Purification and biological activity of oregonin, a novel bioactive diarylheptanoid found in the leaves and bark of *Alnus rubra* (red alder)

by

Carmen Lea
Bachelor of Science, University of Victoria, 2017

A Thesis Submitted in Partial Fulfillment
of the Requirements for the Degree of

MASTER OF SCIENCE

in the Department of Biology

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University of Victoria

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Supervisory Committee

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Supervisory Committee

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Abstract

Red alder (*Alnus rubra*) is the most commercially important hardwood tree species in the Pacific Northwest and has a long history of traditional medicinal use as a source of fungicide and insecticide. Chemical analysis has shown that the diarylheptanoid oregonin ((5S)-1,7-bis(3,4-dihydroxyphenyl)-5-(β-D-xylopyranosyloxy)-heptan-3-one) is the dominant phytochemical contributing to medicinal activity. It was recently discovered that high oregonin concentration in alder leaves is associated with enhanced resistance to western tent caterpillar (*Malacosoma californicum*), a leaf eating lepidopteran herbivore; however, oregonin has never been directly tested on insects, or red alder-associated fungal species. In this thesis, a novel purification method was developed for the preparative extraction of oregonin from red alder leaf and bark material to directly test its biological activity. A battery of insect feeding and toxicity bioassays were carried out with several tree-defoliating caterpillars, and fungal inhibition was tested against a range of plant-associated fungal species, including several alder-associated species. This research represents the first evaluation of oregonin biological activity on insects, plant-associated fungi of the phyla Basidiomycota, and fungal-like pathogens of the phyla Oomycota. Oregonin exhibited promising insect feeding deterrent activity against generalist lepidopteran pests, including cabbage loopers (*Trichoplusia ni)*, white-marked tussock moths (*Orgyia leucostigma*), and fall webworm (*Hyphantria cunea*) at similar concentrations shown to reduce western tent caterpillar herbivory in alder leaf bioassays. The results suggest that oregonin concentration has potential for selection as a breeding trait in managed populations of red alder to improve host resistance to leaf-eating pests.

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List of Abbreviations

[α]	specific rotation
°C	degrees Celsius
Å	ångström
A	absorbance
AE	aqueous extract
ANOVA	analysis of variance
ATR	attenuated total reflectance
AU	absorbance units
β	beta
BC	British Columbia
c	concentration
C	control
CFS	Canadian Forest Service
CI	confidence interval
COSY	^{1}H-^{1}H correlation spectroscopy
D_2O	deuterium oxide
DAD	diode array
DMSO	dimethyl sulfoxide
DW	dry weight
ESI	electrospray ionization
FD	feeding deterrence
FD_{50}	concentration causing 50% feeding deterrence
FDI	feeding deterrence index
g	gram
h	hours
HCl	hydrochloric acid
HMBC	heteronuclear multiple-bond correlation
HPLC	high performance liquid chromatography
HSQC	heteronuclear single-quantum coherence spectroscopy

Hz	hertz
IPS	insect production services
IR	infrared
J	Joule
L	litre
LC-MS	liquid chromatography-mass spectroscopy
M	molar concentration
M/Z	mass-to-charge ratio
mAU	milli absorbance units
MeOH	methanol
min	minute
mm	millimeter
mol	mole
MS	mass spectrometry
n	sample size of subgroup
N	total sample size
nm	nanometer
NMR	nuclear magnetic resonance
OH	Ohio
ON	Ontario
ρ-value	significance level
per. com.	personal communication
ppm	parts per million
PPO	polyphenol oxidase
R^2	coefficient of determination
ROS	reactive oxygen species
RPM	revolutions per minute
RSD	relative standard deviation
RT	retention time
s	seconds
SD	standard deviation

SE	standard error
T=	time
T	treatment
μg	microgram
μL	microliter
μM	micromolar
μm	micrometer
UHPLC	ultra high performance liquid chromatography
USA	United States of America
UV	ultraviolet
V	voltage
v	volume
VIS	visible
W	West
w/w	weight for weight
xyl	xylose
λ_{max}	maximum wavelength
λ_{min}	minimum wavelength

Acknowledgments

I would like to express my sincere gratitude to my supervisors Dr. C. Peter Constabel and Stephen Bradbury for the opportunity to work on this project. Writing this thesis would not have been possible without their continuous guidance and support.

