

TOXICOLOGICAL INVESTIGATION, IDENTIFICATION, AND BIOACTIVITY  
EVALUATION OF STEROIDAL ALKALOIDS DERIVED FROM VERATRUM  
PARVIFLORUM

by

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A thesis

submitted in partial fulfillment

of the requirements for the degree of

Master of Science in Chemistry

Boise State University

August 2022

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BOISE STATE UNIVERSITY GRADUATE COLLEGE

**DEFENSE COMMITTEE AND FINAL READING APPROVALS**

of the thesis submitted by

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Thesis Title: Toxicological Investigation, Identification, and Bioactivity Evaluation of Steroidal Alkaloids Derived from *Veratrum parviflorum*

Date of Final Oral Examination: 11 May 2022

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## ACKNOWLEDGMENTS

The work presented in this thesis was made possible by those who came before me and laid the foundations for this research, provided my training, and worked alongside me. I would like to acknowledge Dr. Matthew Turner for his work regarding the investigation of *Veratrum californicum* steroidal alkaloids. Many of the methods used in this research were adapted from his publications. Thank you to the physicians at Emory University for bringing this project to Dr. Owen McDougal's lab, providing patient and plant samples, and collaborating on a publication for this work. Dr. Shin Pu was an indispensable resource regarding the collection of HPLC-MS data and cell culture. Lastly, I wish to acknowledge the valuable insights and resources provided by the members of my Supervisory Committee, Drs. Owen McDougal, Julia Oxford, and Lisa Warner.

## ABSTRACT

Plants of the *Veratrum* genus have been used throughout history for their emetic properties, rheumatism, and for the treatment of high blood pressure. However, inadvertent consumption of these plants, which resemble wild ramps, induces life threatening side effects attributable to an abundance of steroidal alkaloids. Several of the steroidal alkaloids from *Veratrum* spp. have been investigated for their ability to antagonize the Hedgehog (Hh) signaling pathway, a key pathway for embryonic development and cell proliferation. Uncontrolled activation of this pathway is linked to the development of various cancers, most notably basal cell carcinoma and acute myeloid leukemia. Additional investigation of *Veratrum* spp. may lead to the identification of novel alkaloids with potential to serve as chemotherapeutics. This project aimed to identify steroidal alkaloids in *V. parviflorum*, perform a toxicological investigation for the presence of these alkaloids in the blood and breast milk of patients who ingested *V. parviflorum*, and evaluate the bioactivity of these alkaloids for inhibiting the Hh signaling pathway. Four steroidal alkaloids were identified in the ethanolic extract of *V. parviflorum*: cyclopamine, veratramine, jervine, and muldamine. Cyclopamine, veratramine, and jervine were identified in patient blood while cyclopamine and veratramine were identified in patient breast milk. A bioactivity assessment of the *V. parviflorum* extract using Shh-Light cells suggested that there are additional compounds within the plant that are antagonistic to the Hh signaling pathway.

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## LIST OF ABBREVIATIONS

|           |   |
|-----------|---|
| Hh        | Hedgehog  |
| Smo       | Smoothened  |
| FDA       | Food and Drug Administration                            |
| ITS       | Internal Transcribed Spacers                            |
| TRAIL     | Tumor Necrosis Factor-Related Apoptosis-Inducing Ligand |
| KAAD      | 3-keto, N-aminoethyl aminocaproyl dihydrocinnamoyl      |
| CYP90B27  | Cholesterol 22-hydroxylase                              |
| CYP94N1   | 26-hydroxylase/oxidase                                  |
| GABAT1    | 22-hydroxycholesterol-26-al transaminase                |
| CYP90G1   | 22-hydroxy-26-aminocholesterol 22-oxidase               |
| Shh       | Sonic Hedgehog  |
| HPLC-QTOF | High Performance Liquid Chromatography                  |
| QTOF      | Quadrupole Time-of-Flight                               |
| MS        | Mass Spectrometry                                       |
| IV        | Intravenous   |
| ED        | Emergency Department                                    |
| EKG       | Electrocardiogram                                       |
| DIF       | Digoxin Immune Fab                                      |
| ICU       | Intensive Care Unit                                     |
| BUN       | Blood Urea Nitrogen                                     |

|      |                                  |
|------|----------------------------------|
| SLE  | Supported Liquid Extraction      |
| ESI  | Electrospray Ionization          |
| PVDF | Polyvinylidene Difluoride        |
| LOD  | Limit of Detection               |
| LOQ  | Limit of Quantification          |
| BCC  | Basal Cell Carcinoma             |
| AML  | Acute Myeloid Leukemia           |
| DMEM | Dulbecco's Modified Eagle Medium |
| PTFE | Polytetrafluoroethylene          |





## CHAPTER ONE: VERATRUM PARVIFLORUM: AN UNDEREXPLORED SOURCE FOR BIOACTIVE STEROIDAL ALKALOIDS

### 1. Introduction

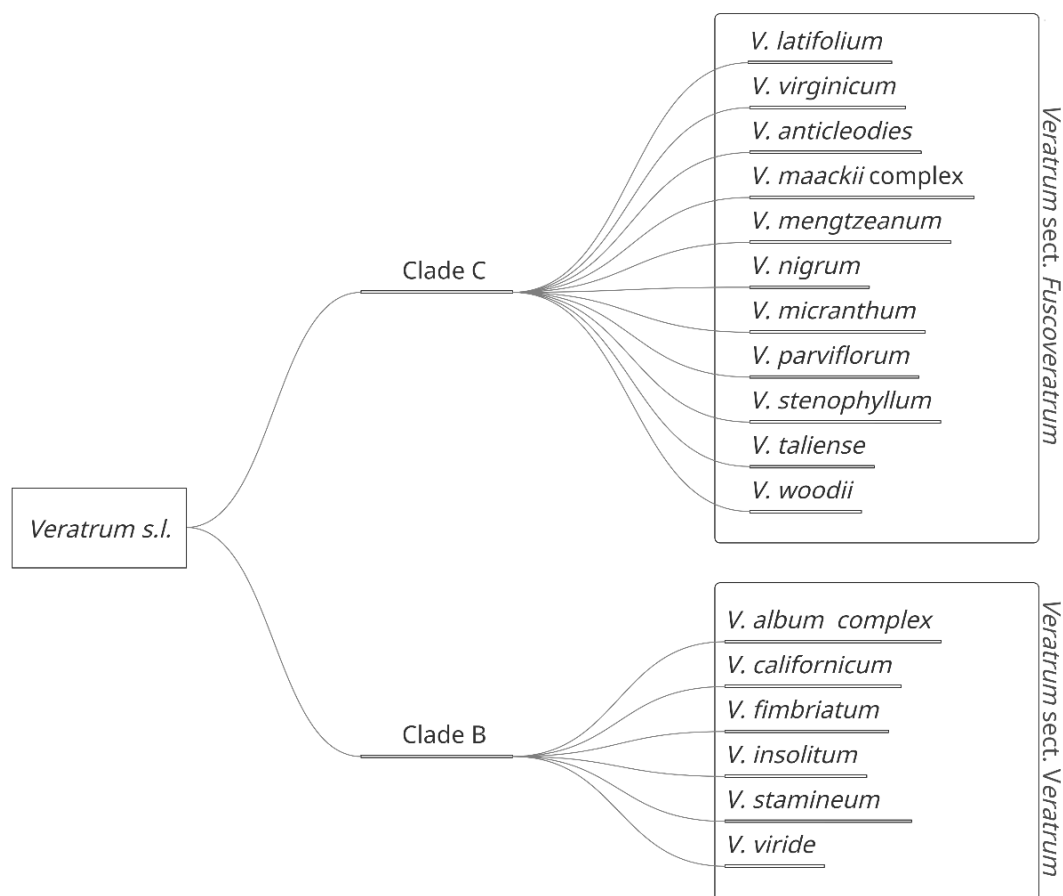
Most modern therapeutics have originated from a treasure trove of secondary metabolites extracted from natural products of terrestrial or marine origin. An estimated 70,000 plant species have been used throughout history for medicinal purposes and more than 3,000 plants are reported to contain compounds with anticancer properties.<sup>1,2</sup> The *Veratrum californicum* derived steroidal alkaloid, cyclopamine, was first isolated in 1965 and later identified as an inhibitor of the protein Smoothed (Smo), which is a critical protein in the Hedgehog signaling pathway.<sup>3,4</sup> Since this discovery, a new class of Food and Drug Administration (FDA) approved chemotherapeutics called Hedgehog pathway inhibitors have been developed for the treatment of cancers, most prominently basal cell carcinoma and acute myeloid leukemia.<sup>5</sup> Plants from the genus *Veratrum*, including *V. viride*, *V. album*, *V. nigrum*, and *V. californicum* have been extensively studied, and found to be rich sources for unique steroidal alkaloids (>100 alkaloids/plant), with approximately 20% of these secondary metabolites being characterized.<sup>6</sup> Here we present a consolidated review of the morphological, ecological, and phytochemical information regarding the sparsely studied *Veratrum* spp., *V. parviflorum*.

## 1.1 Background

### 1.1.1 *Veratrum* Genus

The *Veratrum* genus is comprised of perennial flowering herbs located predominantly in the Northern hemisphere.<sup>7</sup> These plants are found throughout temperate regions of North America and northern temperate to arctic regions in Eurasia.<sup>7</sup>

Depending on taxonomic treatment, the number of species varies between 17-45 species, which can be divided into four species complexes: *V. album* L., *V. nigrum* L., *V. mackii* Regal, and *V. viride* Aiton.<sup>7,8</sup> Wide variability in taxonomic treatment may be attributed to dissimilarity in morphology, including leaves, tepals, and perigonal nectaries, and habitats, including rocky tundra, bogs, meadows, riverbanks, swamps, and deciduous forest slopes.<sup>7</sup> *Veratrum* may be further divided into two major sections: *Veratrum* sect. *Veratrum* [Clade B] and *Veratrum* sect. *Fuscoveratrum* [Clade C] (Figure 1.1).



**Figure 1.1** *Veratrum* spp. separated by classification in *Veratrum* sect. *Fuscoveratrum* and *Veratrum* sect. *Veratrum*.

### 1.1.2 Medicinal Relevance

Traditional medicines have utilized *Veratrum* spp. plants as a source of therapeutically active compounds for centuries.<sup>6,9</sup> Chinese medicine utilized *V. nigrum* in a medicinal concoction, referred to as Li-lu, to treat conditions including aphasia resulting from apoplexy, wind-type dysentery, jaundice, scabies, and chronic malaria.<sup>10</sup> The roots and rhizome of *V. album* subsp. *lobelianum* are described in the first Pharmacopoeia Rossica as a traditional Russian medicine that is made into a tincture or ointment for the treatment of head lice, scabies, neuralgic and rheumatic pain, eczema, or fevers.<sup>11</sup> This plant has also been used as an antiparasitic in cattle against

hypodermatosis.<sup>11</sup> *V. album* has seen widespread use in Eurasia.<sup>12</sup> The Greeks used a powdered form of *V. album* to induce sneezing and for psychological diseases such as depression and epilepsy.<sup>12,13</sup> An alcohol extract of *V. album*'s roots was used in Italy as an antirheumatic.<sup>12</sup> In Iranian folk tradition, a paste of *V. album* roots was used to relieve headaches and neuralgic pains.<sup>12</sup> In North America, Native American tribes including the Shoshone, Bella Coola, Cherokee, Gitksan, Haisla, Hanaksiala, Iroquois, Kitasoo, Okanagan-Colville, Oweekeno, Quinault, Salish Thompson, and Tsimishian used the crushed roots of *V. viride* as an antirheumatic, to treat snake bite wounds, to make a tea for venereal diseases, and as an analgesic for sore throats and colds.<sup>6,12</sup> Table 1.1 presents a comparison of several *Veratrum* spp. including identified steroidal alkaloids and traditional medical applications.

**Table 1.1 A Comparison of Identified Steroidal Alkaloids and Traditional Medicinal Applications for Several *Veratrum* spp.**

| <i>Veratrum</i> spp.                | Alkaloids Identified   | Traditional Medical Applications  | References  |
|-------------------------------------|--|---|-------------|
| <i>V. nigrum</i> <sup>a</sup>       | Epiverazine, veratramine, verazine   | Apoplexy, wind-type dysentery, jaundice, scabies, chronic malaria                       | 10,14       |
| <i>V. album</i> <sup>b</sup>        | Verazine, jervine, pseudojervine, rubijervine, veralosine, veralosidine, verabenzoamine, veratroilzigadenine, 15-O-(2-methylbutyroyl)germine, veralosinine, veratramine, veratridine, cevadine | Head lice, scabies, neuralgic pain, eczema, fever, hypodermatitis, rheumatism, headache | 11,12,13,14 |
| <i>V. viride</i> <sup>b</sup>       | Veratramine, isorubijervosine, pseudojervine, rubijervine  | Rheumatism, venereal diseases, analgesic  | 6,12,14     |
| <i>V. californicum</i> <sup>b</sup> | Cyclopamine, veratramine, muldamine, isorubijervine, cycloposine, veratrosine  | None reported   | 6,14        |
| <i>V. parvilforum</i> <sup>a</sup>  | Cyclopamine, veratramine, veratridine, verazine  | None reported   | 15          |

<sup>a</sup>Species within *Veratrum* sect. *Fuscoveratrum* [Clade C].

<sup>b</sup>Species within *Veratrum* sect. *Veratrum* [Clade B].

## 2. *Veratrum parviflorum*

### 2.1 Taxonomy and Physical Characteristics

*Veratrum (Melanthium) parviflorum*, commonly known as mountain bunchflower, has a complex history regarding its classification in the *Veratrum* and *Melanthium* genera due to variation in morphological constraints set by botanists.<sup>7,16</sup> To provide a more defined taxonomy of *Veratrum* spp., the nuclear ribosomal internal transcribed spacers (ITS) were analyzed and correlated to traditional taxonomic classifications including flower color and geographical location.<sup>7</sup> The strict and bootstrap consensus trees were almost identical, except for *V. parviflorum*. The strict consensus suggested that *V. parviflorum* formed a subclade and was sister to *V. latifolium*, *V. virginicum*, and *V. woodii*, whereas the bootstrap consensus suggested that this species falls outside of the clade, forming a polytomy with the *V. maackii* and *V. micranthum* complexes.<sup>7</sup>

*V. parviflorum* is identified in nature using defined morphological traits. The stem is slender and 2 to 5 feet tall.<sup>16,17</sup> A pseudostem is formed by the overlapping sheaths of the leaves which are broad (2-4 inches wide), petiolate, obscurely plicate, and have a blue tint adaxially.<sup>7,16</sup> The tepals are pale green to olive green, narrowly rhombic oblanceolate, with entire margins, gradually attenuated at base, filaments adnate, gland bilobed, diffuse, and dark (Figure 1.2).<sup>7</sup>



**Figure 1.2** *V. parviflorum* in situ before blooming (left). In the later stages of growth, a stem protrudes from the base of the plant and blooms with pale green flowers.<sup>20</sup>

## 2.2 Geographic Location and Herbivory

*V. parviflorum* is found in the Southeastern regions of North America.<sup>15-19</sup> This species grows in rich deciduous forests (800-2030 m) in the mid-Appalachians, including parts of Alabama, Georgia, Kentucky, North Carolina, South Carolina, Tennessee, Virginia, and West Virginia.<sup>15,16</sup> The states of Alabama, Kentucky, and West Virginia have classified the conservation status of *V. parviflorum* as critically imperiled (S1), imperiled (S2), and vulnerable (S3), respectively.<sup>18,19</sup> This plant is most easily discovered in the spring when it reaches peak germination.<sup>21</sup> Unfortunately, *V. parviflorum* growth season coincides with wild ramps (*Allium tricoccum*), and has led to accidental ingestion of *V. parviflorum*, resulting in cardiac and gastrointestinal toxicity.<sup>15</sup> Despite inducing toxic effects when ingested by humans, white-tailed deer have been observed to consume the entire inflorescences of this species.<sup>16,22</sup>

### 2.3 Toxicity

Cases of *Veratrum* poisoning are found extensively within literature, however, *V. parviflorum* has only been implicated in one case of poisoning in the United States.<sup>15</sup> Symptoms of *Veratrum* poisoning generally include nausea, vomiting, diarrhea, hypotension, bradycardia, hypopnea, paresthesia, or death if medical attention is not received.<sup>15,23-32</sup> Treatment for *Veratrum* poisoning is generally symptomatic and supportive, and may include the administration of atropine, intravenous fluids, vasopressors, activated charcoal, and promethazine.<sup>15,24-30,32</sup> Although digoxin immune Fab has been used for treating symptoms of cardiotoxicity similar to those observed in cases of *Veratrum* poisoning, it has been suggested that medical providers should not unnecessarily administer DigiFab<sup>TM</sup> as they do not bind steroidal alkaloids extracted from *V. viride*.<sup>32</sup> Furthermore, Multigent<sup>TM</sup> digoxin immunoassay reagent antibodies demonstrated cross-reactivity with the alkaloids, resulting in false-positive tests.<sup>32</sup> Table 1.2 presents a summary of treatments for several cases of *Veratrum* poisoning.



