Fall 2020

Preliminary Isolation and Characterization of Root Extract Components of Dalea jamesii

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Senior Capstone

Submitted in Partial Fulfillment of the Requirements for Graduation from

The William O. Douglas Honors College

Central Washington University

November, 2020

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Please note: Signatures have been redacted due to security concerns.

Abstract

The plant genus *Dalea* (Fabaceae) has been found to be prolific in its production of phenolic secondary metabolites including flavonoids, isoflavonoids, chalcones, pterocarpans, stilbenes, and other biosynthetically related compounds. Biological testing has revealed wide-ranging activities for these metabolites in many prior reports. *Dalea jamesii* has not yet been investigated for its chemical content. This study will be conducted with the hypothesis that unique phenolic secondary metabolites will be produced in the roots of *D. jamesii.* It will be of interest to determine if the isolated metabolites exhibit biomedically related activities. It will also be of interest to investigate whether the phenolic secondary metabolites show any bioactivities.

Using various techniques, including vacuum liquid chromatography, Sephadex LH-20 chromatography, and linear gradient or step gradient chromatography, the secondary metabolites from the root portion of *D. jamesii* will be separated. Thin-layer chromatography (TLC) and ^{1}H NMR spectroscopy will be used to combine similar materials for further purification. Structure determination of the pure compounds will be accomplished primarily by extensive 1D and 2D NMR spectroscopy.

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I. Introduction

This research is focused on the isolation and characterization of secondary metabolites produced in *D. jamesii*. This specific plant has not been investigated previously, and a crude extract of it revealed promising anticancer activity in its preliminary testing with glioblastoma cells in vitro.

A natural product is a chemical compound that is found in nature, produced by living organisms. When natural products are isolated from living organisms, they are synthesized in order to be made available in large quantities. One famous example of natural product is taxol (paclitaxel), a complex terpenoid compound, which is a drug used in the treatment of breast, lung, and ovarian cancer. It was first isolated from the bark of *Taxus brevifolia*, the Pacific Yew, and was later mass produced through processes reliant on plant-derived biosynthesis.[1] Similarly, aspirin, a drug used for centuries in the treatment of headaches and fever, was found in the bark of the willow tree (*Salix spp.*).[2]

Secondary metabolites are small molecules that are not directly involved in the normal growth, development, reproduction of the organism, or other primary functions. Rather, they are often responsible for health-maintaining processes, such as in plant defense against herbivory.[3] For example, (±)-sanjuanolide, a secondary metabolite isolated from *D. frutescens*, was found to have anti-inflammatory activity, markedly reducing the production of lipopolysaccharide (LPS)-induced IL-6 and TNF-α with IC_{20} values of 1.1 and 1.6 μM, respectively.[4]

Dalea jamesii (Torr.) Torr. & A. Gray (Fabaceae), commonly known as James' Prairie Clover, is a perennial herb that is predominantly found in dry and rocky plains of the southwestern regions of the United States.[5] *D. jamesii* has bright yellow flowers in clusters that turn reddish brown as they age (Figure 1).

(a) (b)

Figure 1. The whole plant, in flower, *D. jamesii* collected from Sierra Vista scenic overlook, Colorado, in 2017. (a) *D. jamesii* aerial portions. (b) *D. jamesii* aerial and root portion.