Thank you to Dr. Paul De La Bastide and Dr. Rachael Scarth for serving on my committee and providing excellent feedback and advice.

A special thank you to Dr. Kennedy Boateng for his outstanding work on red alder defensive chemistry, and to Dr. Christin Fellenberg for the preliminary determination of oregonin content in red alder leaves, that together provided a starting point for this research. I would also like to acknowledge Dr. Ori Granot from the Department of Chemistry for his guidance and assistance, and Brad Binges from the Centre of Forest Biology, Chris Mackenzie and Tara Zerbe for greenhouse support.

I am grateful to Dr. Chakri Simhadri, and Dr. Jeremy Wulff from the Department of Chemistry for carrying out oregonin characterization and analysis, as well as providing me with technical advice.

I would like to thank Dr. Jacqueline Bede from McGill University and Dr. Murray Isman from University of British Columbia for providing advice on insect rearing and bioassays.

I am also thankful for the past and present members of the Constabel lab, and my colleagues at Ecosafe Natural Products Inc., for their knowledge, encouragement and friendship.

Lastly, I would like to thank my friends, family and partner Mathew Floesser for their love, encouragement, and emotional support when I needed it most.

Dedication

In memory of Roderick Stephen Bradbury, whose mentorship and encouragement laid the foundation for this work. Rod's spirit of adventure with regard to research will continue to inspire me throughout my academic career.

Contributions

The description of the procedure for oregonin characterization by UV and NMR spectroscopy, as well as the accompanying results and discussion (Chapter 2, Sections 2.2.7-2.2.8, and 2.3.3), were written by Dr. Chakravarthi Simhadri, and Dr. Jeremy Wulff from the Department of Chemistry at the University of Victoria for publication in the journal Phytochemical Analysis.

Plant pathogenic and parasitic microbial species were provided by Dr. Paul de la Bastide and Dr. Will Hintz, at the Centre for Forest Biology, University of Victoria. Taxonomic identification of fungal isolates was confirmed by Dr. Paul de la Bastide using sequence analysis of the ITS-rDNA region and comparison to reference sequences available in the NCBI GenBank DNA database (Chapter 4, Section 4.2.3).

An independent lab study was conducted by Dr. Rajagopal Subramaniam (Ottawa Research and Development Centre, at Agri-Food Canada) to test for oregonin fungicidal activity on *Fusarium graminearum* (Chapter 4, section 4.2.5).

Chapter 1. General Introduction

1.1 Red alder (*Alnus rubra* Bong.)

Red alder (*Alnus rubra* Bong.) is the most abundant, and economically important, hardwood tree species in the Pacific Northwest (Harrington, 2006). There are ten species of *Alnus* native to North America, but red alder is the largest and the only one to reach commercial size (Niemiec et al., 1995). Since the 1980s, red alder logs have been rapidly increasing in commercial value (Harrington, 2006). Unlike many slow growing conifers, red alder produces high yields of woody biomass in a short period of time and is regenerated relatively easily (Harrington, 2006). In addition, red alder improves soil fertility due to a symbiotic association with the nitrogen fixing actinomycete *Frankia* (Binkley et al., 1994; Harrington, 2006). This association has made red alder one of the most important nitrogen sources in riparian ecosystems and an integral part of forest succession and regeneration. A growing number of studies also point to potential advantages of growing red alder as a companion species to Douglas fir and Sitka spruce in areas of low nutrient bioavailability (Grotta et al., 2004; Hanley et al., 2006; Radosevich et al., 2006; Deal et al., 2017). As more is learned about the importance of alder in coastal ecosystems, interest in management has increased accordingly. Planting of red alder is expected to exceed a million seedlings per annum over the next few years (Xie, 2008).