**Table 1.2 Summary of Several Cases of *Veratrum* Poisoning Including Causative Plant, Symptoms, and Treatment**

| <i>Veratrum</i> spp.<br>Ingested | Symptoms  | Treatment  | References   |
|----------------------------------|---|--|--------------|
| <i>V. parviflorum</i>            | Nausea, vomiting, hypotension, bradycardia  | Antiemetics, intravenous fluid resuscitation, digoxin immune Fab   | 15           |
| <i>V. viride</i>                 | Nausea, vomiting, diaphoresis, lightheadedness, bilateral retrobulbar headache, leg spasms, hypotension, bradycardia, paresthesia, dyspnea, sluggishly reactive 2-3 mm pupils   | intravenous fluid resuscitation, atropine, promethazine, dopamine infusion   | 23,24, 26,32 |
| <i>V. album</i>                  | Nausea, vomiting, headache, diarrhea, bradycardia, dizziness, paresthesia, blurred vision, abdominal pain, clouded consciousness, pyrosis, atrioventricular dissociation, death | Activated charcoal, antiemetics, intravenous fluid resuscitation, thiethylperazine, atropine, prednisolone, hydrocortisone, tocopherol, unithiol, digoxin immune Fab | 25,27-31     |

The cardiotoxic effects from consuming *Veratrum* spp. are primarily due to the steroidal alkaloids produced by the plant.<sup>33</sup> These steroidal alkaloids are recognized for their tendency to bind to the type 2 receptor site of voltage gated sodium ion channels in vertebrate organisms.<sup>33,34</sup> Once bound, the resting membrane potential is depolarized, causing excitable membranes to fire repetitively.<sup>33-35</sup> Symptoms including bradycardia and hypotension are caused by the alkaloids interacting with cardiac receptors in the left ventricle posterior wall and the baroreceptor area of the coronary sinus while depolarization in the vagus nerve can induce bradycardia, hypotension, and dyspnea.<sup>33,36</sup> Additional symptoms resulting from depolarization of the nerve cells may comprise of paresthesia, numbness, and vomiting.<sup>33</sup> The triad of symptoms including bradycardia, hypotension, and dyspnea caused by *Veratrum* poisoning is referred to as a Bezold-Jarisch reflex.<sup>6,33,37,38</sup>

*Veratrum* steroidal alkaloids are recognized as antagonistic to the Hedgehog signaling pathway.<sup>39-41</sup> In the late 1950s, sheep herders in the South Central and Southwestern alpine meadows of Idaho observed that 1-25% of their lambs were born with cyclopean-type developmental defects.<sup>42</sup> These malformations were originally thought to be congenital, however, further investigations revealed that they resulted from pregnant ewes feeding on *V. californicum* between the 10th and 15th days of gestation.<sup>42,43</sup> The *Veratrum* steroidal alkaloids cyclopamine, jervine, cycloposine, and veratrosine were identified as the causative teratogenic agents.<sup>44</sup> *Veratrum* steroidal alkaloids exert teratogenic effects via antagonism of the Hedgehog signaling pathway by directly binding to the transmembrane protein Smoothed (Smo).<sup>4,39</sup> The binding of the

small molecule to Smo takes place in the deep seven-transmembrane pocket of Smo, inhibiting activation by membrane sterols.<sup>45</sup>

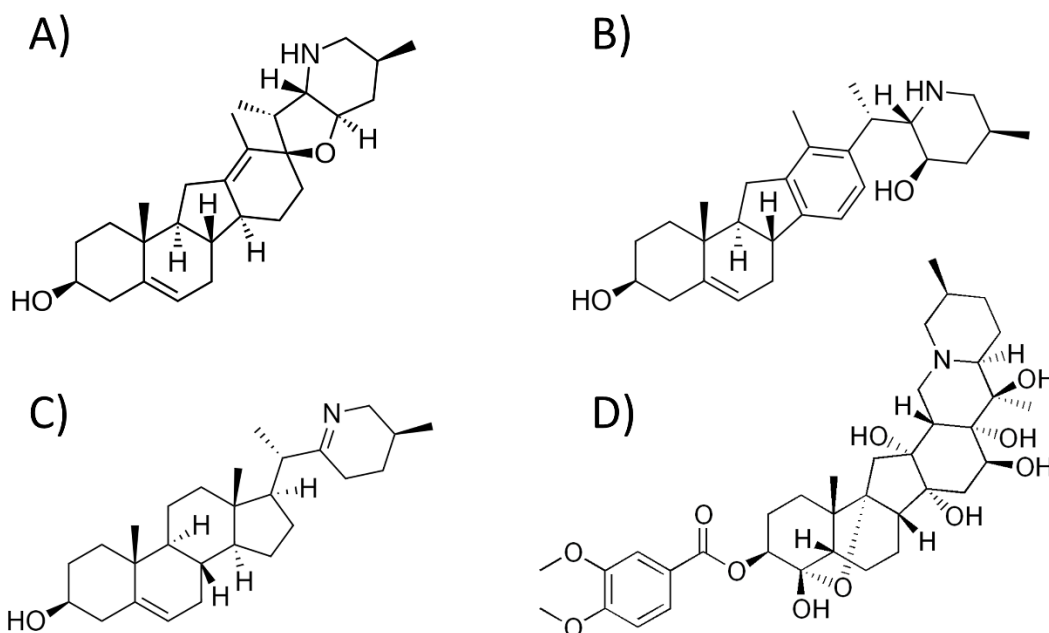
## 2.4 Phytochemistry

There is a lack of published information regarding the steroidal alkaloid content in *V. parviflorum*. Currently, only four alkaloids have been identified in an extract of its roots and rhizome.<sup>15</sup> The four alkaloids including cyclopamine, veratramine, verazine, and veratridine are not novel to the *Veratrum* genus and have been investigated for a variety of bioactive properties.

### 2.4.1 Cyclopamine

Cyclopamine (Figure 1.2A) was isolated from *V. grandiflorum* in 1965 and was the first molecule identified to inhibit the Hedgehog signaling pathway.<sup>3,4,6,46</sup> Since its discovery, cyclopamine has also been identified in *V. californicum* and *V. parviflorum*.<sup>6,14,15,41,44</sup> Cyclopamine is classified as a jervanine type steroidal alkaloid with a C-nor-D-homosteroidal skeleton where the C and D rings of the steroidal backbone are five and six membered rings, respectively.<sup>6</sup> Jervanine type alkaloids feature a tetrahydrofuran E-ring that links the nitrogen containing F-ring to the D-ring through a spiro-carbon at the cyclic ether.<sup>6</sup> This compound has been observed to inhibit Hedgehog signaling in Shh Light II cells, inhibit the growth of breast cancer, induce apoptosis in human prostate cancer, increase the expression of death receptor 5 in tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) resistant gastric cancer cells, induce apoptosis and COX-2 overexpression via PKC activation in HEL and TF1a human erythroleukemia cell lines, and induce growth inhibition in human carcinogenesis of cholangiocarcinoma cell lines.<sup>41,47-51</sup> Cyclopamine has shown promise as a human

chemotherapy, however, it has limited use due to its low solubility in aqueous solutions, and instability in acidic conditions.<sup>52</sup> To address these limitations, semi-synthetic approaches have been undertaken to increase potency and solubility.<sup>53,54</sup> The cyclopamine derivative KAAD-cyclopamine included the addition of a 3-keto,N-aminoethyl aminocaproyl dihydrocinnamoyl (KAAD) functional group to the F-ring nitrogen, resulting in a 10-20 fold increase in potency.<sup>52,53</sup> A cyclopamine-tartrate salt was developed to increase the solubility of the compound in water.<sup>52,54</sup> The cyclopamine-tartrate salt was more soluble in water at about 5 mg/mL, a higher LD<sub>50</sub> of 62.5 mg/kg of body weight compared to that of cyclopamine which is 43.5 mg/kg and had a lower tumor area value in Krt6a-cre: Ptch1<sup>neo/neo</sup> mice.<sup>54</sup> Cyclopamine has been used as a molecular scaffold for the semi-synthetic derivative patidegib, that is currently undergoing phase III clinical trials. Patidegib, formerly known as saridegib and IPI-926, has received orphan drug approval for the treatment of nevoid basal cell carcinoma.<sup>5,55</sup>



**Figure 1.3** Chemical structures of A) cyclopamine, B) veratramine, C) verazine, and D) veratridine.

### 2.4.2 Veratramine

Veratramine (Figure 1.3B) has been identified in *V. parviflorum*, *V. viride*, *V. oxyssepalum*, *V. nigrum* L., *V. californicum*, and *V. grandiflorum*.<sup>15,41,56-59</sup> Similar to cyclopamine, veratramine contains a C-nor-D-homosteroidal skeleton, however, it is further categorized as a veratranine type alkaloid.<sup>6</sup> In comparison to jervanine-type alkaloids, those of the veratranine-type feature an aromatized D-ring and lack a tetrahydrofuran E-ring that connects the piperidine ring to the D-ring. Veratramine is a lipid-soluble alkaloid that exhibits a range of bioactivities.<sup>60</sup> This alkaloid was observed to cause DNA damage in the cerebellum and cerebral cortex of mice in a dose-dependent trend through the generation of reactive oxygen species, inhibit Hedgehog signaling in Shh light II cells, reduce the growth, proliferation, and migration of the PC-3 human metastatic prostate cancer cell line, and induce autophagy-mediated apoptosis by inhibiting PI3K/Akt/mTOR signaling in HepG2 cells.<sup>14,41,60-63</sup> One study regarding the metabolism of veratramine in male Sprague–Dawley rats suggested elimination of the alkaloid primarily occurred through phenyl mono-oxidation, hydroxylation, and methylation.<sup>60</sup> The phenyl-oxidation metabolite of veratramine was proposed to lead to the formation of reactive oxygen species that oxidize DNA and proteins.<sup>60</sup>

### 2.4.3 Verazine

Verazine (Figure 1.3C) is a precursor to steroidal alkaloids, including cyclopamine and veratramine, found across the *Melanthiaceae* and *Solanaceae* plant families.<sup>6,15,64-68</sup> This compound is classified as a verazine type steroidal alkaloid in the cyclopentanophenanthrene skeleton ring system.<sup>6</sup> The cyclopentanophenanthrene skeleton features a ring scaffold typical of cholesterol where the C-ring and D-ring are six

and five membered, respectively.<sup>6</sup> Verazine type alkaloids are differentiated from additional cyclopentanophenanthrene skeleton alkaloids by the presence of an imine containing ring.<sup>6</sup> The importance of verazine to the biosynthesis of *Veratrum* steroidal alkaloids has promoted efforts to elucidate its biosynthetic production from cholesterol.<sup>68</sup> Augustin et al. identified cholesterol 22-hydroxylase (CYP90B27), 22-hydroxycholesterol, 26-hydroxylase/oxidase (CYP94N1), 22-hydroxycholesterol-26-al transaminase (GABAT1), and 22-hydroxy-26-aminocholesterol 22-oxidase (CYP90G1) as the four enzymes that transform cholesterol into verazine.<sup>68</sup> Although these efforts have illustrated how verazine forms, proceeding steps in the biosynthetic formation of additional steroidal alkaloids remain largely unexplored. Kaneko et al. performed a series of studies that observed the conversion of products within the biosynthetic pathway, however, the mechanisms in which these conversions take place remain unknown.<sup>69-76</sup> Verazine has not been studied for potential anticancer properties, however, the alkaloid does exhibit antifungal and melanogenesis inhibitory properties.<sup>66,67</sup> The growth of *Candida albicans* and *Trichophyton rubrum* was inhibited at minimum inhibitory concentrations of 6.2 µg/mL and 3.1 µg/mL, respectively.<sup>67</sup> Furthermore, melanogenesis in B16 F1 mouse melanoma cells were inhibited with an IC<sub>50</sub> of <1 µg/mL.<sup>66</sup> Verazine also showed inhibitory activity for Sc7 yeast, however, it proved to be cytotoxic in an M-109 cell line with an IC<sub>50</sub> of 12.5 µg/mL.<sup>64</sup>

#### 2.4.4 Veratridine

Veratridine (Figure 1.3D) has been identified in *V. album*, *V. viride*, *V. parviflorum*, and *Schoenocaulon officinale*.<sup>15,31,77,78</sup> This compound is classified as a cevanine type alkaloid with a C-nor-D-homosteroidal skeleton.<sup>6</sup> The cevanine alkaloids

are defined by the presence of a six membered E-ring, are highly hydroxylated, and a hemiketal linkage between C4 and C9.<sup>6</sup> This compound is primarily recognized as one of the major alkaloids contributing to the cardiotoxic effects from *Veratrum* poisoning.<sup>15,27,31,79</sup> Veratridine has been identified as an agonist of voltage-gated sodium ion channels.<sup>80</sup> The compound binds to the type 2 receptor of voltage-gated sodium ion channels, leading to membrane depolarization and repetitive firing of the nerve.<sup>33-35,80</sup> Unlike cyclopamine and veratramine, veratridine has not been observed to inhibit Hedgehog signaling through antagonism of Smo. Belgacem and Borodinsky used veratridine to study the effects of a Ca<sup>2+</sup> spike on Gli transcriptional activity.<sup>81</sup> Veratridine selectively inhibits voltage-gated Na<sup>+</sup> ion channels resulting in an increase of Ca<sup>2+</sup> spike activity and diminished Gli levels, which downregulates Sonic hedgehog (Shh) signaling.<sup>81</sup> In contrast, if voltage-gated Na<sup>+</sup> and Ca<sup>2+</sup> ion channels were blocked, Gli transcriptional activity was increased, resulting in the upregulation of Shh signaling.<sup>81</sup> These results suggested that Shh signaling may be selectively regulated by a *Veratrum* steroidal alkaloid in mechanisms other than Smo antagonism.

### 3. Conclusion

*Veratrum* spp. have been investigated for their potential to inhibit the growth of cancers, such as basal cell carcinoma and acute myeloid leukemia, resulting from aberrant activation of the Hedgehog signaling pathway. Although species including *V. viride*, *V. album*, *V. nigrum*, and *V. californicum* have been studied extensively, there is limited information regarding the phytochemistry of *V. parviflorum*. Only four alkaloids (cyclopamine, veratramine, verazine, and veratridine) with known bioactivities have been identified in *V. parviflorum*, however, over forty-three prominent peaks were observed in

the chromatogram of its extract.<sup>15</sup> A combined effort to characterize these unknown alkaloids and assess their capability to antagonize Hh signaling may yield a compound suitable for further study.



## CHAPTER TWO: VERATRUM PARVIFLORUM POISONING: IDENTIFICATION OF STEROIDAL ALKALOIDS IN PATIENT BLOOD AND BREAST MILK

### 1. Abstract

#### 1.1 Introduction

The *Veratrum* genus is composed of plants containing a diverse set of steroidal alkaloids. Ingestion of *Veratrum* plant material has been utilized for centuries as herbal medicines, however the alkaloids have such a low therapeutic index that their use in modern medicine has remained exploratory. Here we report an incident of inadvertent ingestion of *V. parviflorum* by hikers on the Appalachian trail in Georgia that allowed detection, and in several instances identification of alkaloids from the plant, and correlate their presence within patient blood and breast milk specimens.

#### 1.2 Case History

Eight patients, four male and four female, were reported to the Georgia Poison Center in the spring of 2020 and 2021 with symptoms requiring emergent medical attention after ingestion of *Veratrum parviflorum*. All patients believed the plants to be a local native species of wild leek, *Allium tricoccum*, locally known as ramps. Plants were identified using photographs as well as fresh and cooked plant material provided by patients, in consultation with botanists at the University of Georgia Herbarium. Written consent was obtained from all patients for collection of blood and breast milk specimens for laboratory identification of *Veratrum* alkaloids.

### 1.3 Methods

*V. parviflorum* plant material, and patient blood and breast milk were analyzed by high performance liquid chromatography-quadrupole time-of-flight mass spectrometry (HPLC-QTOF) to identify steroidal alkaloids.

### 1.4 Results

The *V. parviflorum* extract was confirmed to contain cyclopamine, veratramine, jervine, and muldamine. Of these alkaloids identified in the plant, cyclopamine, jervine, and veratramine were detected within patient blood, and cyclopamine and veratramine were observed to be present in breast milk.

### 1.5 Discussion

Toxicity resulting from *Veratrum* steroidal alkaloids has primarily been reported from *V. album* and *V. viride*. This is the second report of *V. parviflorum* poisoning. The present work reports for the first time the presence of muldamine and jervine within *V. parviflorum*. This work provides the first instance of identification of *Veratrum* alkaloids in breast milk.

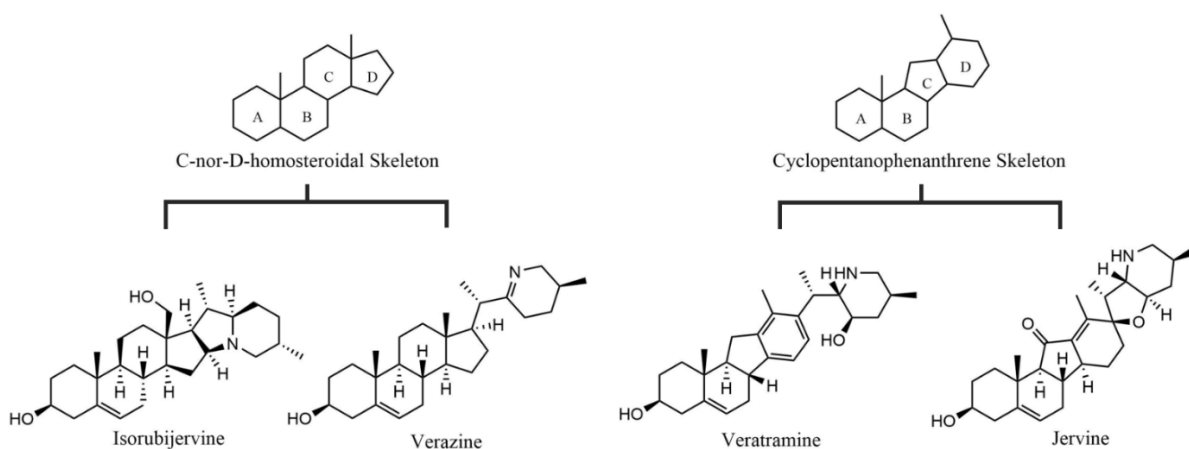
### 1.6 Conclusion

*V. parviflorum* toxicity was observed to cause nausea, vomiting, hypotension, bradycardia, abdominal pain, light-headedness, blurred vision, and tingling in the arms. Treatment included the administration of digoxin immune Fab, intravenous (IV) fluids, antiemetics, atropine, norepinephrine, ondansetron, and dopamine.