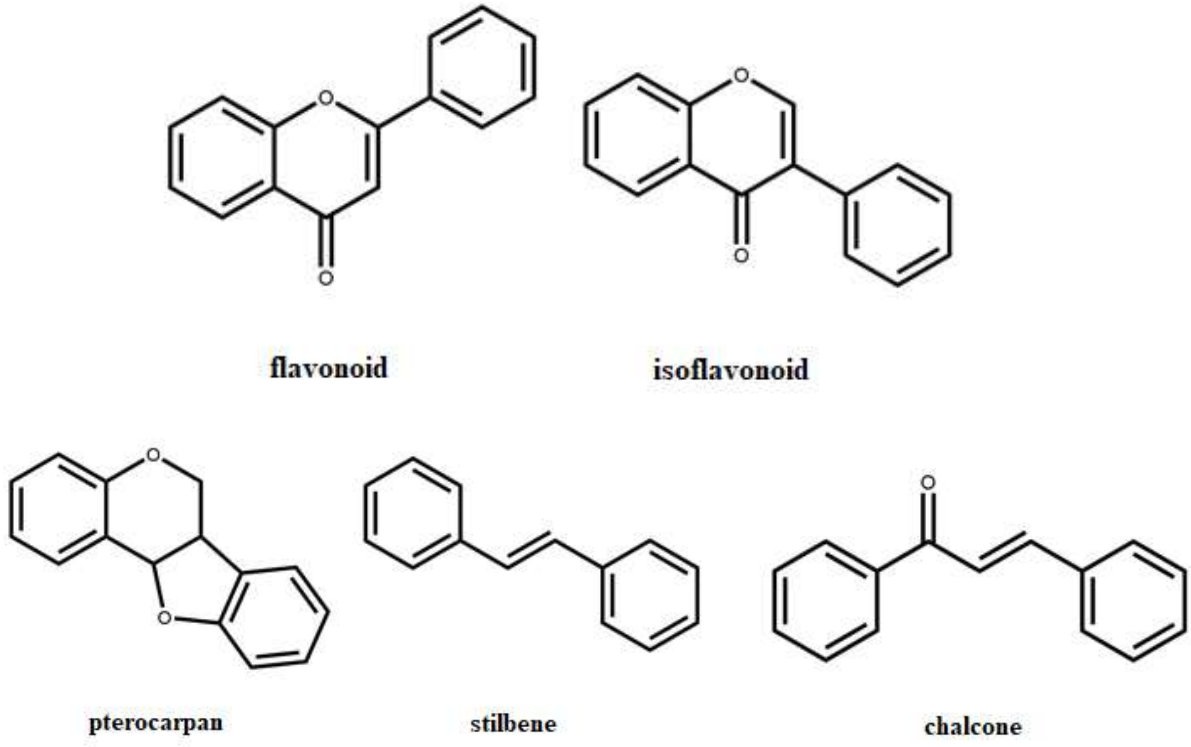

Figure 2. Core structures of flavonoids, isoflavonoids, chalcones, pterocarpans, and stilbenes.

It is reasonable to assume that compounds similar to those shown in Figure 2 will be found in our investigation of *D. jamesii*.

There is a growing body of knowledge from prior investigations of *Dalea* species. An investigation of the roots of *D. filiciformis* revealed a new pterocarpan, daleformis, the structure of which (Figure 3) was determined by interpretation of NMR spectroscopic data and by X-ray diffraction analysis. Bioassays revealed daleformis inhibited endothelin-converting enzyme with a half maximal inhibitory concentration (IC_{50}) of 9 μM.[6]

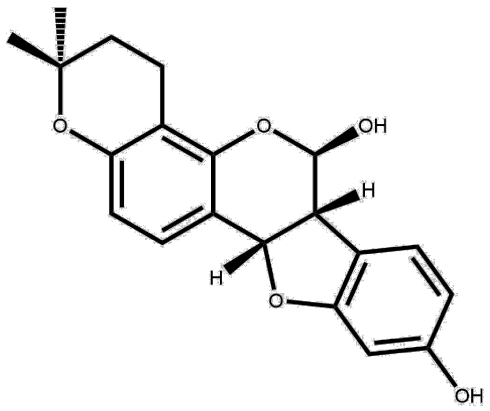

Figure 3. The pterocarpan daleformis was isolated from *D. filiciformis*.

From the whole plant *D. versicolor*, a new prenylated isoflavanone, dalversinol A (Figure 4), and a number of known phenolic compounds, including prenylated flavanones, a chalcone, a pterocarpan, and a stilbene, were isolated. Structures of the purified compounds were determined by extensive 1D, and 2D NMR spectroscopy, and high resolution mass spectrometric (HRMS) methods. Dalversinol A and three other isolated compounds were found to exhibit direct or synergistic activity toward the human pathogens *Staphylococcus aureus* and *Bacillus cereus*.[7]