The anticipated increase in seedling plantation and high commercial log value has led to renewed interest in red alder improvement programs. To date, the majority of research is focused on wood quality and growth potential, and there are only limited data on phytochemical resistance to insects or fungi (Grotta et al., 2004; Xie, 2008; Porter et al., 2013). Although insect damage is generally considered to have low impact on red alder productivity, defoliating insect pests such as tent caterpillars, alder flea beetle, and saw flies can cause substantial growth reduction (Harrington, 2006). Furthermore, weakened and aging alder stands become increasingly susceptible to alder bark beetle (*Alniphagus aspericollis*), a boring insect that selectively colonizes trees of impaired vigor (Borden, 1969). Although infestations are usually endemic, alder bark beetle can attack healthy trees, resulting in mass mortality events at high population density (Borden, 1969). The number of potential insect and fungal pests affecting red alder is likely to increase in future years due to the expansion of alder silviculture aiming to increase seedling volume and suitable habitat. In addition, climate change has allowed for the range expansion and population increases of invasive tree-feeding pests (Suckling et al., 2017).

The determination of potential mechanisms of red alder pest resistance is therefore becoming increasingly important in species management.

Several groups of secondary metabolites have been identified and isolated from various *Alnus* species. When compared to many other shrubs and broadleaf tree species in the Pacific Northwest, red alder leaf and bark material has been found to contain considerably elevated levels of phenolic secondary metabolites (McArthur et al., 1993), which are thought to contribute the majority of bioactivity (Sati et al., 2011). Only a few studies, however, have investigated concentrations of phenolics in alder in the context of pest resistance, and have largely focused on how these chemicals may contribute to a reduction in herbivory by browsing ruminants (McArthur et al., 1993; González-Hernández et al., 2000). To date, no studies have investigated red alder phenolics in the context of insect resistance, or to red alder-associated fungal plant pathogens.

1.2 Phenolic metabolites in plant pest resistance

In addition to primary metabolites, which are any metabolites essential for primary growth and development, plants have evolved a broad spectrum of substances called secondary metabolites, which have no direct metabolic functions, but are important in an ecological context (Heldt, 2005). Many of these metabolites are thought to protect against damage from insects, fungi, and other pests; however, the complete functional spectrum of the numerous chemicals described to date is not fully understood (Vickery and Vickery, 1981). The most widespread groups of secondary metabolites, ubiquitous in plants, are the phenylpropanoids. All phenylpropanoids are biosynthesized starting with either phenylalanine or tyrosine through the phenylpropanoid biosynthetic pathway, and comprise subgroups such as the flavonoids, stilbenes, diarylheptanoids, coumarins, and tannins (Heldt, 2005). Because all phenylpropanoids are derived from amino acids characterized by the presence of an aromatic ring, these subgroups are known collectively as phenolics. In biosynthesis of many compounds, for example the stilbenes, flavonoids, and tannins, an additional aromatic ring is generated by the chalcone synthase or stilbene synthase enzyme from three molecules of malonyl-CoA (Heldt, 2005). Phenolics may be further polymerized, glycosylated, hydroxylated or otherwise derivatized to create a huge diversity of structures, which perform a wide range of functions in the plant environment.