## **2. Introduction**

The *Veratrum* genus is comprised of 14-45 distinct species of woodland and alpine perennial herbs located throughout temperate regions of the northern

hemisphere.<sup>6,7,14</sup> Nine species, or species complexes, of *Veratrum* may be found in North American meadows, swamps, alpine forests, and riverbanks, including *V. album*, *V. californicum*, *V. viride*, *V. parviflorum*, and *V. tenuipetalum*.<sup>6,7,14,82</sup> These plants have been incorporated into traditional medicines for centuries due to powerful physiological effects resulting from their diverse mixture of steroidal alkaloids. Over 200 unique steroidal alkaloids have been identified in *Veratrum* spp. and can be separated into two distinct structural skeletons: C-nor-D-homosteroidal skeleton and cyclopentanophenanthrene skeleton (Figure 2.1).<sup>6,82</sup>



**Figure 2.1** Representative steroidal alkaloids from *Veratrum* spp. categorized by structure template.

*Veratrum* steroidal alkaloids are primarily recognized for their propensity to bind voltage gated sodium channels in vertebrate organisms.<sup>33</sup> German physiologists Albert von Bezold and Ludwig Hirt first observed the hypotensive and bradycardic effects of veratrine administration on the heart of a rabbit in 1867, however it took until 1939 before Jarisch and Richter confirmed the mechanism by which *Veratrum* steroidal alkaloids act on heart muscle.<sup>6,36,38</sup> In their definitive work, Jarisch and Richter identified that the effects of veratrine, a mixture of *Veratrum* derived alkaloids, resulted from reflex

action in the ventricles of the heart and transmission by the afferent and efferent pathways of the vagus nerve.<sup>36</sup> The symptoms of bradycardia, vasodilation, and hypotension resulting from cardiac receptor stimulation is now termed the Bezold-Jarisch reflex.<sup>6,33,37</sup>

Indigenous communities of North America are reported to have developed medicines utilizing *Veratrum* plant material. Most notably, *V. viride* has been used to treat a variety of conditions including boils, ulcers, pain, rheumatism, and venereal diseases.<sup>6,83</sup> Interestingly, a non-medicinal application of *Veratrum* plants was observed by Charles Osgood in 1835. As part of the election of Native American leaders, the rhizome of *V. viride* was made into a concoction that was ingested by candidates and those who resisted the extract's emetic effects the longest were considered most fit to lead.<sup>84</sup>

Despite recognition as an ingredient in potent traditional medicines, the history of *Veratrum* spp. is rife with accidental poisoning. *Veratrum* intoxication is most often due to inadvertent consumption as a result of misidentification during foraging.<sup>15,23-30</sup> Cases of *V. viride* poisoning are reported to have occurred due to mistaking the plant for *Symplocarpus foetidus* (skunk cabbage), *Phytolacca americana* (pokeweed), *Allium ampeloprasum* (wild onion), and *Allium tricoccum* (ramps).<sup>23,24,26</sup> Likewise, *V. album*, a species most prominently found in northern Eurasia and localized regions of Alaska, is reported to have been misidentified as *Allium ursinum* (wild onion) and *Gentiana lutea* (yellow gentian).<sup>27,30</sup> Most cases of poisoning manifest with symptoms including diarrhea, nausea, vomiting, and a Bezold-Jarisch reflex (bradycardia, hypopnea, and hypotension). Patients with mild symptoms typically respond well to symptomatic and

supportive treatments with intravenous fluids and antiemetics, but those with symptomatic bradycardia or hypotension may require treatment with atropine or vasopressors.<sup>15,23-30</sup>

*V. parviflorum* (Appalachian Bunchflower) may be found in the moist, deciduous forested slopes (800-2000 m) of the southern Appalachian Mountains. Characteristics of this plant include shortened rhizome (0.3-6 cm), narrow stem with lengths of 0.5-1.5 m, broadly oblanceolate to obovate leaves, and green-yellow to dark green flowers (Figure 2.2).<sup>8,15,17</sup>



**Figure 2.2** In situ *Veratrum parviflorum* (left) and plant material collected in April 2020 for steroidal alkaloid analysis (right).<sup>20</sup>

In April 2015, two patients presented to a Georgia hospital with *Veratrum* poisoning resulting from erroneous identification of *V. parviflorum* as *Allium tricoccum*.<sup>15</sup> In addition to a Bezold-Jarisch reflex, neurological symptoms including taste disturbance, vertigo, dysarthria, and vision changes were observed. These neurological

symptoms have not been reported for previous cases of toxicity with *V. viride* or *V. album*.<sup>23-27,29,30</sup> Analysis of *V. parviflorum* biomass identified verazine, veratramine, veratridine, and cyclopamine, all of which have been previously observed in *Veratrum* species. Due to the appearance of atypical neurological symptoms and a lack of information regarding the phytochemical profile of *V. parviflorum*, it was hypothesized that additional steroidal alkaloids, beyond those previously detected from patients, may be observed. The present study investigated eight cases of *V. parviflorum* poisoning resulting from the misidentification of plant material. Patient serum and breast milk was collected over the course of inpatient treatment and analyzed using high performance liquid chromatography-quadrupole time of flight mass spectrometry (HPLC-QTOF).

### 3. Case History

Eight patients were reported to the regional poison center in the spring of 2020 and 2021 with symptoms requiring emergent medical attention after ingestion of *Veratrum parviflorum*. All patients believed the plants to be a local native species of wild leek, *Allium tricoccum*, locally known as ramps. Plants were identified using photographs as well as fresh and cooked plant material provided by patients, in consultation with botanists at the University of Georgia Herbarium. Written consent was obtained from all patients for collection of blood and breast milk specimens for laboratory identification of *Veratrum* alkaloids. Clinical findings are summarized in Table 2.1.

A 34-year-old man (patient 1) and 34-year-old woman (patient 2) presented to a community emergency department (ED) in April 2020 after ingesting plants thought to be ramps (*Allium tricoccum*) that they foraged in Union County, Georgia and sauteed in oil.

Patient 1 developed abdominal cramping with nausea and profuse vomiting approximately one hour after ingesting a half cup of cooked plants. He also reported visual disturbances, including a yellow tint to his vision and halos. His blood pressure on arrival was 78/40, and heart rate was 43 beats per minute, occasionally dropping to the 30s. A 12-lead EKG showed sinus bradycardia with a rate of 54.

Patient 2 developed nausea and vomiting approximately two hours after ingestion of approximately one tablespoon of cooked plants. Her initial blood pressure was 90/52, and heart rate was 60 beats per minute. Her 12-lead EKG showed sinus bradycardia with heart rate of 56, with incomplete right bundle branch block.

Both patients were treated with IV fluid resuscitation and antiemetics. Their clinical presentation was concerning for cardiac glycoside ingestion, and the plant leaf material was initially noted to resemble lily of the valley (*Convallaria majalis*). Both patients were treated empirically with five vials of digoxin immune Fab (DIF) (200 mg) IV. Serum digoxin levels were undetectable in both patients.

Patient 1 did not improve after receiving DIF. He was unable to receive more DIF as the hospital pharmacy had no more in stock. He received atropine 0.4 mg IV and a norepinephrine infusion for persistent symptomatic bradycardia and hypotension. His hemodynamics improved with vasopressor support, and his nausea and vomiting resolved. He was transferred to an intensive care unit (ICU) at a tertiary care hospital, where he was observed overnight. He remained hemodynamically stable and norepinephrine was weaned off. All symptoms resolved approximately 12 hours after arrival, and he was discharged home.

Patient 2 responded well to ondansetron, IV fluids, and DIF with full resolution of her symptoms. She was observed in the ED for approximately five hours and remained hemodynamically stable. She was discharged home. As she was breastfeeding at the time, she agreed to provide breast milk samples from the time of exposure for further analysis, in addition to blood samples collected at the hospital.

Patients 3-6 were a family of four who presented in April 2020, consisting of a 50-year-old man (patient 3), 53-year-old woman (patient 4), 15-year-old daughter (patient 5), and 18-year-old son (patient 6). They foraged and ate plants thought to be ramps found along the Benton MacKaye Trail on Flat Top Mountain in Epworth, Fannin County, Georgia. Approximately six to seven whole plants including the leaves, stem, and root material were sauteed with olive oil and consumed. All family members developed symptoms within 3.5 hours of ingestion and presented to an ED approximately 14 hours after ingestion.

Patient 3 developed nausea with multiple bouts of profuse vomiting with epigastric pain 3.5 hours after ingestion of two plants. He also developed generalized weakness and lightheadedness that was worse with exertion. Initial vitals showed blood pressure of 117/88 and heart rate 52. EKG showed sinus bradycardia with rate of 44 and first degree block. Labs were significant for elevated serum creatinine of 1.92 mg/dL (reference range 0.7 – 1.3), total bilirubin of 1.3 mg/dL (reference range 0.3-1), and undetectable digoxin level. He was treated symptomatically with ondansetron and fluids. He remained stable and symptoms resolved. Serum creatinine improved to 1.52 mg/dL the following day, although total bilirubin increased to 1.6 mg/dL. He was discharged home approximately 36 hours after ingestion.



Patient 4 ate one leaf and developed nausea with mild epigastric discomfort approximately one to two hours after ingestion. Her symptoms persisted for approximately 16 hours before resolving spontaneously. On arrival at the ED, blood pressure was 134/75, heart rate 105. EKG showed normal sinus rhythm with a rate of 75. Lab studies showed no acute abnormalities, with undetectable digoxin level.

Patient 5 reported eating one plant that included three leaves, stalk, and roots. She developed nausea and diffuse abdominal pain 2.5 hours after ingestion, but had no vomiting or other symptoms. Initial blood pressure in the ED was 139/66, with heart rate of 84. EKG showed sinus bradycardia with a ventricular rate of 53. Blood lab analyses revealed a positive digoxin assay with a reported level of 0.4 ng/mL (reference <2 ng/mL).

Patient 6 developed nausea two hours after ingesting six leaves, followed by three episodes of profuse vomiting. He also reported palpitations with lightheadedness, diaphoresis, and blurred vision as well as one bout of loose stool. Initial blood pressure in the ED was 104/51, and heart rate was 58. EKG showed normal sinus rhythm, rate 63. Lab studies showed slightly elevated total bilirubin of 1.7 mg/dL, but otherwise normal with negative digoxin assay. He received ondansetron and IV fluid hydration, with subsequent resolution of nausea and lightheadedness.

Patient 7 is a 57-year-old female who picked *V. parviflorum* plants mistaken for ramps in Hiwassee, Cherokee County, North Carolina. She ate above-ground leafy material from two plants that were blanched in water and seasoned with a dressing. She developed nausea, vomiting, diarrhea, and cramping epigastric pain within four hours of ingestion. She received oral promethazine and ondansetron from her primary care

physician, but symptoms persisted. She presented to the ED eight hours after ingestion and was hypotensive, with blood pressure of 84/49, heart rate 91. Labs showed mild hypokalemia with potassium of 3.3 mEq/L, and undetectable digoxin. She continued to have intractable vomiting with persistent hypotension, with lowest blood pressure of 78/41, but no bradycardia. She was admitted and treated with IV fluids and metoclopramide, but did not require vasopressors. EKG showed normal sinus rhythm with rate of 96 and normal intervals. Her symptoms resolved with supportive care and she was discharged 42 hours after ED presentation.

Patient 8 is a 58-year-old female who also consumed false hellebore leaves boiled in water. She developed vomiting, lightheadedness, chest discomfort, generalized weakness, and a sensation of pressure with tingling in the right arm 1.5 hours after the meal. She initially presented to an urgent care clinic where she was noted to be hypotensive, and transferred to an ED. Blood pressure on arrival was 80/50, with a heart rate of 46. She received atropine 0.5 mg IV with transient improvement of her heart rate to 80, and blood pressure of 130/70. Her heart rate decreased to the 40s again in the ED and blood pressure was 80/50. She received another dose of atropine 0.5 mg IV and a 1 L IV fluid bolus. Labs showed no acute abnormalities, with undetectable digoxin. EKG revealed sinus bradycardia. She was started on a dopamine infusion at 15 mcg/min and admitted to the ICU. She remained hemodynamically stable with heart rate in the 60s and 70s with normal blood pressure overnight. The dopamine drip was weaned off over 24 hours, with full resolution of all symptoms.

Table 2.1 Summary of Patients and Clinical Presentation

| Patient | Age/Gender | Clinical Effects  | Time to Symptom Onset | Lab Findings*  | EKG  | Treatment   |
|---------|------------|---|-----------------------|--|--|---|
| 1       | 34M        | Nausea, vomiting, abdominal cramping, hypotension, bradycardia                        | 1 hour                | No acute abnormalities   | Sinus bradycardia, rate 54                                       | Digoxin immune Fab, IV fluids, promethazine, atropine, norepinephrine |
| 2       | 34F        | Nausea, vomiting, hypotension, bradycardia  | 2 hours               | No acute abnormalities   | Sinus bradycardia, rate 56, incomplete right bundle branch block | Digoxin immune Fab, IV fluids, ondansetron                            |
| 3       | 50M        | Nausea, vomiting, epigastric pain, generalized weakness, lightheadedness, bradycardia | 3.5 hours             | Creatinine 1.92 mg/dL (ref 0.7-1.3), total bilirubin 1.6 ng/dL (ref 0.3-1) | Sinus bradycardia, rate 44, 1st degree AV block                  | IV fluids, ondansetron  |

|   |     |  |           |                                       |                              |                               |
|---|-----|--|-----------|---------------------------------------|------------------------------|-------------------------------|
| 4 | 53F | Nausea, mild epigastric discomfort   | 1-2 hours | No acute abnormalities                | Normal sinus rhythm, rate 75 | None                          |
| 5 | 15F | Nausea, diffuse abdominal pain, bradycardia  | 2.5 hours | Digoxin level 0.4 ng/mL (ref <2)      | Sinus bradycardia, rate 53   | None                          |
| 6 | 18M | Nausea, vomiting, palpitations, light-headedness, diaphoresis, blurred vision, loose stool                         | 2 hours   | Total bilirubin 1.7 ng/dL (ref 0.3-1) | Normal sinus rhythm, rate 63 | IV fluids, ondansetron        |
| 7 | 57F | Nausea, vomiting, diarrhea, epigastric pain, hypotension   | 4 hours   | Potassium 3.3 mEq/L (ref 3.5-5)       | Normal sinus rhythm, rate 96 | IV fluids, metoclopramide     |
| 8 | 58F | Nausea, vomiting, light-headedness, chest discomfort, generalized weakness, arm tingling, hypotension, bradycardia | 1.5 hours | No acute abnormalities                | Sinus bradycardia            | Atropine, IV fluids, dopamine |

\* All patients had lab tests including serum electrolytes, BUN/creatinine, liver enzymes, complete blood count, and digoxin level. Normal results are not listed in this table.

## 4. Materials and Methods

### 4.1 Root and Rhizome Extraction

Whole *V. parviflorum* plants were collected by the treating medical toxicology physicians at the same site where patients 3, 4, 5, and 6 gathered the plants they consumed. Roots and rhizomes were separated from the plant, diced, and lyophilized for 24 hrs before being ground into a fine powder. Ethanol (100 mL of 95%, Decon Laboratories, Inc.) was added to 10 g of dried *V. parviflorum* powder to form a slurry that was sonicated for 30 min and stirred for 24 hrs at room temperature. The mixture was filtered to produce a clear and brown supernatant that was then concentrated via rotary evaporation. Removal of solvent resulted in a viscous dark brown extract to which 2 mL of 100% ethanol (Decon Laboratories, Inc.) was added. This extract was filtered through a 0.45  $\mu\text{m}$  nylon syringe filter and placed in an autosampler vial for steroidal alkaloid characterization via HPLC-QTOF.

### 4.2 Blood Extraction

Chloroform was used to extract the steroidal alkaloids from patient serum. A biphasic mixture containing 1 mL patient serum and 0.4 mL chloroform was vortexed for 1 min then centrifuged for 5 min at 14000 rpm. A 0.3 mL aliquot of the chloroform layer was removed and evaporated to dryness under a stream of nitrogen. The remaining residue was dissolved in 0.1 mL ethanol (100%) for HPLC-QTOF analysis. A calibration curve consisting of 5, 1, 0.5, 0.1, and 0.05  $\mu\text{g/mL}$  of standard was formed using commercially available cyclopamine ( $\geq 95\%$ , PhytoLab GmbH & Co. KG), veratramine ( $>98\%$ , TCI America), veratridine ( $\geq 98\%$ , Tocris Bioscience), muldamine (99%, Logan

Natural Products), and jervine ( $\geq 95\%$ , PhytoLab GmbH & Co. KG) to quantify steroidal alkaloids in patient serum.

#### 4.3 Breast Milk Extraction

A 0.5 mL aliquot of patient breast milk was diluted with 0.5 mL ammonium hydroxide (29%) and loaded onto a supported liquid extraction (SLE) column (Agilent Chem Elut S) and allowed to adsorb onto the solid phase for 10 min. Following adsorption, 10 mL of chloroform was added to the column and eluted on a vacuum manifold with -0.2 bar applied vacuum for 30 sec. The eluted solvent was filtered through a 0.45  $\mu\text{m}$  PVDF syringe filter and evaporated to dryness under a stream of nitrogen. Remaining residue was dissolved in 0.1 mL ethanol (100%) and analyzed via HPLC-QTOF. Quantification of alkaloids in breast milk was performed using a calibration curve consisting of 5, 1, 0.5, 0.1, and 0.05  $\mu\text{g/mL}$  of alkaloid standards.

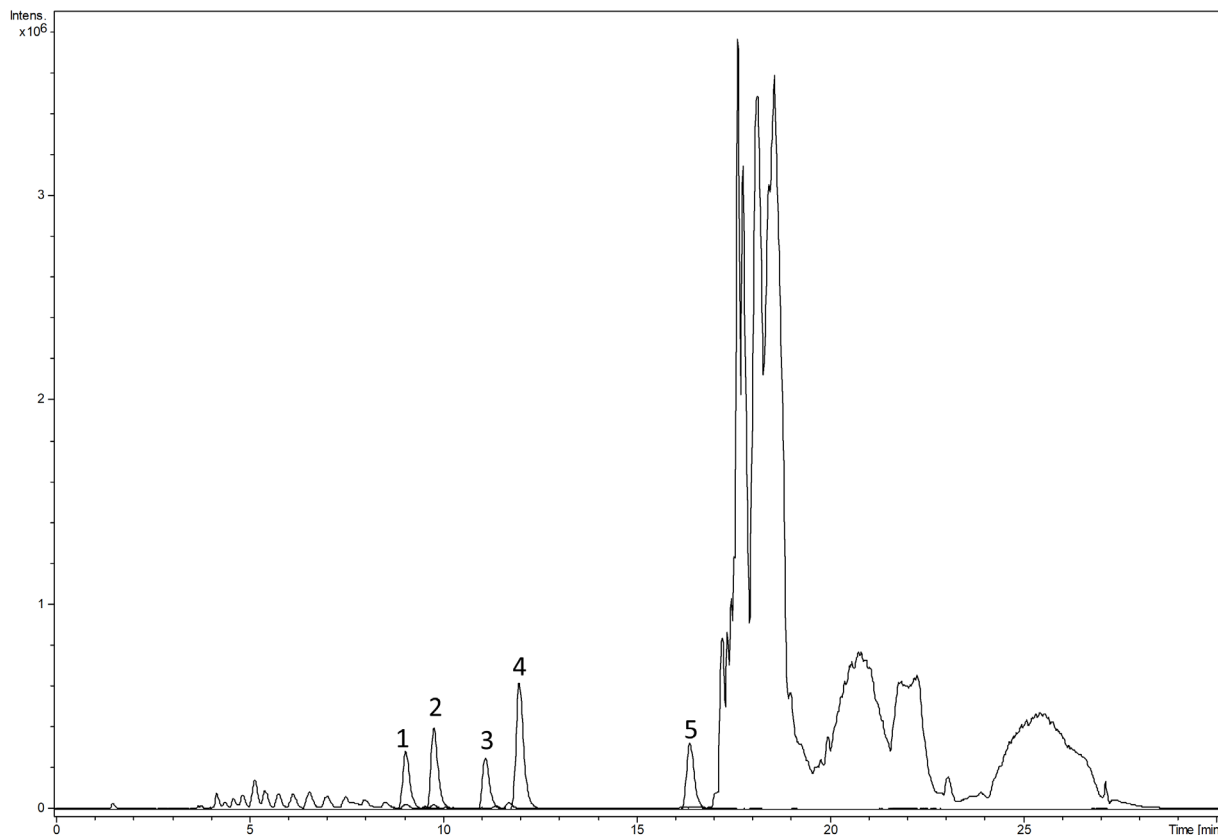
#### 4.4 Steroidal Alkaloid Identification

*Veratrum* steroidal alkaloids were identified in blood, breastmilk, and root/rhizome extracts using HPLC-QTOF and commercially available standards. Analysis was performed on a Bruker maXis ESI Q-TOF mass spectrometer (Bruker Corporation, Billerica, MA) coupled with a Dionex Ultimate 3000 LC system (Thermo Scientific, Waltham, MA). The samples were injected onto a Waters Xterra MS C<sub>18</sub> column (5  $\mu\text{m}$ , 2.1 x 150 mm) maintained at 40 °C at an injection volume of 5  $\mu\text{L}$ . A gradient elution consisting of 0.1% formic acid in water (solvent A) and 0.1% formic acid in acetonitrile (solvent B) was used with a 300  $\mu\text{L/min}$  flowrate. The elution method began at 10% solvent B before increasing to 20% solvent B after 1 min. Between minute 1 and 16, solvent B was increased to 40%. After 16.1 min, solvent B was increased to

100% and maintained until minute 20. Solvent B was decreased to 5% at 20.1 min and held constant until minute 25. The electrospray ionization (ESI) source was operated under the following conditions: positive ion mode, 4000 to -500 V voltage between capillary and end plate offset, 10.0 L/min flow rate of N<sub>2</sub> drying gas at 200 °C.