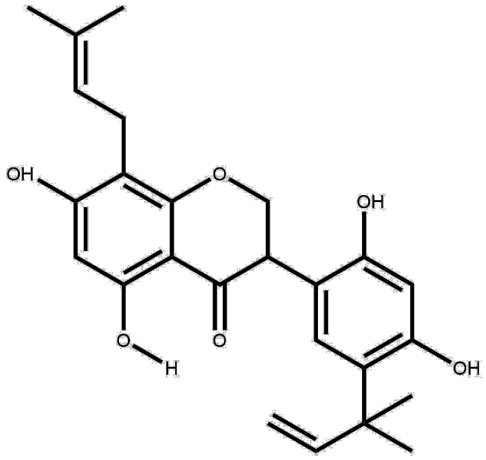

Figure 4. The prenylated isoflavanone dalversinol A was isolated from *D. versicolor.*

D. searlsiae was investigated for its chemical content and from the roots, new prenylated and geranylated flavanones, named malheurans, and known flavanones were isolated.[8] From the aerial portions, known rotenoids and isoflavones, were isolated (Figure 5). Structures of purified compounds were determined primarily by extensive 1D- and 2D-NMR spectroscopy. The absolute configurations of compounds were assigned using electronic circular dichroism spectroscopy. Bioassays revealed significant antimicrobial bioactivity concentrated in the plant roots.[8]

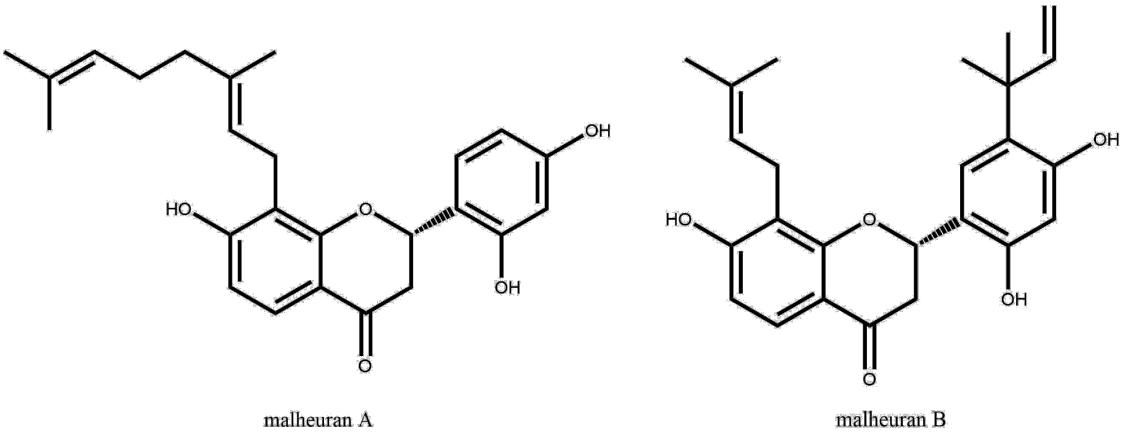

Figure 5. New prenylated and geranylated flavanones, malheurans, isolated from *Dalea searlsiae.*

Preliminary in vitro assays of the root extract of *D. jamesii* (tested at 10 mg/mL) caused a reduction (<90%) in cell viability of U87MG glioblastoma cells. Specific cell counts are not yet available, but this extract was one of six listed as exhibiting relatively potent activity in this recent testing. Glioblastoma are malignant brain tumor cells that also colonize niches outside the central nervous system (CNS).[9] It has high proliferative rate, aggressive invasiveness and insensitivity to radiotherapy and chemotherapy, resulting in short patient survival periods. It is difficult to inhibit glioblastoma cells because they are heterogeneous, containing various populations of cells. As a result, a drug may work for some cells but have no effect on others. The tumor grows behind the blood-brain barrier, a highly selective, semipermeable border that protects the brain from toxic chemicals. This requires that drugs have certain limited characteristics to be able to cross this barrier and reach the tumor.[10] The current treatments of glioblastoma tumors include surgery, radiation, and chemotherapy with temozolomide, Platinol (cisplatin), BiCNU (carmustine), and Gleostine (lomustine). None of these compounds are

flavonoids or natural products. These treatments can slow the tumor growth but are unlikely to eradicate the tumor.[11] Another synthetic compound KHS101, a complex heterocyclic amine, has showed promising in vivo activity in mice via disruption of mitochondrial respiration.[12]

