et al., 2019a) where hupB was primarily found in genotype 3.

The present study shows that the same plant species could differ qualitatively and quantitatively in its chemical fingerprints between genotypes. Therefore, it is recommended that precautions should be taken in plant identification before phytochemical studies are initiated, to ensure data consistency (Xu, Heidmarsson, De Boer, Kool, & Olafsdottir, 2019c). The separation of plant genotypes using chemical fingerprint is also an interesting chemotaxonomic tool, which enables subspecies or genotype level plant classification.

# 6. CONCLUSIONS

This study has determined quantitative variation of hupA between *H. selago* genotypes in Iceland. A large variation was found from 41 to 649 ug/g d.w., where genotype 3 contains the highest amount of hupA. Genotype 3 can be considered as the preferred source of natural hupA for further studies. Alkaloid fingerprinting and PCA can distinguish between the three genotypes of *H. selago*, and reveals a quantitative and qualitative difference in their alkaloid composition. The study highlights the importance of chemical fingerprinting and thorough plant identification for selection of medicinal plant raw material with the highest pharmaceutical interest.

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