## 5. Results

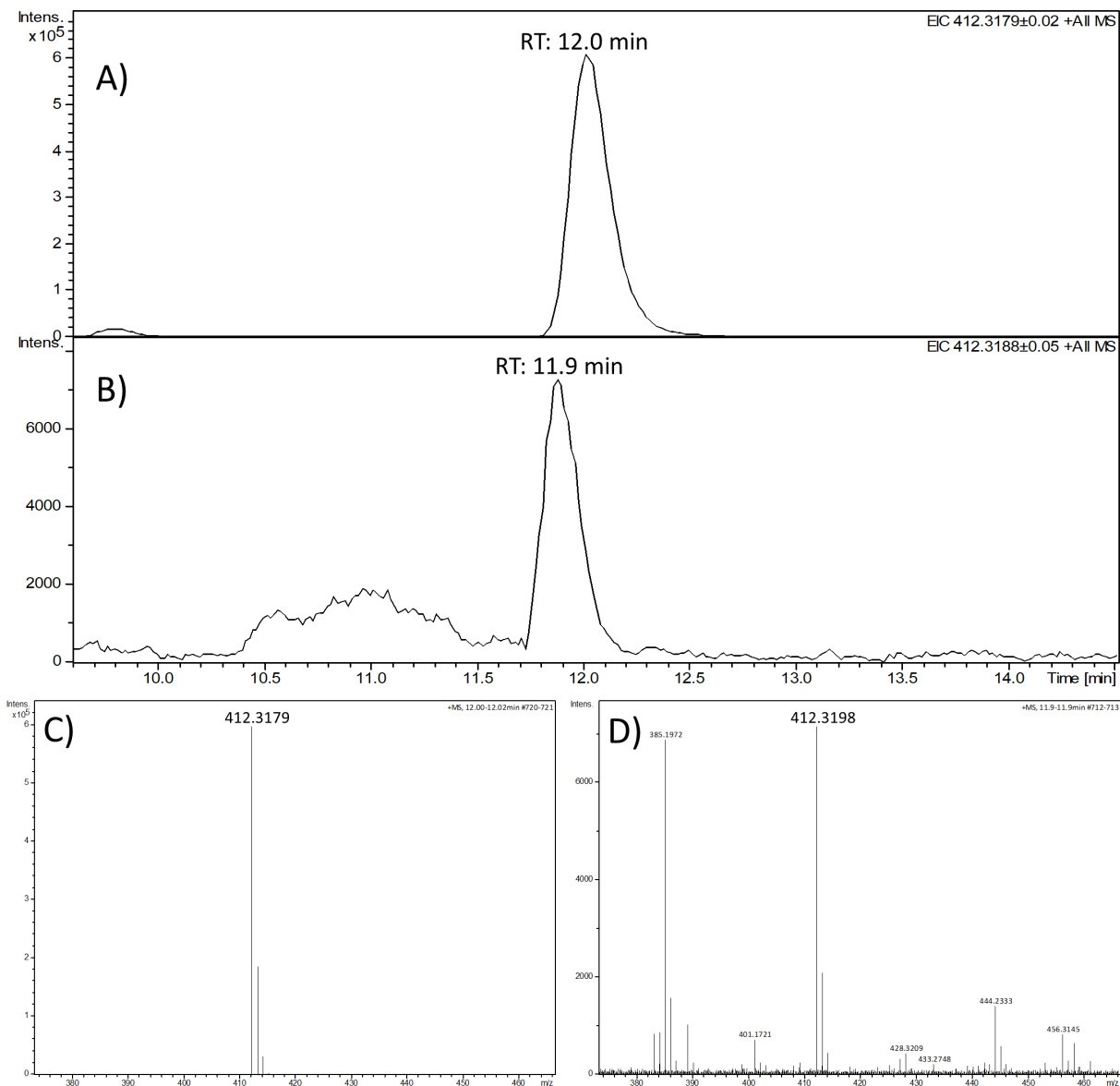
To identify the presence of *Veratrum* steroidal alkaloids within patient blood samples, commercially available human serum was spiked at 1, 10, and 50 mg/L with five steroidal alkaloid standards: jervine, veratramine, veratridine, cyclopamine, and muldamine. The spiked serum underwent the described extraction procedure to not only determine the retention time and ions produced, but to also assess the limit of detection (LOD) and limit of quantification (LOQ) for the alkaloids (Figure 2.3). The LOD for jervine, veratramine, veratridine, cyclopamine, and muldamine was 44, 16, 63, 45, and 38 ng/mL, respectively, while the LOQ were 134, 48, 191, 136, and 115 ng/mL, respectively.



**Figure 2.3** Base peak chromatogram for human serum extracts. Base peak chromatogram for patient #1 overlaid with extracted ion chromatograms for commercially available steroidal alkaloids that were spiked to 10 mg/L into and extracted from a human serum standard. Peaks 1-5 were identified as jervine, veratramine, veratridine, cyclopamine, and muldamine, respectively.

The retention time for cyclopamine in the standard and patient extracts were 12.0 and 11.9 minutes, respectively (Figure 2.4). An  $[M+H]^+$  parent ion for cyclopamine ( $412.32 \pm 0.02$  m/z) was identified in the patient #1 extract (Figure 2.4C). Jervine, veratramine, veratridine, and muldamine were not detected in the blood extract of patient #1.





**Figure 2.4** Serum extracts for patient #1 were compared to a cyclopamine standard. **A)** Extracted ion chromatogram for cyclopamine ( $412.32 \pm 0.02$  m/z) extracted from commercially available human serum. **B)** Extracted ion chromatogram for  $412.32 \pm 0.05$  m/z. **C)** Mass spectrum of the peak (RT: 12.0 min) in chromatogram A where the observed  $[M+H]^+$  ion for cyclopamine ( $412.32 \pm 0.02$  m/z) has been identified. **D)** Mass spectrum of the peak (RT: 11.9 min) in chromatogram B where the observed  $[M+H]^+$  ion for cyclopamine ( $412.32 \pm 0.02$  m/z) has been identified.

Qualitative analysis of the blood extract of patient #4 recognized jervine and cyclopamine (Figures A.1 and A.2). The retention time for jervine was 9.1 minutes (Figures A.1A and A.1B) and the  $[M+H]^+$  parent ion ( $426.30 \pm 0.05$  m/z) was identified

(Figure A.1C). Cyclopamine was identified in the blood of patient #4 by the presence of the  $[M+H]^+$  parent ion ( $412.32 \pm 0.05$  m/z) (Figure A.2C). Veratramine was identified in the blood extract of patient #7 by the observation of the  $[M+H]^+$  parent ion ( $410.31 \pm 0.05$  m/z) (Figure A.3) and retention time of 10.0 min. *Veratrum* alkaloids were not detected in blood extracts of patients 2, 3, 5, 6, and 8.

**Table 2.2 Identity and Concentration of Alkaloids Identified in Patient Blood Extracts**

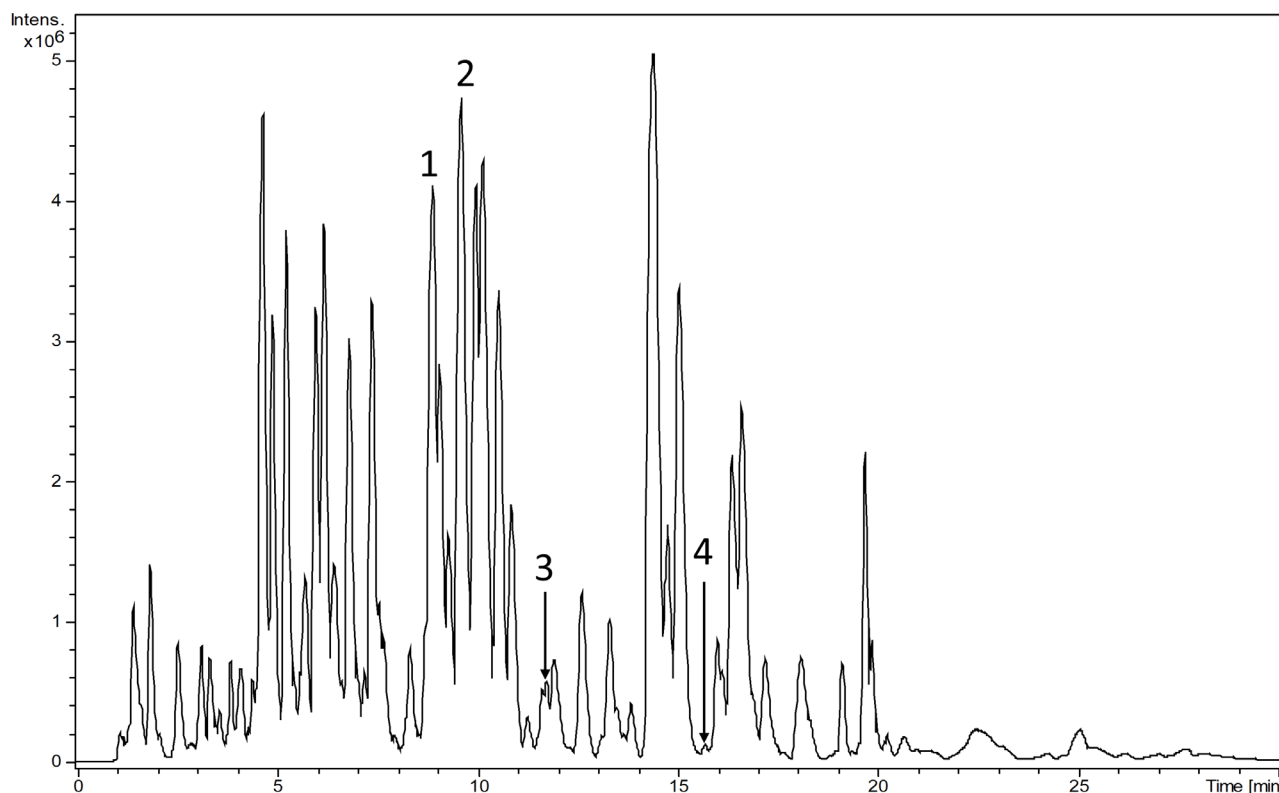
| Patient # | Alkaloids Identified   |
|-----------|------------------------|
| 1         | Cyclopamine            |
| 4         | Cyclopamine<br>Jervine |
| 7         | Veratramine            |

Veratramine and cyclopamine were the only steroidal alkaloids identified in an extract of the breast milk of patient #2 (Figures A.4-A.6). Veratramine was identified to have a retention time of 9.4 min and a  $[M+H]^+$  parent ion of  $410.31 \pm 0.02$  m/z. Cyclopamine was identified with a retention time of 11.6 min and a  $[M+H]^+$  parent ion of  $412.32 \pm 0.02$  m/z. The concentration of veratramine (LOD = 11 ng/mL; LOQ = 34 ng/mL) in the milk was observed to decrease in the second sample, then increase in the third sample to a lower concentration than initially observed. The concentration of cyclopamine (LOD = 11 ng/mL; LOQ = 32 ng/mL) was below the LOQ in all samples. The fourth breast milk sample collected on April 9 at 17:00 did not contain a detectable level of veratramine or cyclopamine (Table 2.3).

**Table 2.3 Identity and Concentration of Steroidal Alkaloids in Patient Breast Milk Extracts**

| Patient # | Date/Time of Collection | Alkaloids Identified | Concentration (ng/mL) |
|-----------|-------------------------|----------------------|-----------------------|
| 2         | April 8 2020 at 11:30   | Veratramine          | 405                   |
|           |                         | Cyclopamine          | -                     |
|           | April 8 2020 at 16:20   | Veratramine          | 136                   |
|           |                         | Cyclopamine          | -                     |
|           | April 9 2020 at 8:00    | Veratramine          | 303                   |
|           |                         | Cyclopamine          | -                     |
|           | April 9 2020 at 17:00   | -                    | -                     |

The root and rhizome extract of *V. parviflorum* yielded a chromatogram with over 53 peaks representing unique constituents (Figure 2.5). Commercially available steroidal alkaloid standards for jervine, veratramine, veratridine, cyclopamine, and muldamine were used to identify known alkaloids within the *V. parviflorum* extract. All steroidal alkaloid standards were present in the plant extract with the exception of veratridine (Figure A.7-A.10).



**Figure 2.5** *V. parviflorum* root and rhizome ethanolic extract. Peaks labelled 1-4 correspond to jervine, veratramine, cyclopamine, and muldamine, respectively.

## 6. Discussion

*Veratrum* steroidal alkaloids are recognized for their toxicity and have been implicated in multiple cases of inadvertent poisoning.<sup>15, 23-30</sup> An ethanolic extract of the roots and rhizomes of *V. parviflorum* resulted in the identification of four common *Veratrum* steroidal alkaloids: cyclopamine, veratramine, jervine, and muldamine. Contrary to the results presented by Anwar et al., where cyclopamine, veratramine, verazine, and veratridine were identified, veratridine was not identified in the present extract of *V. parviflorum*.<sup>15</sup> A standard of verazine could not be obtained, however, an EIC for 398.3553 m/z presented several peaks suggesting that the previously identified alkaloid may still be observed given the appropriate reference material. Variation in the presence of veratridine in *V. parviflorum* may be due to differences in growth stage,

harvest time, or harvest location.<sup>85</sup> The growth stage, time of harvest, harvest location, and part of the plant for *V. californicum* has shown to influence the alkaloid composition in plant material.<sup>85</sup> Although the poisoning described by Anwar et al. also occurred in April, it is possible that the *V. parviflorum* used in that study was harvested at an earlier date, in a different location, or at a different stage of growth.

This study provides the first evidence for the presence of muldamine and jervine within *V. parviflorum*. Jervine has previously been isolated from *Veratrum* spp. including *V. nigrum* L., *V. californicum*, *V. album*, and *V. viride*, whereas muldamine has only been isolated from *V. californicum*.<sup>44,56,85-88</sup> Symptoms exhibited by the patients in the present study, including nausea, vomiting, hypotension, and bradycardia, were consistent with those commonly observed for *Veratrum* toxicity. Additionally, the time between ingestion and the appearance of symptoms was consistent with prior cases.<sup>15,23-30</sup>

The identification of veratramine and cyclopamine in breast milk suggests that the indirect exposure of an infant via ingestion is possible, although the clinical significance is unknown. The alkaloid profiles and concentrations observed in breast milk may differ from blood. In samples from patient 2, *Veratrum* alkaloids were only detected in the breast milk, but not blood, even though the first milk sample was collected the morning after her ED visit. Veratramine and cyclopamine were identified, although the cyclopamine concentrations were below the LOQ. Lipophilicity may account for the presence of these alkaloids in breast milk even after they are no longer detectable in the blood. SwissADME was used to predict the partition coefficients for each of the *Veratrum* steroidal alkaloids. As predicted by SwissADME, veratramine is the most

lipophilic of the three alkaloids we identified ( $\text{LogP}_{o/w} = 4.30$ ), followed by cyclopamine ( $\text{LogP}_{o/w} = 4.16$ ), with jervine being the least lipophilic ( $\text{LogP}_{o/w} = 3.53$ ).

Veratramine has been identified to exert bradycardic and teratogenic effects through the inhibition of  $\text{Na}^+$  ion channels and Hedgehog signaling, respectively, with an  $\text{LD}_{50}$  of 15.9 mg/kg when administered to Kunming mice intragastrically.<sup>58,60</sup> Furthermore, veratramine is a releaser and uptake inhibitor of 5-HT.<sup>58,60</sup> Cyclopamine has also been identified as an inhibitor of the Hedgehog signaling pathway with an  $\text{LD}_{50}$  of 43.5 mg/kg when administered to 129S11/SvImj mice intraperitoneally.<sup>6,54,82</sup> Excretion of xenobiotics in breast milk can depend on many factors such as plasma levels and physical properties including lipophilicity, protein binding, and ionization. Concentrations of *Veratrum* alkaloids in milk may also vary with different collection times. We observed higher veratramine concentrations in milk samples collected in the morning compared to the afternoon. The patient reported that she had not nursed or pumped milk for 16 hours prior to collection of the first sample included in our analysis. It is possible that more *Veratrum* alkaloids accumulated overnight due to less frequent nursing and pumping, leading to higher concentrations in milk expressed in the morning compared to other times during the day. Although breast milk samples were collected four times over a 29.5 hour period, additional time points over a longer collection period are required to determine the half-life of veratramine and cyclopamine more accurately. Variation in observed concentrations of veratramine may be due to acute changes in the breast milk composition and chemical properties of the small molecule.<sup>89,90</sup> Additional study regarding the pharmacokinetics of *Veratrum* steroidal alkaloids in relation to concentration in breast milk may be beneficial.

Three of the eight patients had detectable levels of *Veratrum* alkaloids in the blood. Due to the small sample size, we were unable to characterize associations between the presence of certain alkaloids or their concentrations with severity of illness. As the blood specimens were convenience samples left over from routine clinical testing, there were limitations including differences in collection times, storage conditions, and the prolonged time in storage before analysis.

Cross-reactivity with digoxin clinical chemistry assays have been reported in patients who have ingested *Veratrum* species.<sup>32</sup> Only one patient in our study had a positive digoxin assay, although she had mild symptoms. There was no discernable association between symptom severity and digoxin assay results.