Our prior work has revealed *Dalea* spp. to be prolific producers of phenolic secondary metabolites including flavonoids, isoflavonoids, chalcones, pterocarpans, stilbenes, and other biosynthetically related compounds. There have been reports of *Dalea* compounds with antitumor activities. The flavanone 6PP, isolated from *D. elegans,* showed antitumor activity in a rat liver cell line.[13] Perhaps similar in action to KHS101, 6PP had disruptive effects on mitochondria. Another study showed the chalcone (R)-sanjuanolide, synthesized (R)-configuration from the isolated (±)-sanjuanolide from *D. frutescens*, to have antiproliferative and cytotoxic activities, with a half maximal inhibitory concentration (IC_{50}) of 11 ±4 mM and 7 ±3 mM toward the prostate cancer cell lines PC-3 and DU 145, respectively.[14] Therefore, flavonoids may have potential for the treatment of glioblastoma and other cancers.

Dalea spp. are native to geographic regions that, in future years, are expected to see increasing frequency and severity of climate-change induced drought and heat; a concern to the scientific community because these are regions of relatively high biodiversity.[15,16] While only several are currently listed as threatened or endangered,[5] *Dalea* spp. are C_3 photosynthetic plants,[17] and are likely to be more susceptible to drought than C_4 or CAM photosynthetic plants like grasses and cacti, respectively.[16,18]

II. Research Questions & Hypothesis

Main research question:

- Are new phenolic secondary metabolites, including flavonoids, isoflavonoids, chalcones, pterocarpans, stilbenes, and other biosynthetically related chemotypes, produced in the roots of *Dalea jamesii*?

Additional questions:

- Do the phenolic secondary metabolites show antitumor activities?
- Do the phenolic secondary metabolites show other bioactivities?
- Are any of the phenolic secondary metabolites new? If known, where were they produced?
- For any new compounds…are there any similar compounds from prior reports? Do they have similar functional groups? Is there any similarity in bioactivity?

Based on previous investigations on *Dalea* spp., phenolic secondary metabolites of higher interest were produced in the roots; therefore, it is hypothesized that phenolic secondary metabolites will be produced in the roots of *D. jamesii.* Also, preliminary testing of the root extract exhibited cytotoxic activity to U87MG glioblastoma cells. Therefore, it is hypothesized that phenolic secondary metabolites will show antitumor activities.

III. Research Plan

In this research, methods of extraction and chromatography will be used to isolate the compounds. The collected *D. jamesii* was divided into aerial and root portions. Preparation of the crude extracts of these was first done on a small scale for biological testing by blending with methanol. Preliminary testing of the resulting root extract revealed cytotoxic activity to U87MG glioblastoma cells, while the crude extract of the aerial portions did not exhibit any activity. The subsequent crude extract was first separated using vacuum liquid chromatography (VLC) over silica gel, eluting with varying proportions of hexane, ethyl acetate, dichloromethane, and methanol solvents in increasing polarity. Then, VLC fractions will be further separated using Sephadex LH-20 chromatography with isocratic elution using 3:1:1 hexane-toluene-methanol. Pure methanol will be used as a final wash. Combinations of certain of the resulting fractions will be then successively separated by linear gradient or step gradient chromatography over silica gel until pure compounds are isolated.

Thin-layer chromatography (TLC) will be used at each stage of the purification process to evaluate the purity and polarity of the compounds in each fraction. Fractions of similar composition, according to TLC and, as purity increases, ^{1}H NMR spectroscopy, will be combined for further purification. Structure determination of the pure compounds will be accomplished primarily by extensive 1D- and 2D-NMR spectroscopy.

There are some limitations in this research. First, the lack of certain instrumentation in the Chemistry Department is one major limitation. Central Washington University does not have an ECD (electronic circular dichroism) spectrometer, for obtaining absolute configuration of certain molecules, or a high-resolution mass spectrometer, for supporting the molecular formula derived from

NMR. Therefore, samples will be sent out to other facilities that obtain these data. When samples are sent out, it takes time to get the results back, which is inconvenient.

The Chemistry Department is awaiting the delivery and installation of a new NMR spectrometer at the start of the 2020-21 academic year. Once this happens, we will have access to important methods that were previously unavailable, like NOESY NMR, used for obtaining relative configuration. However, there is still uncertainty as to whether the new NMR will be available for use prior to my own graduation. Finally, despite the research mentor's earnest desire to spend more time in the lab with his mentees, he has much less teaching release time (granted by the College of the Sciences) than in the past.