Many phenolic secondary metabolites are implicated in plant defense against herbivorous insects and microorganisms (War et al., 2012). The tannins are a widespread group of

polyphenolic compounds able to bind and precipitate proteins. Tannins can be divided into two classes: the hydrolysable tannins derived from galloyl glucose, and flavonoid polymers known as condensed tannins (Boeckler et al., 2014). Many tannins are known to have direct negative effects on herbivores as feeding deterrents, inhibitors of digestion, and pro-or anti-oxidants, although the effects seem to be highly dependent on the target organism (Barbehenn and Peter Constabel, 2011). A group of phenylpropanoid derivatives strongly linked to herbivore defense are the coumarins, which have a distinctive phenolic structure consisting of a benzene ring linked to a pyrone ring (Stringlis et al., 2019). Coumarins are thought to possess an abundance of biological activities including insecticidal, antimicrobial, and antifeedant properties (Berenbaum and Zangerl, 2006; Al-Majedy et al., 2016; Stringlis et al., 2019). Phenolic metabolites may also be conjugated to sugar units to form phenolic glycosides. A subset of phenolic glycosides known as the salicinoids, found the family Salicaceae, are characterized by the glycosylation and esterification of salicyl alcohol. Salicinoids are thought to act as feeding and oviposition deterrents to generalist insects, and reduce insect performance (Lindroth and Pajutee, 1987; Boeckler et al., 2011; Boeckler et al., 2016). Diarylheptanoids, a small but interesting subgroup of phenylpropanoid derivatives, described in more detail in the following section, are thought to be the primary bioactive constituent of plants of the genus *Alnus* and *Betula* (Ren et al., 2017), although their ecological function is largely unknown.

The toxicity of phenolics can result from several modes of action, and also depends on the physiochemical conditions in which they occur (Appel and Martin, 1990). Under certain conditions, phenolics may participate in molecular binding to nutrients, lipids, metals and carbohydrates inhibiting herbivore digestion. Phenolics may also be oxidized or hydrolyzed to form superoxide anion radicals, hydroxyl radicals, peroxidases and quinones, which may covalently bind with proteins, or result in the destabilization of membranes and breakage of DNA strands (Appel and Martin, 1990). The biological activity of phenolics, therefore, may be potentiated by enzymes, such as oxidases or hydrolases, and may depend on the redox conditions of the plant environment or digestive the tract of herbivores. In the acidic to neutral pH of most organisms, phenolics may bind to and precipitate dietary proteins, resulting in digestive inhibition. In the alkaline gut condition (> pH 9) of many lepidopteran insects, hydrogen bond formation may be inhibited, and oxidative reactions potentiated (Appel, 1993; Hemming and Lindroth, 2000; Barbehenn et al., 2008).

1.3 Diarylheptanoids

Several members of the diarylheptanoid family of phytochemicals have been investigated due to their wide range of biological properties, including antioxidant, fungicidal, and insecticidal activities (Lee et al., 2005; Novaković et al., 2015; De Souza Tavares et al., 2016; Dong et al., 2017). Since 1815, when curcumin was discovered in the rhizomes of turmeric (*Curcuma longa*), almost 500 additional diarylheptanoids have been identified, and despite their structural diversity, have almost all have been isolated from a small number of plants comprising the genera *Zingiber, Curcuma, Alpinia, Alnus, Betula,* and *Myrica* (Lv and She, 2012; Kunnumakkara et al., 2017). Members of the diarylheptanoid family occur most frequently in angiosperms, and can typically be found in the stem and bark of trees and shrubs (Novaković et al., 2015), but have also been discovered in the extracts of leaves and rhizomes (Yang et al., 2011).