## 7. Conclusion

This study sought to identify toxic steroidal alkaloids in the blood of eight patients and breast milk for one of them that inadvertently ingested *V. parviflorum*. Four alkaloids were identified in an ethanolic extract from the roots of *V. parviflorum*: jervine, veratramine, cyclopamine, and muldamine. An efficient and effective method was developed for the extraction of steroidal alkaloids from patient samples, leading to the identification of cyclopamine, jervine, and veratramine in patient blood, and veratramine and cyclopamine in patient breast milk. The presence of steroidal alkaloids in breast milk suggests that the indirect poisoning of a breastfeeding infant should be considered in cases of suspected *Veratrum* poisonings.

## CHAPTER THREE: ASSESSMENT OF HEDGEHOG SIGNALING INHIBITION BY STEROIDAL ALKALOIDS IN VERATRUM PARVIFLORUM

### 1. Introduction

Natural products derived from marine and terrestrial sources have been used for the treatment of various illnesses and conditions for millennia.<sup>91</sup> Mesopotamian clay tablets dating from around 2600 BCE were inscribed with an estimated 100 plant-derived therapeutics used to treat symptoms including inflammation, parasitic infections, and coughing.<sup>92,93</sup> Similarly, ancient Chinese medicines dating from around 1100 BCE described herbal remedies for the prevention and treatment of various diseases.<sup>92,94</sup> These records indicate that plants have been a major source for the development of sophisticated medical formulations.<sup>92</sup> The broad medical applications of plant-derived therapeutics result from an abundance of structurally unique and chemically diverse compounds.<sup>95</sup> These compounds are commonly secondary metabolites that have evolved in plants through millions of years of evolution, driven by various ecological and physiological stressors, including defense from herbivores, pathogens, and abiotic conditions. Plant secondary metabolites facilitate interactions with pollinators, plant-plant signaling, and attracting the predators of herbivores.<sup>96</sup>

The *Veratrum* genus is composed of perennial flowering herbs reported to have been incorporated into Eastern and Western traditional herbal medicines. These herbal treatments have been used for conditions such as rheumatism, sore throat, aphasia, chronic malaria, and venereal diseases.<sup>7,10</sup> For over a century, there has been great



interest in utilizing the steroidal alkaloids produced by the *Veratrum* spp. in modern medicine due to their potent physiological effects. Initial investigations performed in 1867 by Albert von Bezold and Ludwig Hirt identified that the onset of hypotension and bradycardia occurred after the administration of veratrine, a mixture of *Veratrum* derived alkaloids, to a rabbit.<sup>38</sup> Jarisch and Richter later identified that the hypotensive and bradycardic effects of veratrine were a result of the transmission of a reflex action in the ventricles of the heart through the afferent and efferent pathways of the vagus nerve.<sup>6,36</sup> Dr. Charles Osgood noted the similar physiological effects of veratrine to *V. viride*, a *Veratrum* spp. native to North America, and recommended the use of its extract for inflammatory diseases. Preparations of *V. viride* extracts were commonly prescribed for high blood pressure from the late 19<sup>th</sup> to mid-20<sup>th</sup> century. However, by the 1970s, *V. viride* alkaloids were abandoned as a prescribed therapeutic due to an unreliable supply, clinical limitations, and the development of more effective antihypertensive drugs.<sup>6</sup>

In the 1950s, sheep herders observed that between 1-25% of lambs born from ewes bred in the alpine meadows of South Central and Southwestern Idaho were beset with cyclopean-type developmental defects.<sup>42</sup> The malformations were originally thought to be congenital, however, further investigations identified that the deformations resulted from ewes feeding on *Veratrum californicum*, also known as false hellebore or corn lily, between day 10 and 15 of gestation.<sup>42,43</sup> Keeler and Binns later identified that the steroidal alkaloids cyclopamine, cycloposine, jervine, and veratrosine were teratogenic agents contributing to the cyclopean malformations.<sup>44</sup> The mechanism in which these alkaloids exert the teratogenic effect is through inhibition of the Hedgehog (Hh) signaling pathway by directly binding to the transmembrane protein Smoothed (Smo).<sup>4,39</sup> The Hh

pathway is vital to embryo development and cell proliferation, however, uncontrolled activation is linked to the development of over 20 cancers, most notably basal cell carcinoma and acute myeloid leukemia.<sup>5,97</sup>

As a result of this relationship, *Veratrum*-derived steroidal alkaloids have been investigated for their ability to antagonize Hh signaling. A number of these steroidal alkaloids have been evaluated for Hh antagonism and proven effective in reducing cancer activity.<sup>4,39-41,62,98-100</sup> The jervanine type alkaloid cyclopamine has received the most study and remains as one of the most potent naturally occurring Hh inhibitors.<sup>6,62</sup> This alkaloid has been observed to reduce Hh signaling in Shh light II cells, block the proliferation of murine medulloblastoma in vitro, and induce cell death in human medulloblastomas.<sup>62,99</sup> One study observed that the treatment of cyclopamine in combination with paclitaxel induced improved tumor apoptosis in comparison to treatments only consisting of paclitaxel.<sup>98</sup> These results suggested that a Hedgehog signaling could be a prospective drug target for cancer cells that have developed a resistance to mitotic inhibitor chemotherapies.<sup>98</sup> Despite the observed bioactivity, *Veratrum* alkaloids such as cyclopamine have limited therapeutic use due to poor aqueous solubility and instability in acidic chemical environments.<sup>6</sup> The pharmaceutical industry has since developed more potent synthetic and semi-synthetic drugs including vismodegib, sonidegib, glasdegib, and patidegib (IPI-926) that antagonize Smo. With the exception of IPI-926, which is currently undergoing Phase III clinical trials, these drugs have been approved by the United States Food and Drug Administration (FDA) for the treatment of basal cell carcinoma (BCC) and acute myeloid leukemia (AML).<sup>5,101</sup> Although proven to be effective anticancer drugs, recurrence after treatment is common

due to mutations in Smo.<sup>102-104</sup> Additional investigation of *Veratrum* spp. may lead to the identification of novel alkaloids and alkaloid mixtures with potential to serve as chemotherapeutics or as guides for the development of semi-synthetic and synthetic drugs.

*V. parviflorum*, commonly known as the Appalachian bunchflower, is a lesser-known species of the *Veratrum* genus native to the mountain slopes of the Southeastern United States. In contrast to other *Veratrum* spp., such as *V. album* and *V. viride*, *V. parviflorum* has not been reported to be used in traditional medicines.<sup>6</sup> Instead, this plant has been implicated in the accidental poisoning of foragers who mistake the plant for wild leeks (*Allium tricoccum*) in its adolescent stages (Figure 3.1).<sup>15</sup> *Veratrum* poisoning may be characterized by gastrointestinal unrest and a Bezold-Jarisch reflex resulting from neuronal sodium channel hyperpermeability.<sup>15,33</sup> Cardiovascular toxicity is generally observed in patients due to the propensity of the alkaloids to bind to voltage-gated sodium ion channels, causing the channels to remain open.<sup>14,15,33,105</sup>



**Figure 3.1** In situ *Veratrum parviflorum*(Appalachian bunchflower) (Top) and *Allium tricoccum* (wild leek) (Bottom).<sup>20,106</sup>

A qualitative investigation of *V. parviflorum* identified four known alkaloids from an ethanolic extract of the roots and rhizome: verazine, veratramine, veratridine, and cyclopamine.<sup>15</sup> Of these alkaloids, cyclopamine and veratramine have been identified as a Hh pathway inhibitor.<sup>41,107,108</sup> The study of *Veratrum* sect. *Fuscoveratrum* species including *V. mackii*, *V. mengtzeanum*, *V. stenophyllum*, and *V. taliense* has led to the discovery of alkaloids thought to be unique to specific species.<sup>7</sup> Due to the classification of *V. parviflorum* in sect. *Fuscoveratrum* and the lack of information regarding its phytochemistry, additional study of the steroidal alkaloids extracted from the root and rhizome may yield novel alkaloids that promote inhibition of Hh pathway signaling. In the present study, HPLC-MS was used to identify steroidal alkaloids within an extract of the roots and rhizomes of *V. parviflorum*. Following identification, a series of treatments containing alkaloids found within the plant were made using commercially available standards and assessed using a Shh-Light II cell bioactivity assay. Combinations of these steroidal alkaloid standards were developed at concentrations that matched those found within the plant extract. A comparison of bioactivity measurements for alkaloid mixtures and the *V. parviflorum* extract may provide insights into the presence of less abundant alkaloids that are antagonistic to the Hh pathway.

## **2. Materials and Methods**

### **2.1 Chemicals and Solvents**

Acetonitrile (>99% purity), 95% ethanol, 100% ethanol, trifluoroacetic acid (>99.9% purity), Dulbecco's Modified Eagle Medium, Geneticin, Zeocin, Penicillin-Streptomycin (10,000 U/mL), and fetal bovine serum were purchased from ThermoFisher Scientific. Recombinant mouse Sonic Hedgehog/Shh, N-terminus protein was purchased

from R&D Systems. The Dual-Luciferase Reporter Assay System was purchased from Promega. Cyclopamine ( $\geq 95\%$  purity) and jervine ( $\geq 95\%$  purity) were purchased from PhytoLab GmbH & Co. KG, veratramine ( $> 98\%$  purity) was purchased from TCI America, veratridine ( $\geq 98\%$  purity) was purchased from Tocris Bioscience, and muldamine (99% purity) was purchased from Logan Natural Products.

## 2.2 Alkaloid Extraction

The roots and rhizome of *V. parviflorum* were separated and lyophilized for 24 hrs then ground into a fine powder. One hundred milliliters of 95% ethanol were added to 10 g of dried *V. parviflorum* powder. The resulting slurry was sonicated for 30 min and stirred at room temperature for 24 hrs. The mixture was vacuum filtered, producing a clear and brown filtrate, and concentrated by rotary evaporation. Removal of the extraction solvent resulted in a viscous extract which was then dissolved in 2 mL of 100% ethanol. The extract was filtered through a 0.45  $\mu\text{m}$  PTFE syringe filter into an autosampler vial for HPLC-QTOF analysis.

## 2.3 Alkaloid Identification

*Veratrum* steroidal alkaloids were identified in the root/rhizome extracts using HPLC-QTOF and commercially available standards. Analysis was performed on a Bruker maXis ESI Q-TOF mass spectrometer (Bruker Corporation, Billerica, MA) coupled with a Dionex Ultimate 3000 LC system (Thermo Scientific, Waltham, MA). The samples were injected onto a Waters Xterra MS C<sub>18</sub> column (5  $\mu\text{m}$ , 2.1 x 150 mm) maintained at 40 °C at an injection volume of 5  $\mu\text{L}$ . A gradient elution consisting of 0.1% formic acid in water (solvent A) and 0.1% formic acid in acetonitrile (solvent B) was used with a 300  $\mu\text{L}/\text{min}$  flowrate. The elution method began at 10% solvent B before

increasing to 20% solvent B after 1 min. Between minute 1 and 16, solvent B was increased to 40%. After 16.1 min, solvent B was increased to 100% and maintained until minute 20. Solvent B was decreased to 5% at 20.1 min and held constant until minute 25. The electrospray ionization (ESI) source was operated under the following conditions: positive ion mode, 4000 to -500 V voltage between capillary and end plate offset, 10.0 L/min flow rate of N<sub>2</sub> drying gas at 200 °C.

#### 2.4 Alkaloid Quantification

Alkaloids identified within the extract were quantified using commercially available standards. A calibration curve consisting of five points between 65 and 6.88 mg/L was formed for cycloamine, veratramine, jervine, and muldamine. An Agilent InfinityLab LC/MSD. The sample was injected onto a Waters Xterra MS C<sub>18</sub> column (5 µm, 2.1 x 150 mm) maintained at 40 °C at a volume of 10 µL. The ESI source was operated with a capillary voltage set to 4000 V. The N<sub>2</sub> drying gas temperature was set to 300 °C with a flow rate of 7.0 L/min and nebulizer pressure of 15 psi. The same solvent gradient was used as described in the previously.

#### 2.5 Cell Culture and Bioactivity Assay

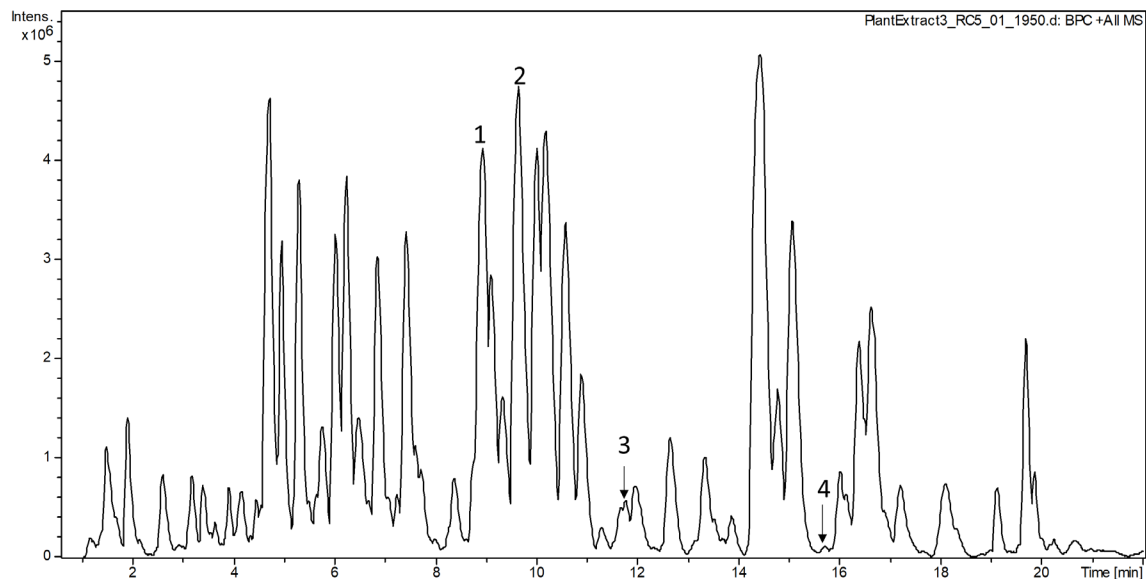
Shh Light II cells (JHU-068) were grown in DMEM supplemented with 0.4 mg/mL geneticin, 0.15 mg/mL Zeocin<sup>TM</sup>, 1% Penicillin-Streptomycin, and 10 % fetal bovine serum. The cells were grown at 37 °C in an atmosphere of 5% CO<sub>2</sub> in air, and 100% relative humidity. Shh Light II cells were seeded into a 95-well plate at a seeding density of 10,000 cells/well and grown to confluence in the previously described growth media. Alkaloid treatments were dissolved in 100% ethanol and added to DMEM containing 0.5% fetal bovine serum and 1% Penicillin-Streptomycin with a final ethanol

concentration of 1%. After the cells become confluent, the growth media was replaced with DMEM supplemented with 0.5% fetal bovine serum and 1% Penicillin-Streptomycin (starvation media), 10 ng of N-terminal mouse recombinant Shh dissolved in DMEM, and the alkaloid treatment. The positive control was treated with 10 ng of N-terminal mouse recombinant Shh dissolved in DMEM while the negative control only contained starvation media. Gli activity was assessed 48 hours following treatment with the Dual-Luciferase® Reporter Assay System from Promega. Luminescence resulting from the transcription of the luciferase reporter with eight copies of the Gli binding site was measured using a Synergy H1 Hybrid Multi-Mode Reader. Each treatment was performed five times.

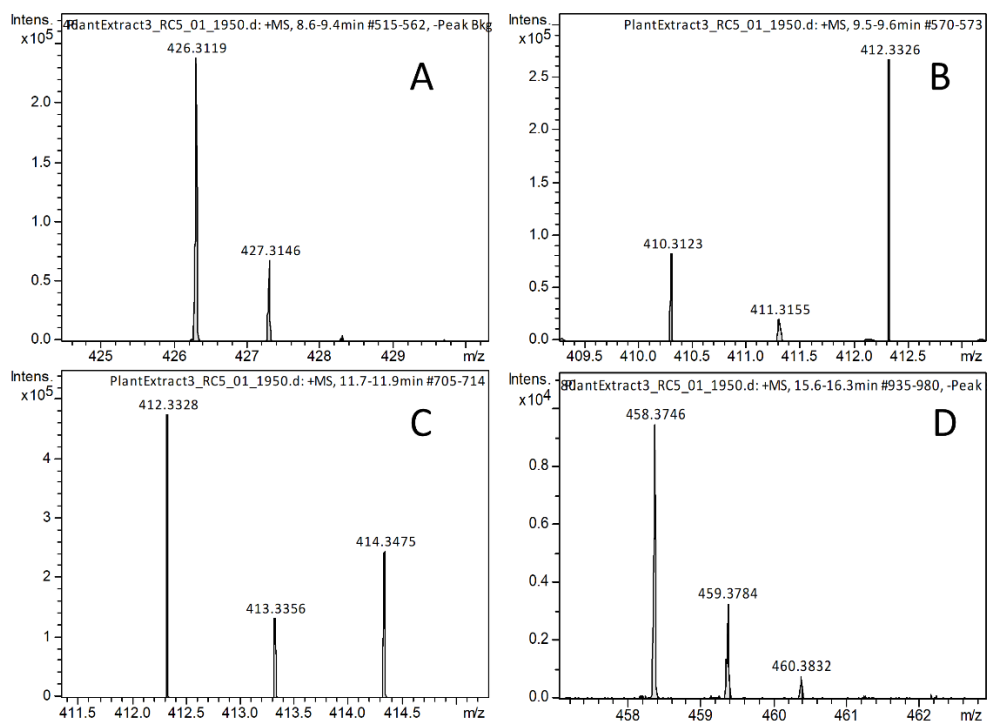
### 3. Results

#### 3.1 Alkaloid Identification and Quantification

Qualitative analysis of the *V. parviflorum* extract revealed four alkaloids: cyclopamine, veratramine, muldamine, and jervine (Figure 3.2). These alkaloids were identified by comparing the respective retention times and mass spectra to commercially available standards. Cyclopamine, veratramine, muldamine, and jervine had retention times of 11.5, 9.4, 15.7, and 8.8 min, respectively. The mass spectra presented in Figure 3.3 depict the  $[M+H]^+$  ions for each alkaloid in the extract. The  $[M+H]^+$  ions for cyclopamine, veratramine, muldamine, and jervine of  $412.32 \pm 0.02$ ,  $410.30 \pm 0.02$ ,  $458.36 \pm 0.02$ ,  $426.30 \pm 0.02$  m/z, respectively.



**Figure 3.2** Chromatogram of *V. parviflorum* extract collected the HPLC-QTOF. Peaks labeled 1-4 were identified as jervine, veratramine, cyclopamine, and muldamine, respectively.