Time Table

Week 1	Grind *D. jamesii* roots in methanol to prepare crude extraction (already done)
Week 2	Run vacuum liquid chromatography (VLC) over silica gel, eluting with varying portions of hexane, ethyl acetate, dichloromethane, and methanol solvents in increasing polarity.
Week 3	TLC and determine which fractions will be loaded onto Sephadex LH-20.
Week 4	Run size-exclusion column chromatography (Sephadex LH-20) with isocratic elution using 3:1:1 hexane-toluene-methanol.
Week 5	TLC and determine which fractions will be further purified.

Week 6-8	Linear gradient or step gradient chromatography over silica gel until pure compounds are isolated. Thin-layer chromatography (TLC) will be used to evaluate the purity and polarity of the compounds in each fraction.
Week 9, 10	Using NMR spectroscopy, the structures of isolated pure compounds will be determined.

IV. Results

A Bruker 400 MHz NMR spectrometer was used exclusively for determining the structure of the newly discovered compound LA-1-21-F2. A broadband probe was used in the acquisition of ^{1}H NMR data (Figure 6), ^{13}C NMR data (Figure 7), DEPT 90, DEPT 135 and COSY NMR experiments. An inverse probe was used for acquisition of 2D spectra, HSQC, and HMBC (Figure 6), experiments. Another compound, LA-1-32-F4, was also isolated and identified as a known compound, previously isolated from *D. wrightii* and named JC-1-13-F2 (Figure 8).

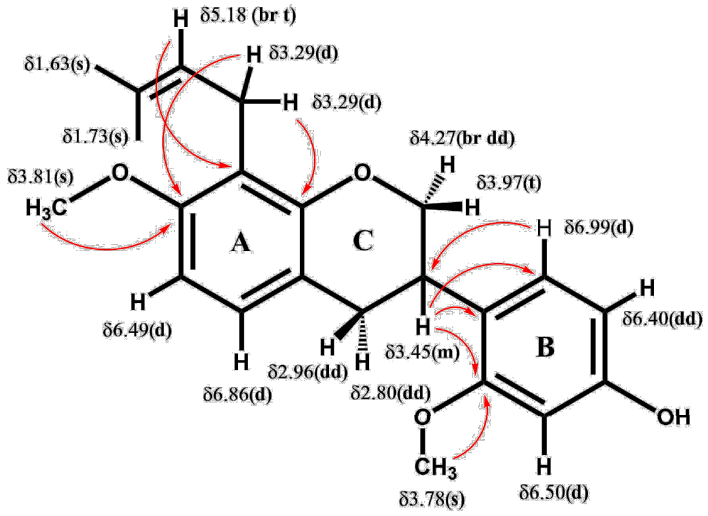

Figure 6. ^{1}H NMR assignments and selected HMBC correlations for the isoflavan LA-1-21-F2. Key HMBC correlations are represented by red arrows from hydrogen nuclei to corresponding carbon nuclei.

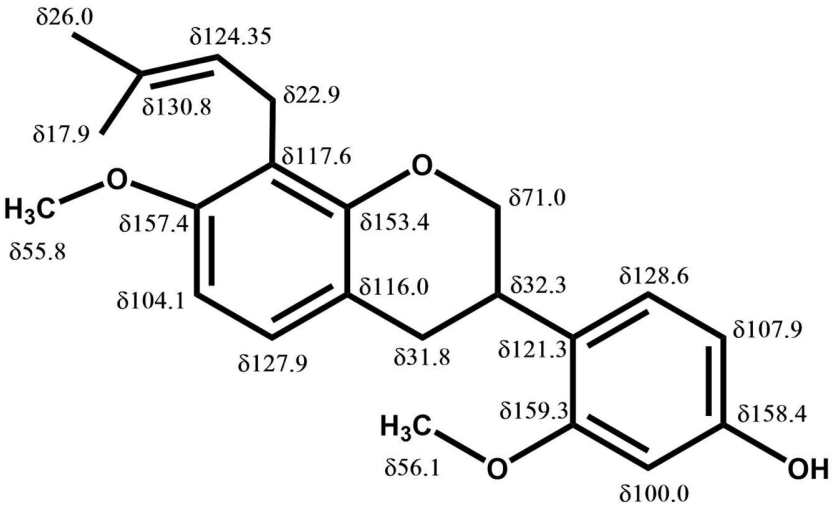

Figure 7. ^{13}C NMR spectroscopic assignments for the isoflavan LA-1-21-F2.