In ginger and turmeric, diarylheptanoid biosynthesis has been partially elucidated and shown to branch from the phenylpropanoid biosynthetic pathway (Koo et al., 2013). Effectively no work has been done to disentangle diarylheptanoid biosynthetic pathways in other species, including red alder. All diarylheptanoids are characterized by a 1,7-diphenylheptane skeleton, and may be linear or cyclic in form (Lv and She, 2012; Koo et al., 2013). Cyclic diarylheptanoids are further classified as diarylether-type or biaryl-type cyclic diarylheptanoids depending on the nature of the connection between the two aromatic groups (Figure 1.1). The 7-carbon chain in any category may be saturated or can have up to three double bonds, while the phenolic rings are frequently hydroxylated, methoxylated, acetylated or glycosylated. Other structural variations may occur along the 7-carbon skeleton, including substitutions of the C-3 and/or C-5 carbons with methyl, acetyl, sulfate or glycosyl functional groups (Alberti et al., 2018). Patterns of diarylheptanoid saturation and desaturation have been observed and seem to depend on the species from which they are isolated. For example, linear diarylheptanoids are more abundant in *Zingiber, Alpinia, Alnus,* and *Betula* while cyclic diarylheptanoids are more frequently distributed in *Myrica, Carpinus, Juglans,* and *Acer* (Alberti et al., 2018).

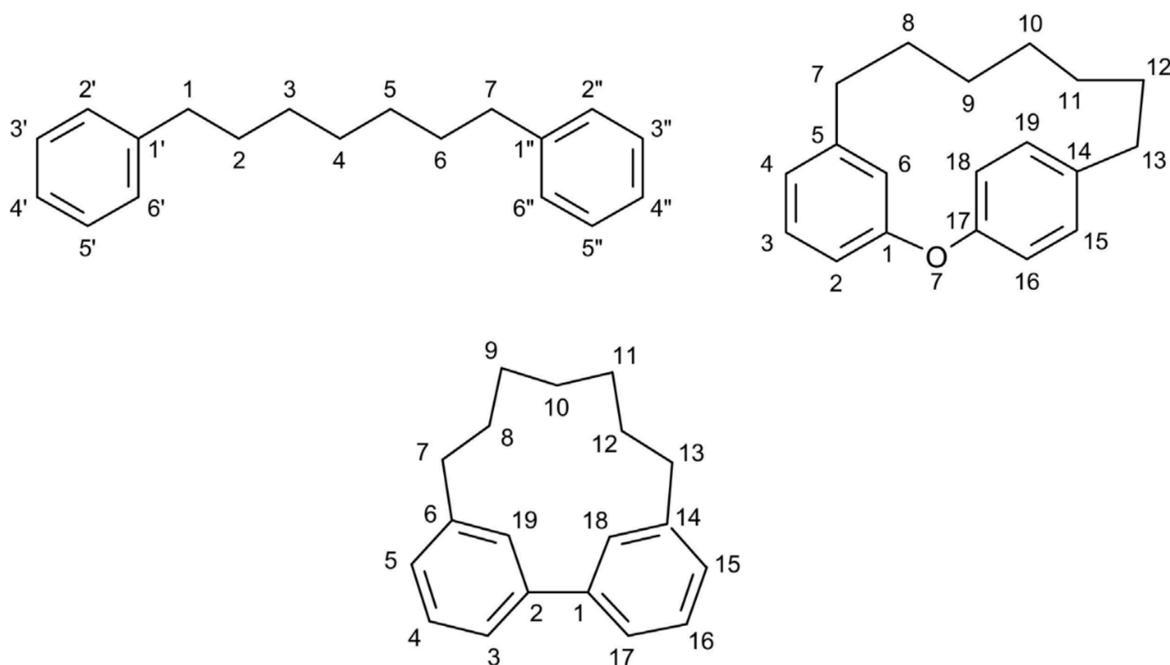


Figure 1.1 Chemical structure (left to right, top to bottom) of linear, diarylether-type, or biaryl-type cyclic diarylheptanoids (Alberti et al., 2018)