**Figure 3.3** Mass spectra for identified steroidal alkaloids in *V. parviflorum* extract. Panels A-D provide the mass spectra for jervine, veratramine, cyclopamine, and muldamine, respectively. Jervine, veratramine, cyclopamine, and muldamine were identified by the observed  $[M+H]^+$  ions of  $426.31 \pm 0.02$ ,  $410.33 \pm 0.02$ ,  $412.33 \pm 0.02$ , and  $458.37 \pm 0.02$ , respectively.



The concentration of the steroidal alkaloids in *V. parviflorum* were calculated using calibration curves with  $R^2 > 0.99$ . The concentration of cyclopamine, veratramine, muldamine, and jervine in the root and rhizome extract were 10.2, 4.9, 0.4, and 3.6 mg/L, respectively (Table 3.1). These concentrations were then replicated using commercially available standards and used as a treatment condition in the Shh-Light II cell bioactivity assay.

**Table 3.1** Concentration of Identified Steroidal Alkaloids in *V. parviflorum* Extract

| Steroidal Alkaloid Identified | Concentration (mg/L) |
|-------------------------------|----------------------|
| Cyclopamine                   | 10.2                 |
| Veratramine                   | 4.9                  |
| Muldamine                     | 0.4                  |
| Jervine                       | 3.6                  |

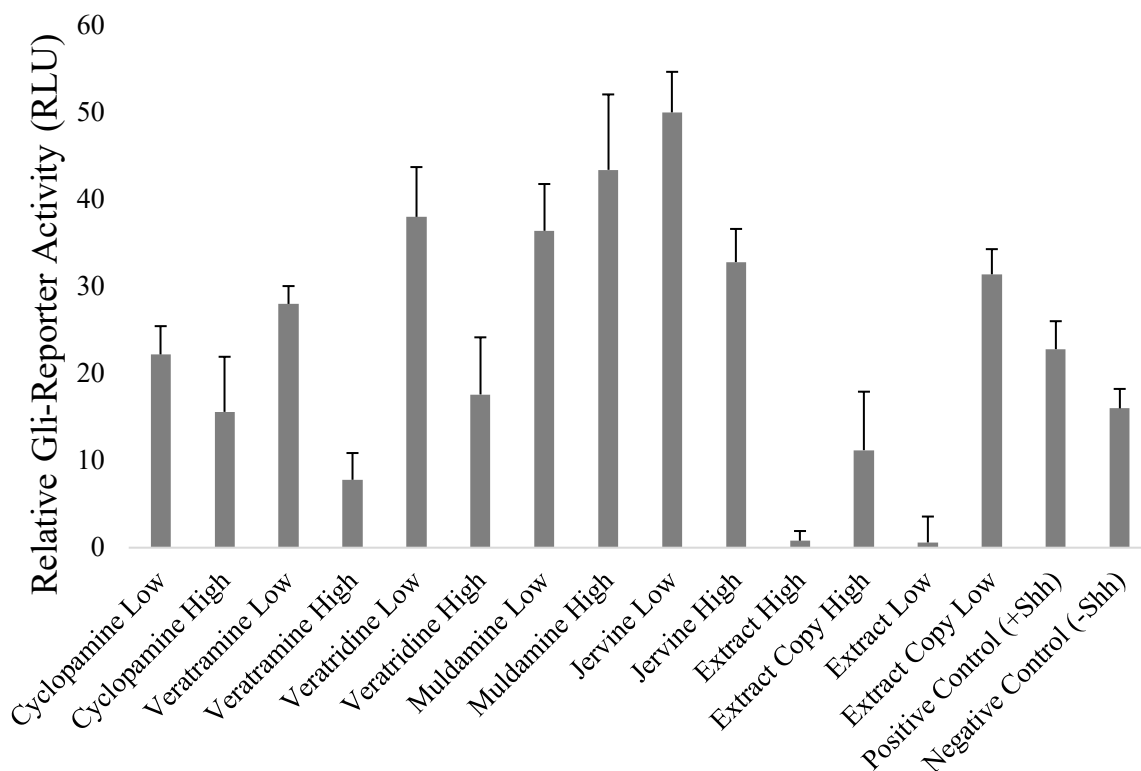
### 3.2 Bioactivity Assessment

Steroidal alkaloid treatments were made using commercially available standards (Table 3.2). The extract copy high and low treatments consisted of steroidal alkaloid concentrations that matched those found within the plant extract. The purpose of evaluating Hh signaling using this treatment was to identify if there were additional alkaloids within the extract with Hh inhibitory properties. The bioactivity evaluation of the *V. parviflorum* steroidal alkaloids identified that there was no statistically significant difference between the low and high treatments for cyclopamine and muldamine (Figure 3.4). At low concentrations, cyclopamine was the most potent inhibitor while veratramine was the most effective inhibitor at high concentrations. A comparison of the low copy extract to the low extract treatment indicated that there was a statistically significant

difference ( $p < 0.01$ ). This suggests that there are additional components within the *V. parviflorum* extract that contribute to enhanced Hh inhibition.

**Table 3.2 Alkaloid Treatments for Shh-Light II Cell Assay**

| <b>Treatment Condition</b> | <b>Cyclopamine (<math>\mu\text{M}</math>)</b> | <b>Veratramine (<math>\mu\text{M}</math>)</b> | <b>Veratridine (<math>\mu\text{M}</math>)</b> | <b>Muldamine (<math>\mu\text{M}</math>)</b> | <b>Jervine (<math>\mu\text{M}</math>)</b> |
|----------------------------|---|---|---|---|---|
| Cyclopamine High           | 1   | -   | -   | -   | -   |
| Cyclopamine Low            | 0.1   | -   | -   | -   | -   |
| Veratramine High           | -   | 1   | -   | -   | -   |
| Veratramine Low            | -   | 0.1   | -   | -   | -   |
| Veratridine High           | -   | -   | 1   | -   | -   |
| Veratridine Low            | -   | -   | 0.1   | -   | -   |
| Muldamine High             | -   | -   | -   | 1   | -   |
| Muldamine Low              | -   | -   | -   | 0.1   | -   |
| Jervine High               | -   | -   | -   | -   | 1   |
| Jervine Low                | -   | -   | -   | -   | 0.1                                       |
| Extract Copy High          | 0.2   | 0.1   | -   | 0.01  | 0.08                                      |
| Extract Copy Low           | 0.02  | 0.01  | -   | 0.001                                       | 0.008                                     |



**Figure 3.4 Bioactivity results for the Shh-Light II cell assay. There was a statistically significant difference between the high and low treatment conditions for veratramine ( $p < 0.01$ ), veratridine ( $p < 0.05$ ), jervine ( $p < 0.05$ ), and the extract copies ( $p < 0.05$ ). The extract low treatment exhibited lower relative Gli-reporter activity than the low extract copy treatment ( $p < 0.01$ ).**

#### 4. Discussion

The two primary objectives of the current study were to 1) identify steroidal alkaloids with an extract of *V. parviflorum* and evaluate their ability to inhibit the Hh signaling pathway, and 2) determine if there were additional alkaloids within the *V. parviflorum* extract by comparing the Hh inhibition of the extract to standard mixtures of alkaloids consisting of identical concentrations of cyclopamine, veratramine, muldamine, and jervine.

The qualitative investigation regarding the steroidal alkaloid content of *V. parviflorum* resulted in the identification of cyclopamine, veratramine, jervine, and

muldamine. Cyclopamine and veratramine have been previously identified within the plant, however, jervine and muldamine are novel to this species.<sup>15</sup> Jervine has been previously isolated in *V. nigrum* L., *V. californicum*, *V. album*, and *V. viride*, while muldamine has only been isolated from *V. californicum*.<sup>44,56,85-88</sup> In addition to cyclopamine and veratramine, Anwar et al. identified verazine and veratridine within the root and rhizome extract.<sup>15</sup> An extracted ion chromatogram for the expected [M+H]<sup>+</sup> ion for verazine (398.3423 m/z) presents several peaks suggesting that the compound may be present but remains unidentified due to the unavailability of a commercially available standard. The absence of veratridine in the *V. parviflorum* extract may be due to differences in time of harvest, growth stage, geographic location.<sup>85</sup> One study on *V. californicum* observed variation in steroidal alkaloid content given these factors.<sup>85</sup>

The inhibition of Hh signaling in Shh-Light II cells was evaluated using luciferase reporter assay. Results from this assessment suggested that there was a statistically significant difference between the high and low treatments for veratramine ( $p < 0.01$ ), veratridine ( $p < 0.05$ ), jervine ( $p < 0.05$ ), and the extract copies ( $p < 0.05$ ). There was a general trend of lower relative Gli-reporter activity in the high concentration treatments compared to the low concentration treatments. Previous work identified that cyclopamine, veratramine, jervine, and muldamine inhibit Hh signaling.<sup>41,63,87,107,108</sup> The copy extract high treatment provided a lower Gli-reporter activity than observed for the alkaloid treatments consisting of a single analyte. Although lower Gli-reporter activity was observed, enhancement of Hh inhibition seems to be additive rather than synergistic. Combinations of steroidal alkaloids from *V. californicum* have been observed to improve Hh signaling inhibition in Shh-Light II cells in an additive trend.<sup>41</sup> A comparison of the

high and low copy extract treatments to the high and low extract treatments indicated that there may be additional compounds within the extract of *V. parviflorum* with Hh antagonistic properties. Further studies of the *V. parviflorum* extract may include the isolation, characterization, and bioactivity evaluation of less abundant steroidal alkaloids.

## 5. Conclusion

*V. parviflorum* is an underexplored plant species that has been identified to contain steroidal alkaloids that inhibit the Hh signaling pathway. The present study sought to identify alkaloids within an ethanolic extract of *V. parviflorum*'s roots and rhizome and assess the bioactivity of these alkaloids for Hh inhibition. Cyclopamine, veratramine, jervine, and muldamine were identified in the extract of *V. parviflorum* and evaluated for Hh inhibition using Shh-Light II cells in a Dual-Luciferase® Reporter Assay System. There was no statistically significant difference in Hh inhibition between the different alkaloid treatments consisting of a single analyte, however, combinations of these alkaloids at concentrations that match those found within the plant extract provided enhanced inhibition. Furthermore, the plant extract showed much lower levels of Gli-reporter activity than the copy extract treatments indicating that there may be less abundant alkaloids within the extract that inhibit Hh signaling.

## CHAPTER FOUR: CONCLUSION AND FUTURE DIRECTIONS

The foundation for modern therapeutic development has been established by secondary metabolites originating from marine and terrestrial sources. Out of an estimated 70,000 plant species used throughout history for medicinal purposes, more than 3,000 plants are reported to contain compounds with anticancer properties.<sup>1,2</sup> The *Veratrum* genus of plants has played a critical role in the development of a class of cancer

therapeutics, called Hedgehog pathway inhibitors, that target cancers including basal cell carcinoma and acute myeloid leukemia.<sup>5</sup> Although proven effective at treating cancers, recurrence after treatment is common due to mutations in Smo.<sup>102-104</sup> Investigating underexplored *Veratrum* spp. may yield novel steroidal alkaloids with anticancer properties

This project investigated the underexplored plant species *V. parviflorum* following the accidental poisoning of eight individuals. Prior to this work, only four steroidal alkaloids had been identified in an extract of this plant's roots and rhizome: cyclopamine, veratramine, veratridine,<sup>5</sup> and verazine. An analysis of patient blood and breast milk revealed the presence of cyclopamine and jervine in the blood, and cyclopamine and veratramine in the breast milk. Investigation into the alkaloid content within the roots and rhizome of *V. parviflorum* resulted in the identification of two alkaloids novel to this *Veratrum* spp., muldamine and jervine. The bioactivity assessment of the steroidal alkaloids identified in *V. parviflorum* suggests there may be additional steroidal alkaloids that contribute to Hh inhibition in Shh-Light II cells.

Future work could be directed towards the isolation, characterization, and bioactivity evaluation of steroidal alkaloids in *V. parviflorum* that do not have commercially available standards. Although the current study revealed two alkaloids new to this species, these alkaloids have already been studied for their ability to antagonize the Hh pathway.<sup>41,87,107,108</sup> A comparison of the bioactivity results for the plant extract and the extract copy that contains cyclopamine, veratramine, jervine, and muldamine at the same concentrations as found in the plant extract suggested that there are additional alkaloids that inhibit Hh signaling to a greater extent.

In addition to identifying novel steroidal alkaloids, there remains great area to investigate regarding the biosynthesis of *Veratrum* steroidal alkaloids. Augustin et al. identified four enzymes that convert cholesterol to verazine, however, the proceeding biosynthetic steps leading to compounds such as cyclopamine, veratramine, veratridine, jervine, and muldamine remain unknown.<sup>68</sup> Elucidating these biosynthetic pathways would not only provide insights into how such structurally diverse compounds with wide ranges of bioactivities form, but also into how appreciable amounts of *Veratrum* steroidal alkaloids may be generated. Currently, most commercially available *Veratrum* steroidal alkaloids are extracted and isolated from plant material. There have been efforts directed towards the synthesis of these compounds, however, such endeavors require complex reactions and provide low yields. For example, the biomimetic and diastereoselective synthesis of cyclopamine from dehydroepiandrosterone involves twenty steps with a 1% overall yield.<sup>109</sup> If the enzymes involved in the biosynthesis of *Veratrum* steroidal alkaloids are identified, chemoenzymatic syntheses could offer one method for producing larger quantities of these compounds for use in experiments pertinent to drug discovery such as bioactivity assays or pharmacokinetic studies.

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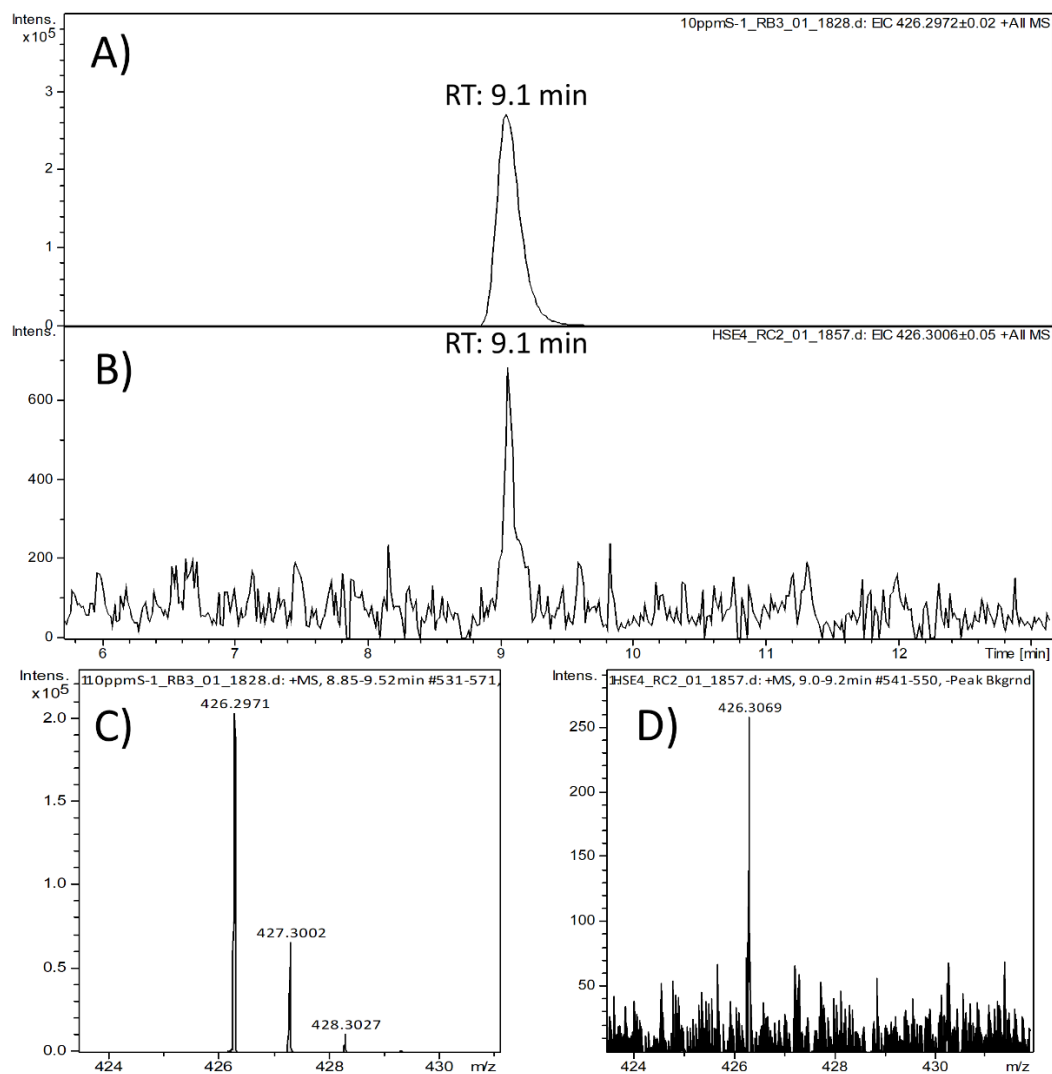
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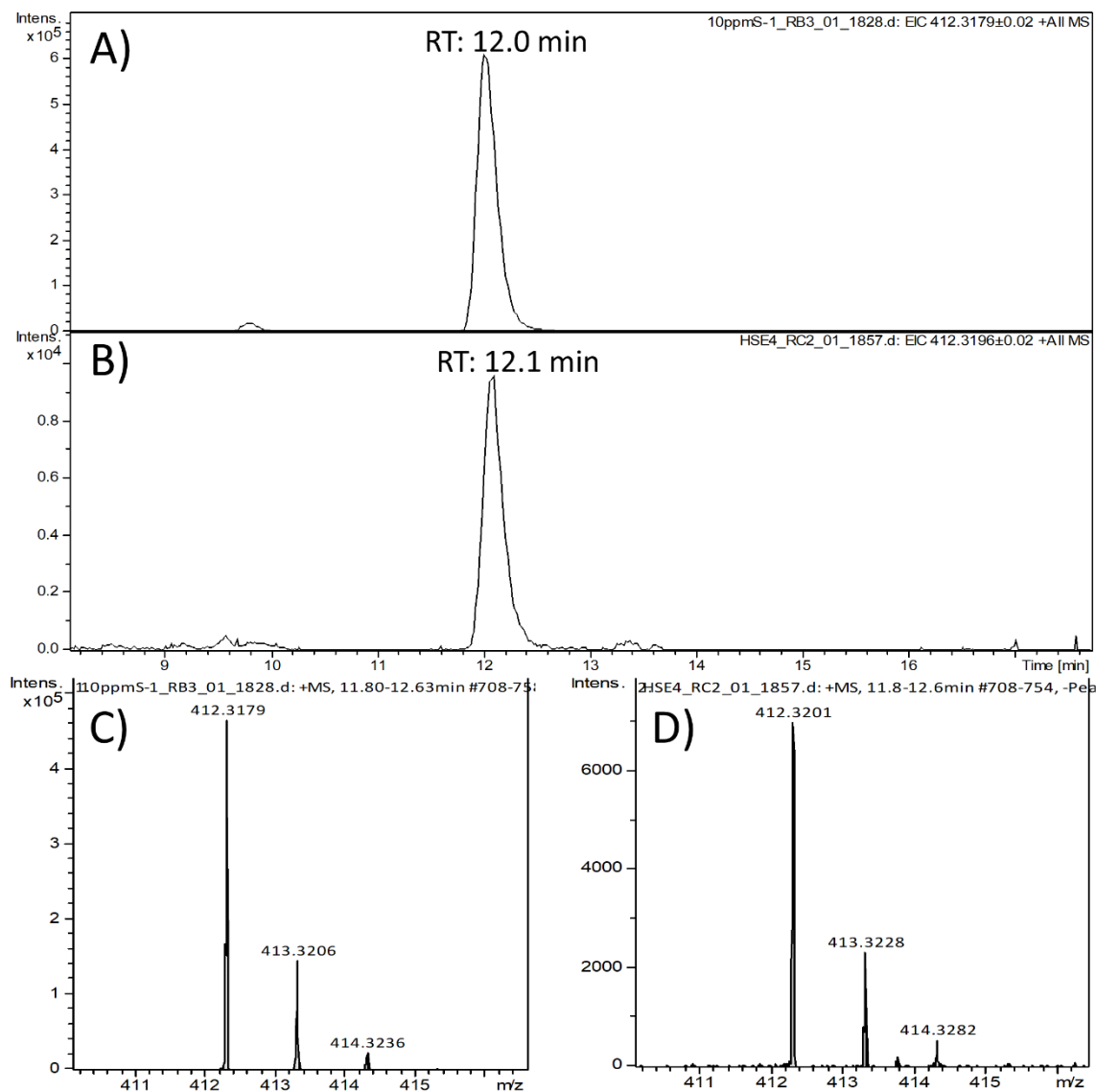
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APPENDIX A