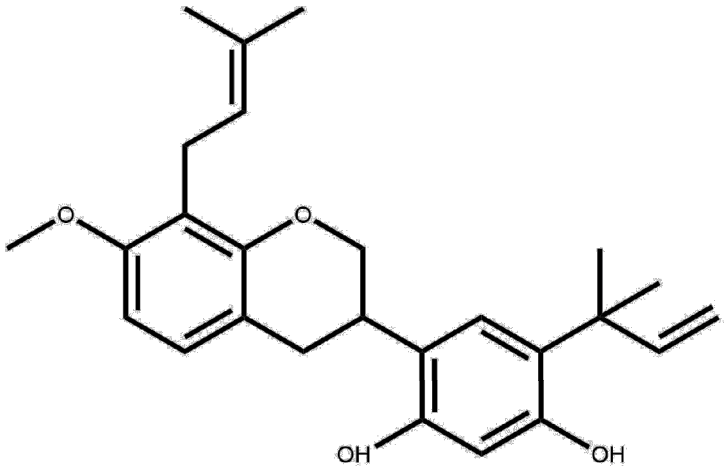

Figure 8. The isoflavan JC-1-13-F2.

V. Discussion

The 1D NMR spectra were predominantly used for initial comparisons of LA-1-21-F2 to compounds of our in-house NMR spectroscopic library. These data also allowed us to identify certain sub-structures that are characteristic of metabolites of *Dalea* spp. The 2D NMR spectra were critical for the determination of the overall structure of LA-1-21-F2. The HSQC and HMBC (heteronuclear multiple bond correlation) experiments in particular, helped to establish the connectivity between rings B and C, and the placement of methoxy and prenyl substituents of ring A (Figure 6). LA-1-21-F2 was found to be a new compound. The most similar known compound (Figure 9) was reported 32 years ago from the tropical tree *Diphysa robinioides.*[19]

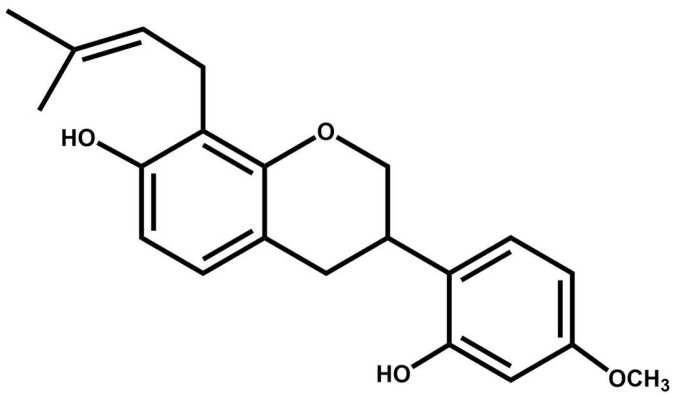

Figure 9. The most similar known compound to LA-1-21-F2 is (-)-4'-O-methylpreglabridin.[19]

VI. Conclusion

Two compounds were isolated and identified. LA-1-21-F2 was newly discovered while LA-1-21-F2 were identified to be identical to a previously discovered compound, JC-1-13-F2. Further purification of fractions collected from *Sephadex LH-20 chromatography,* followed by further NMR spectroscopic characterization is needed. The determination of absolute configuration of LA-1-21-F2 will be done using electronic circular dichroism spectroscopy (ECD). In vitro testing toward the glioblastoma cells will be performed for the pure compound, LA-1-21-F2, and all other pure isolates.

VII. Acknowledgements

I would like to thank Dr. Gil Belofsky for leading me through this project and Dr. JoAnn Peters for helping me organize my thesis for the William O. Douglas Honor College. Thanks to Ms. Cindy White, our NMR technician for her assistance in training and in the acquisition of our NMR spectroscopic data. I am grateful for a grant from the National Institutes of Health, National Center for Complementary and Integrative Health (R15AT008546) under which the collection of *D. jamesii* was originated. Thanks to Dr. David Giblin of the University of Washington's Burke Herbarium for authentication of *D. jamesii* samples, and thanks to Ms. Valentyna and Mr. Luther Belofsky for assistance in plant collection.

VIII. References

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