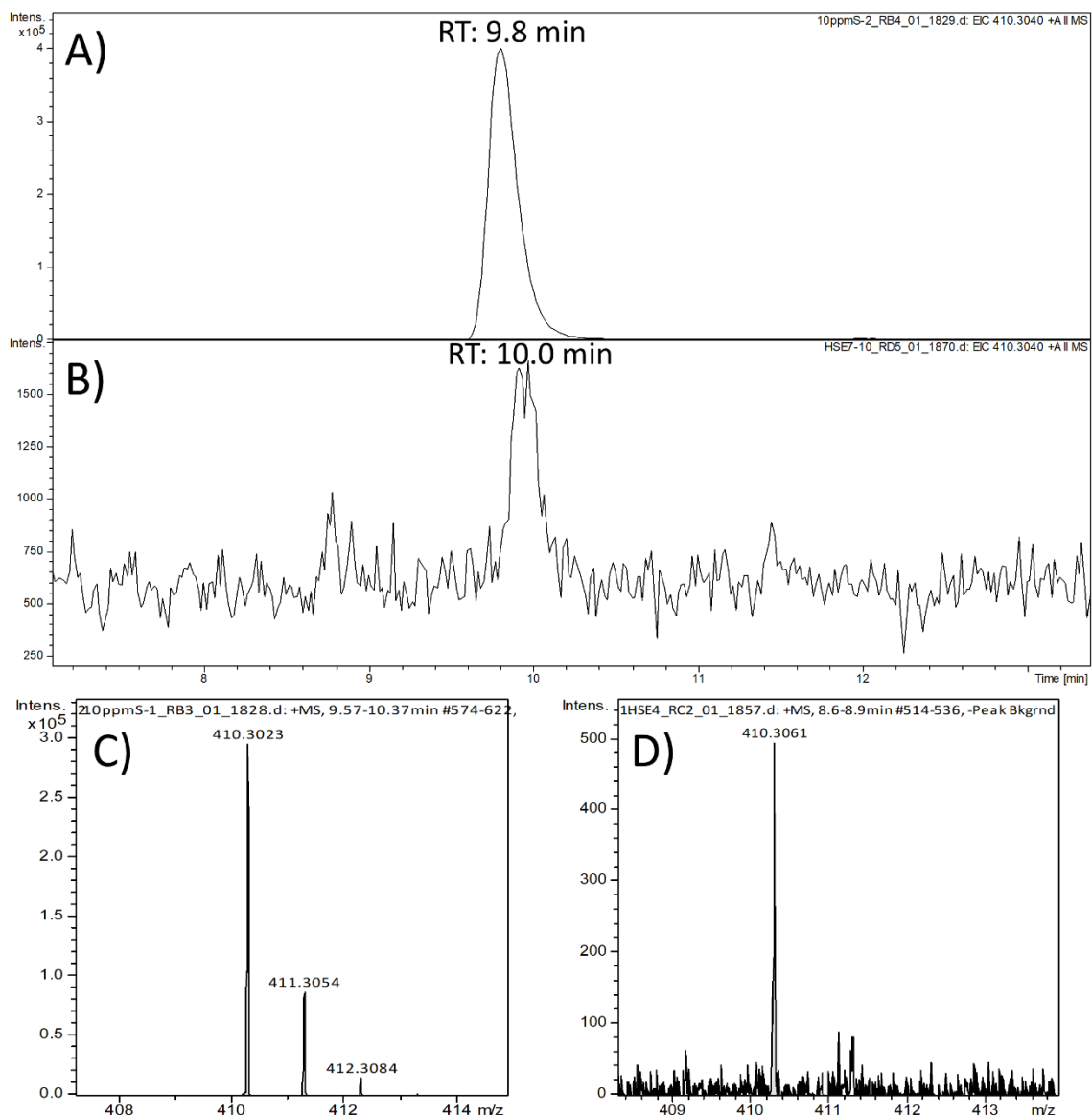
**Chapter Two Supplemental Figures**



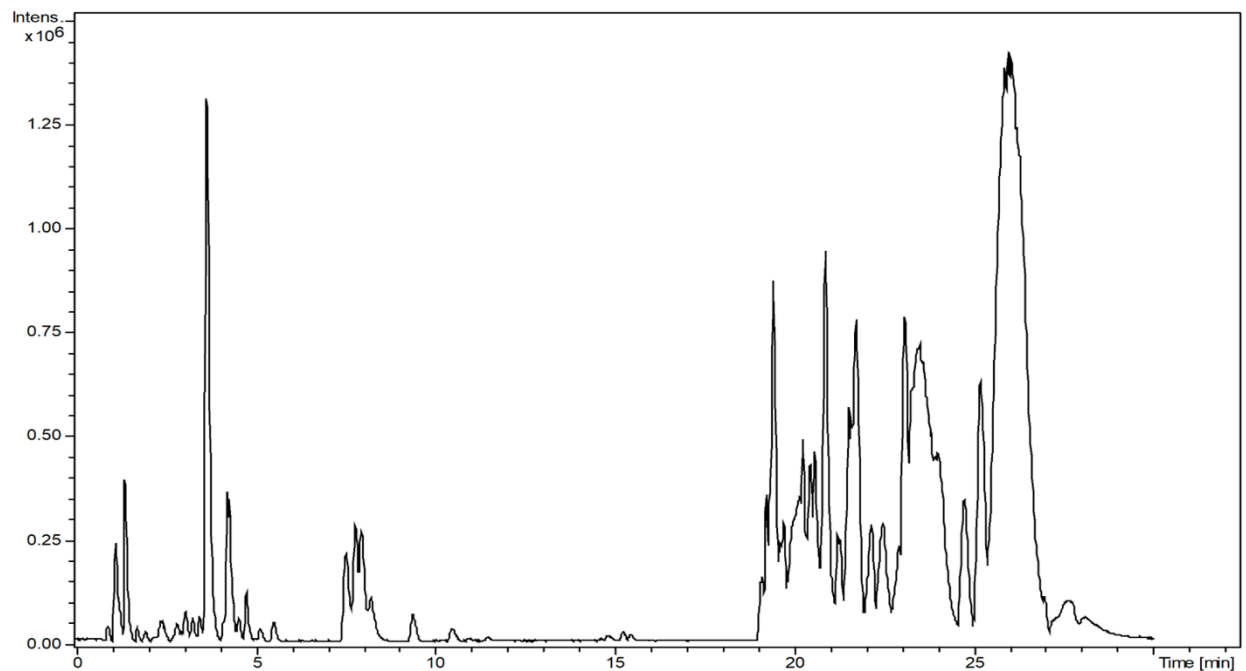
**Figure A.1** Serum extracts for patient #2 were compared to a jervine standard. **A)** Extracted ion chromatogram for jervine extracted from commercially available human serum. **B)** Extracted ion chromatogram for 426.30  $\pm$  0.05  $m/z$ . **C)** Mass spectrum of the peak (RT: 9.1 min) in chromatogram A where the observed  $[M+H]^+$  ion for jervine (426.30  $\pm$  0.05  $m/z$ ) has been identified. **D)** Mass spectrum of the peak (RT: 9.1 min) in chromatogram B where the observed  $[M+H]^+$  ion for jervine (426.30  $\pm$  0.05  $m/z$ ) has been identified.



**Figure A.2** Serum extracts for patient #2 were compared to a cyclopamine standard. A) Extracted ion chromatogram for cyclopamine extracted from commercially available human serum. B) Extracted ion chromatogram for  $412.32 \pm 0.02$  m/z. C) Mass spectrum of the peak (RT: 12.0 min) in chromatogram A where the observed  $[M+H]^+$  ion for cyclopamine ( $412.32 \pm 0.02$  m/z) has been identified. D) Mass spectrum of the peak (RT: 12.1 min) in chromatogram B where the observed  $[M+H]^+$  ion for cyclopamine ( $412.32 \pm 0.02$  m/z) has been identified.

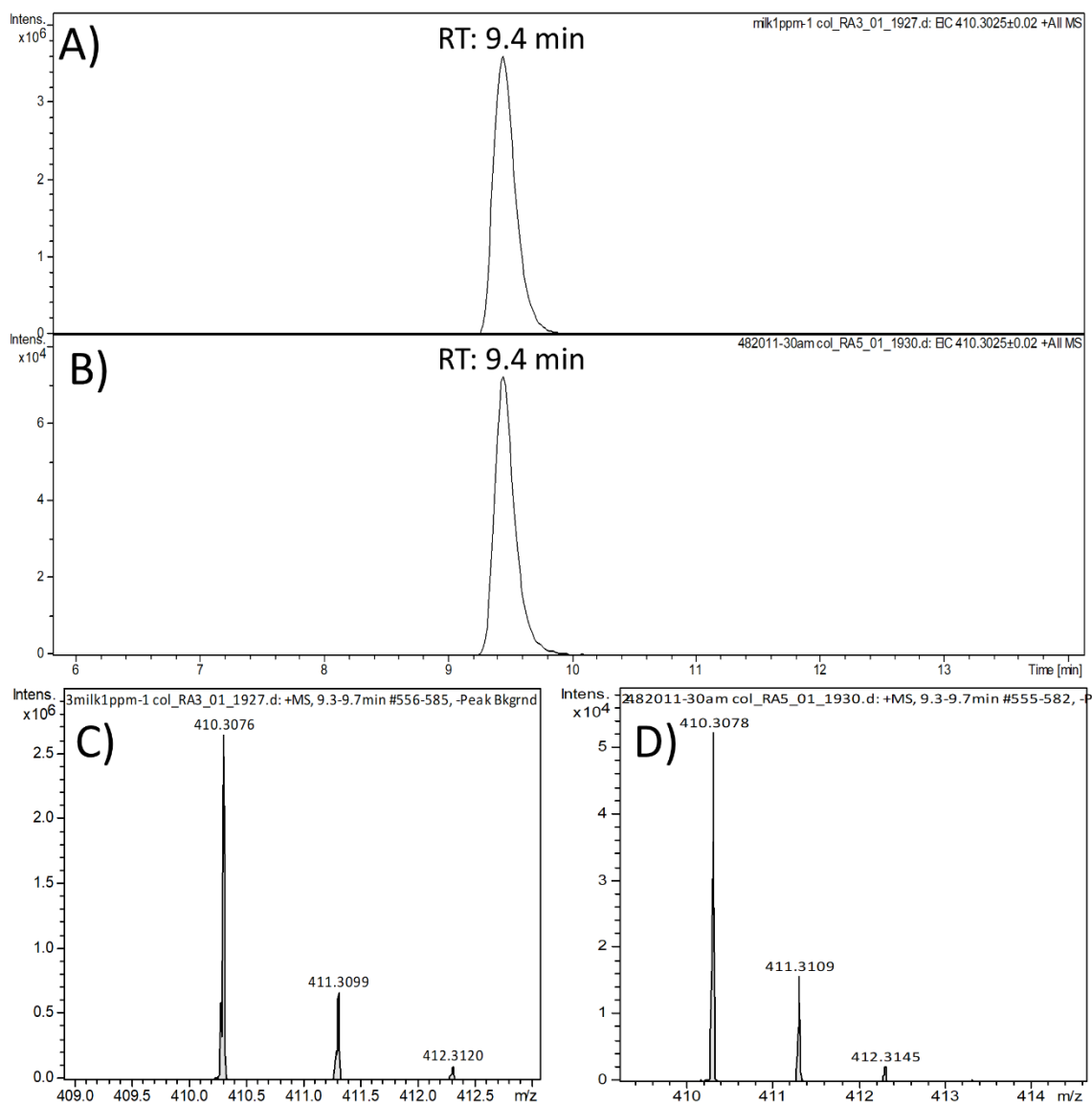


**Figure A.3** Serum extracts for patient #7 were compared to a veratramine standard. A) Extracted ion chromatogram for veratramine extracted from commercially available human serum. B) Extracted ion chromatogram for  $410.30 \pm 0.02$   $m/z$ . C) Mass spectrum of the peak (RT: 9.8 min) in chromatogram A where the observed  $[M+H]^+$  ion for veratramine ( $410.30 \pm 0.02$   $m/z$ ) has been identified. D) Mass spectrum of the peak (RT: 10.0 min) in chromatogram B where the observed  $[M+H]^+$  ion for veratramine ( $410.30 \pm 0.02$   $m/z$ ) has been identified.

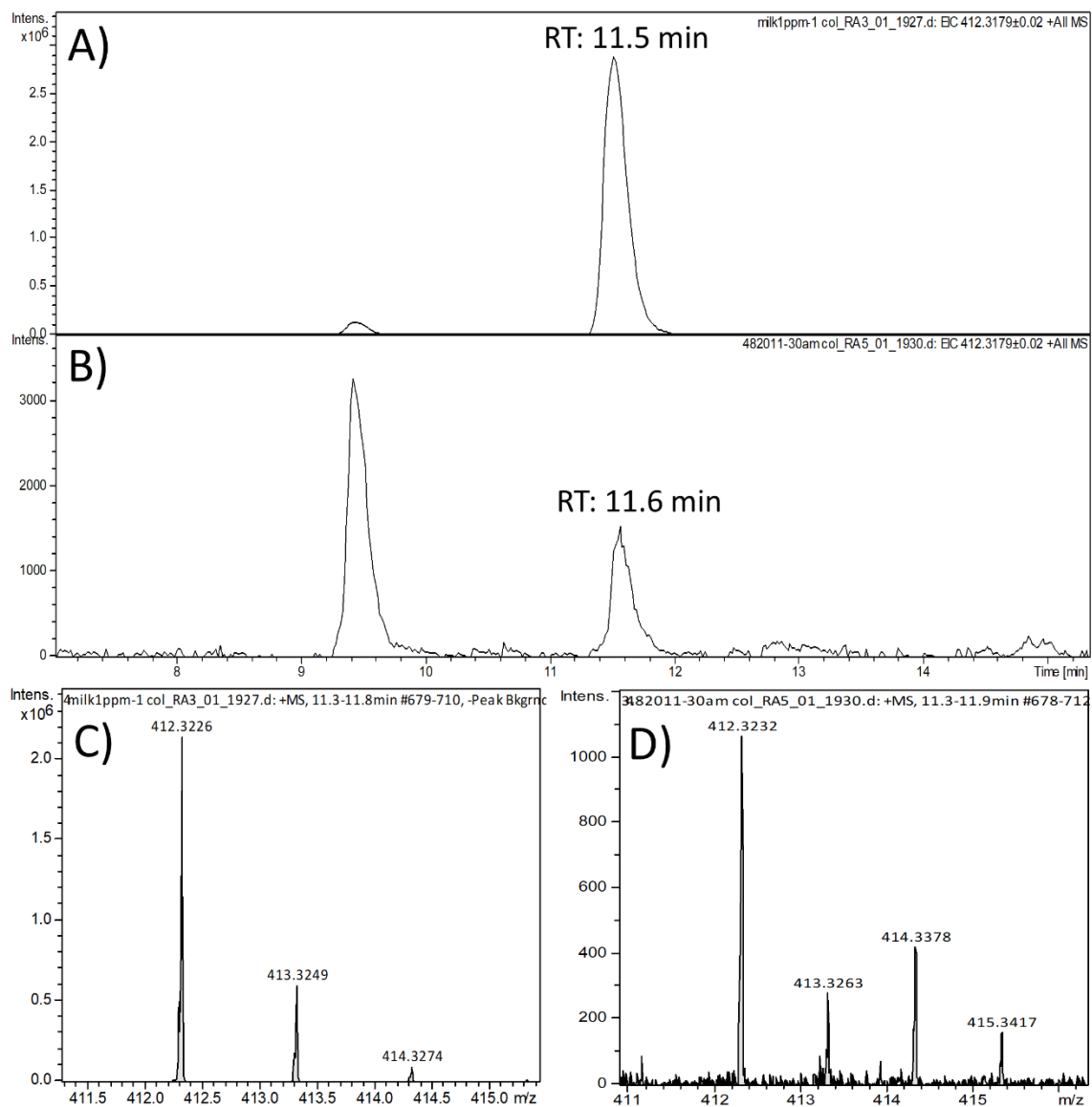


**Figure A.4** Base peak chromatogram of the extract from the breast milk sample collected on April 8, 2020 at 11:30.

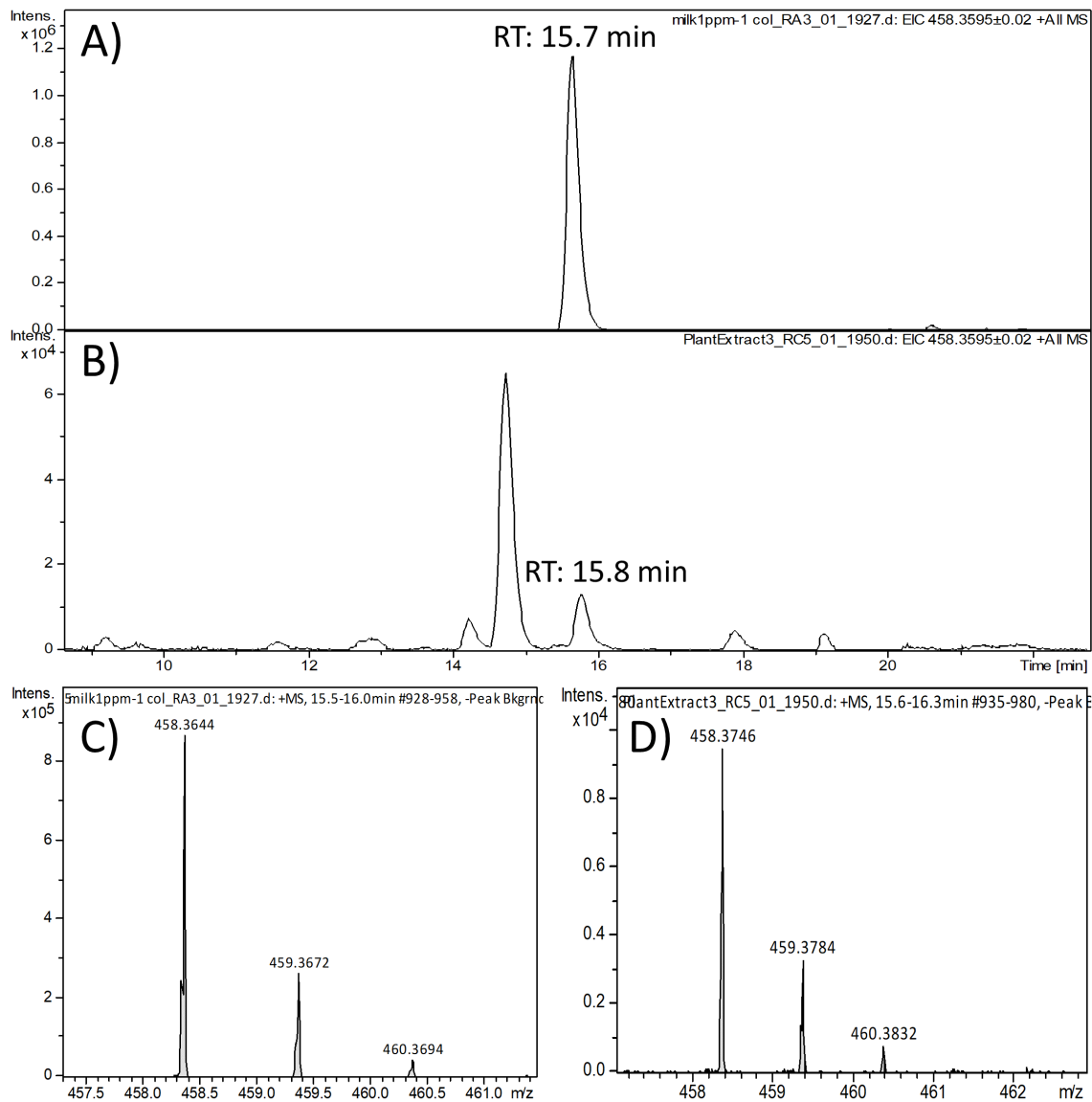




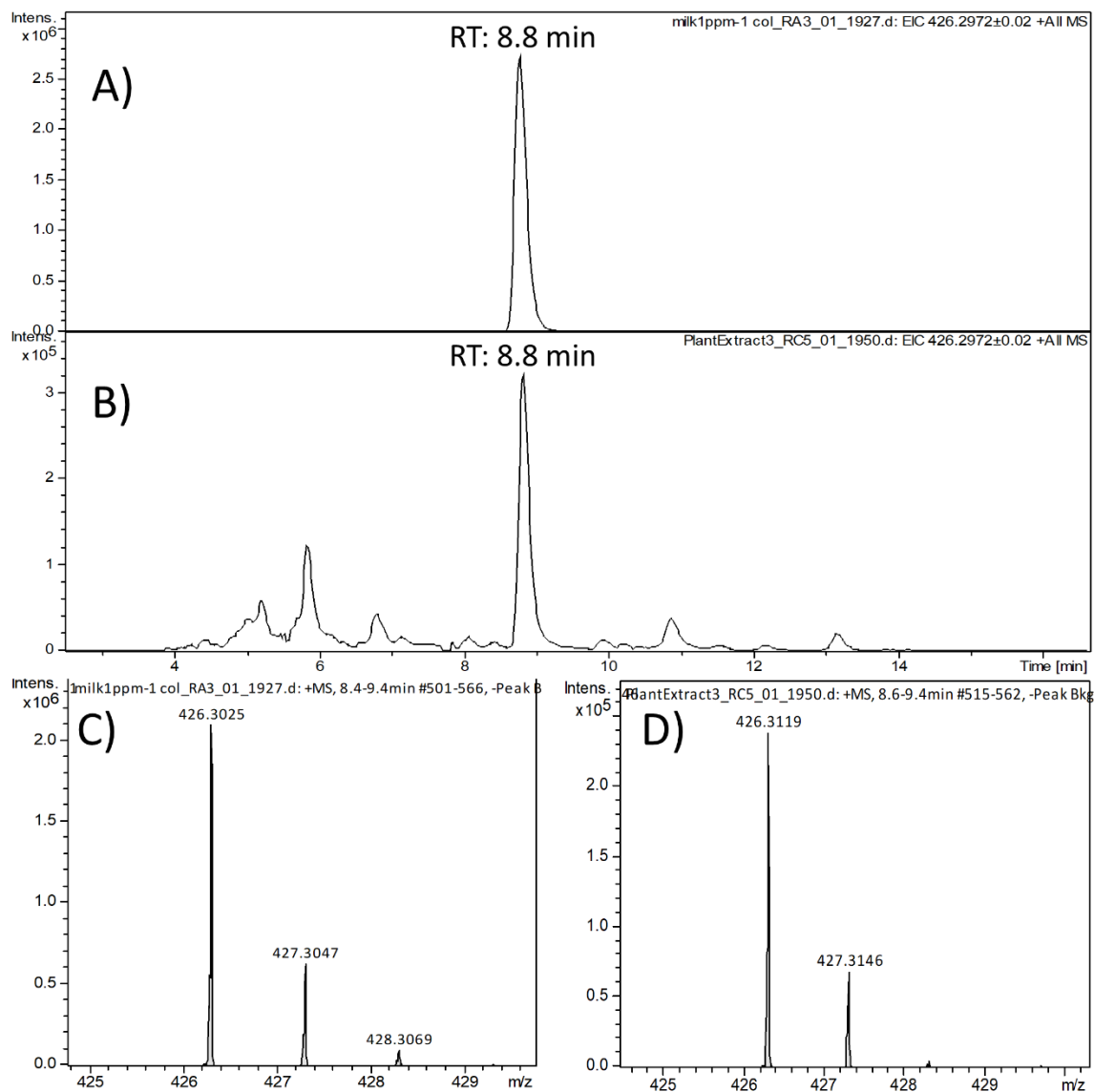
**Figure A.5** Breast milk extracts for patient #2 were compared to a veratramine standard that was extracted from breast milk spiked at 1 mg/L. A) Extracted ion chromatogram for veratramine standard extracted from spiked breast milk. B) Extracted ion chromatogram for 410.30 ± 0.02 m/z. C) Mass spectrum of the peak (RT: 9.4 min) in chromatogram A where the observed [M+H]<sup>+</sup> ion for veratramine (410.31 ± 0.02 m/z) has been identified. D) Mass spectrum of the peak (RT: 9.4 min) in chromatogram B where the observed [M+H]<sup>+</sup> ion for veratramine (410.31 ± 0.02 m/z) has been identified.



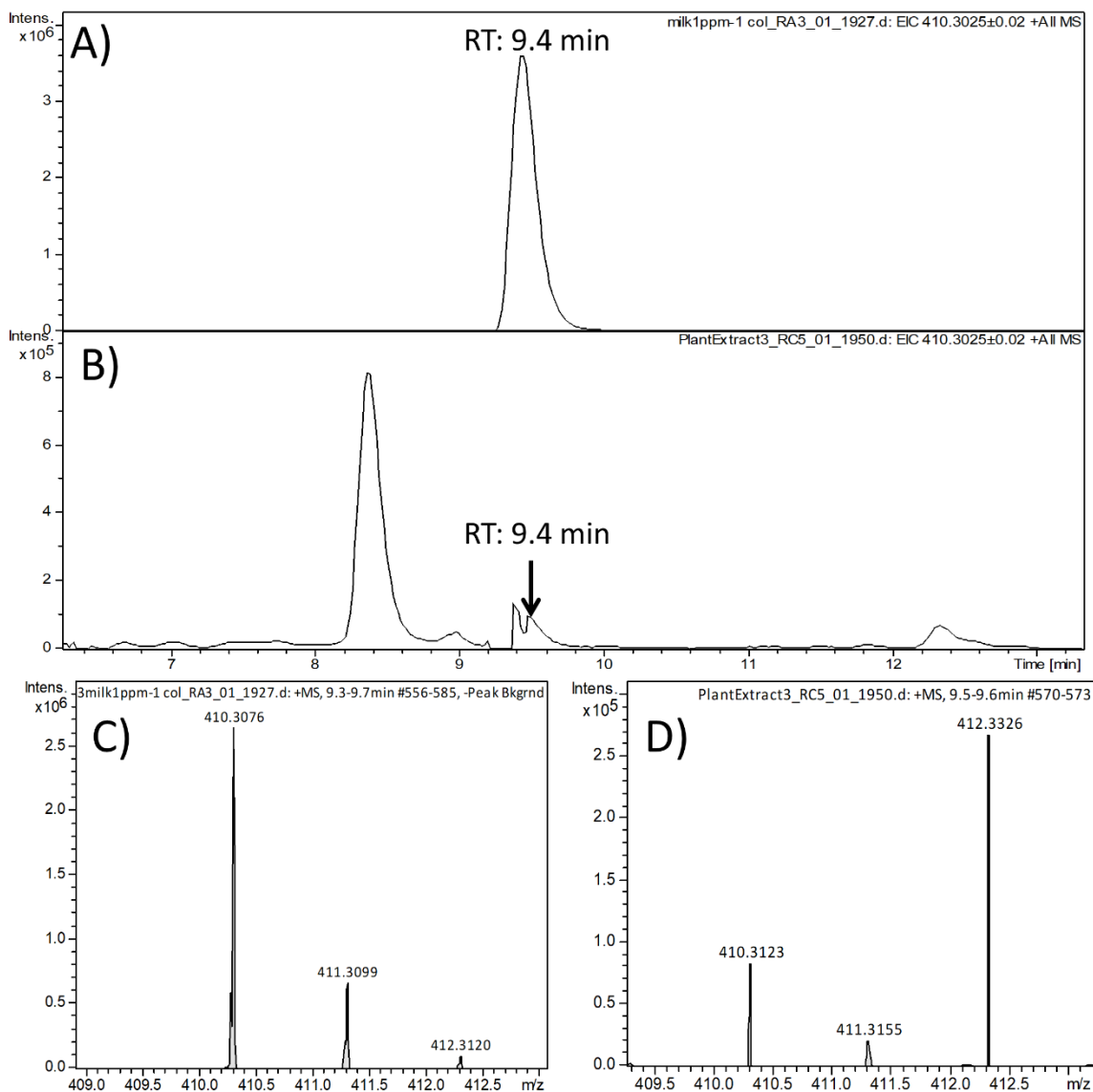
**Figure A.6** Breast milk extracts for patient #2 were compared to a cycloamine standard that was extracted from breast milk spiked at 1 ppm. A) Extracted ion chromatogram for cycloamine standard extracted from spiked breast milk. B) Extracted ion chromatogram for 412.32 ± 0.02 m/z. C) Mass spectrum of the peak (RT: 11.5 min) in chromatogram A where the observed [M+H]<sup>+</sup> ion for cycloamine (412.32 ± 0.02 m/z) has been identified. D) Mass spectrum of the peak (RT: 11.6 min) in chromatogram B where the observed [M+H]<sup>+</sup> ion for cycloamine (412.32 ± 0.02 m/z) has been identified.



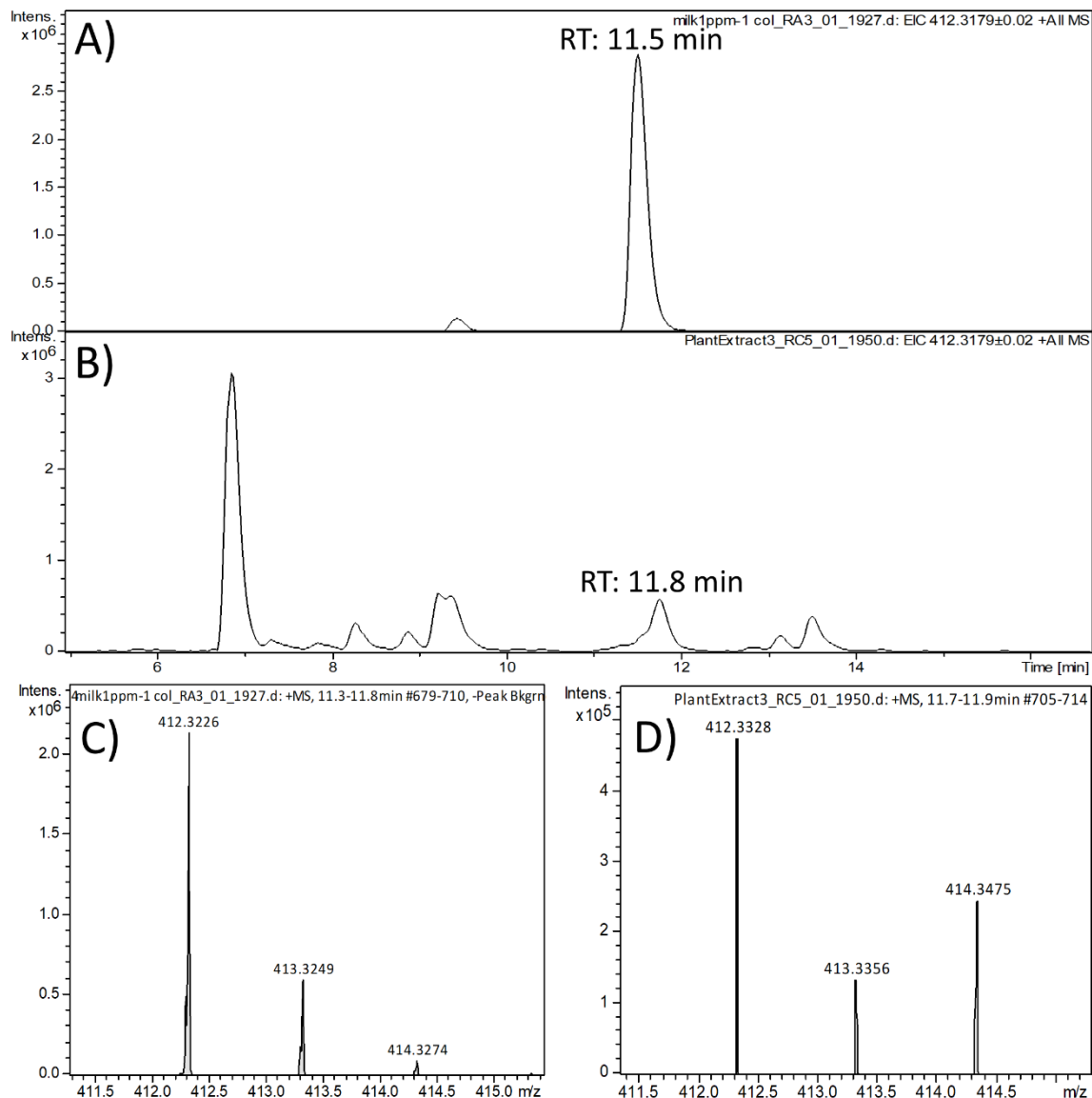
**Figure A.7** *V. parviflorum* ethanolic extract alkaloid at  $R_t$  of 15.7-15.8 min corresponding to muldamine. A) Extracted ion chromatogram for standard muldamine (RT: 15.7). B) Plant extract extracted ion chromatogram for  $458.36 \pm 0.02$  m/z. C) Mass spectrum of the peak (RT: 15.7 min) in chromatogram A where the observed  $[M+H]^+$  ion for muldamine ( $458.36 \pm 0.02$  m/z) has been identified. D) Mass spectrum of the peak (RT: 15.8 min) in chromatogram B where the observed  $[M+H]^+$  ion for muldamine ( $458.37 \pm 0.02$  m/z) has been identified.



**Figure A.8** *V. parviflorum* ethanolic extract alkaloid at  $R_t$  of 8.8 min corresponding to jervine. A) Extracted ion chromatogram for standard jervine (RT: 8.8). B) Plant extract extracted ion chromatogram for  $426.30 \pm 0.02$  m/z. C) Mass spectrum of the peak (RT: 8.8 min) in chromatogram A where the observed  $[M+H]^+$  ion for jervine ( $426.30 \pm 0.02$  m/z) has been identified. D) Mass spectrum of the peak (RT: 8.8 min) in chromatogram B where the observed  $[M+H]^+$  ion for jervine ( $426.30 \pm 0.02$  m/z) has been identified.



**Figure A.9** *V. parviflorum* ethanolic extract alkaloid at  $R_t$  of 9.4-9.5 min corresponding to veratramine. **A)** Extracted ion chromatogram for standard veratramine (RT: 9.4). **B)** Plant extract extracted ion chromatogram for  $410.30 \pm 0.02$  m/z. **C)** Mass spectrum of the peak (RT: 9.4 min) in chromatogram A where the observed  $[M+H]^+$  ion for veratramine ( $410.30 \pm 0.02$  m/z) has been identified. **D)** Mass spectrum of the peak (RT: 9.5 min) in chromatogram B where the observed  $[M+H]^+$  ion for veratramine ( $410.30 \pm 0.02$  m/z) has been identified.



**Figure A.10** *V. parviflorum* ethanolic extract alkaloid at  $R_t$  of 11.5-11.8 min corresponding to cyclopamine. A) Extracted ion chromatogram for standard cyclopamine (RT: 11.5). B) Plant extract extracted ion chromatogram for  $412.32 \pm 0.02$  m/z. C) Mass spectrum of the peak (RT: 11.5 min) in chromatogram A where the observed  $[M+H]^+$  ion for cyclopamine ( $412.32 \pm 0.02$  m/z) has been identified. D) Mass spectrum of the peak (RT: 11.8 min) in chromatogram B where the observed  $[M+H]^+$  ion for cyclopamine ( $412.32 \pm 0.02$  m/z) has been identified.