Extracts from diarylheptanoid containing plants have a long history of traditional use in medicine and hygiene. Turmeric, for example, is used as a herbal medicine for indigestion, inflammation and infection, and contains high concentrations of the diarylheptanoid curcumin (Bhowmik et al., 2011)(see Figure 1.2 for structures of described diarylheptanoids). Curcumin has also received attention in modern medical literature, due to the discovery of its anti-inflammatory, neuroprotective and anti-cancer properties (Esatbeyoglu et al., 2012). The diarylheptanoid platyphylloside is thought to be the major bioactive constituent in extracts and tinctures made from silver birch bark (*Betula platyphylla*), which are used in traditional Chinese medicine for their anti-inflammatory and healing properties (Lee et al., 2019). The vinegar extract of red alder bark is used in a similar manner to treat head-lice, scabies, and mouth or throat infections. Indigenous healers along the Pacific Northwest of North America use red alder bark for medicinal teas to treat indigestion and fever, and apply a paste of red alder leaves to cuts, burns, and other wounds to expedite healing (Sati et al., 2011; Ren et al., 2017). Biochemical profiling has suggested that the diarylheptanoid oregonin may be the dominant bioactive constituent in red alder tissue; however, despite its relatively high abundance in red alder leaves and bark (4-9%), its ecological function has not been established (González-Hernández et al., 2000; Sati et al., 2011; Telysheva et al., 2011)

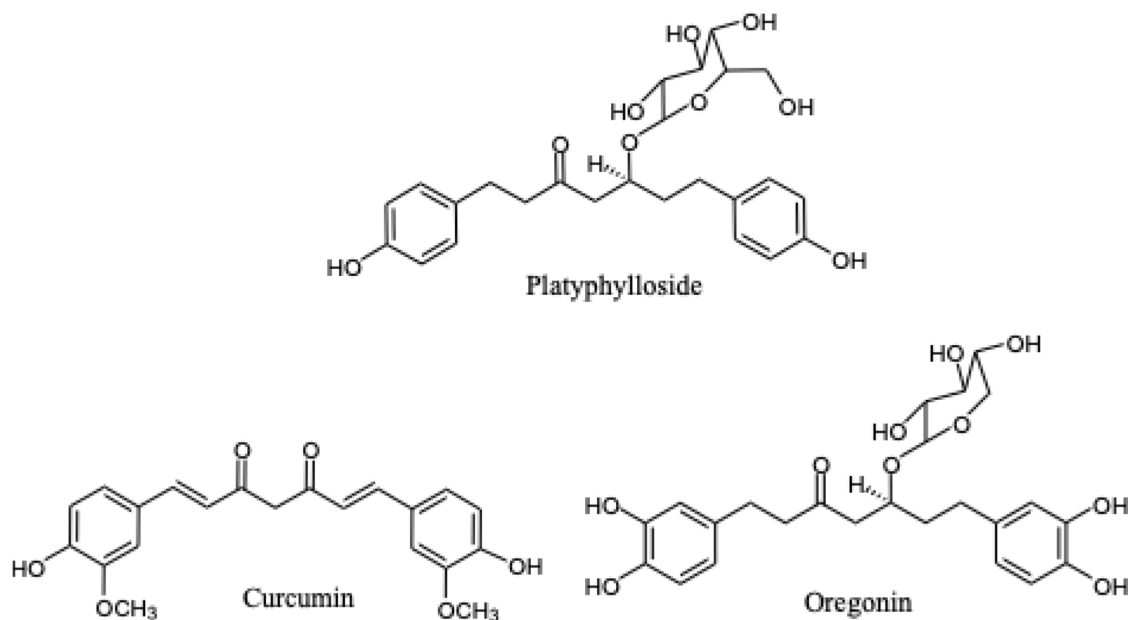


Figure 1.2 Chemical structures of described diarylheptanoids

1.4 Oregonin

Red alder gets its common name due to the staining phenomenon of alder bark upon exposure to oxygen (Figure 1.3). Interest in red alder bark staining led to the discovery of the diarylheptanoid xyloside oregonin in 1974, which forms red-orange products on oxidation and hydrolysis (Karchesy et al., 1974). Since its discovery in red alder, oregonin has been characterized in several other *Alnus* species, and has generated biomedical interest due to the discovery of its strong potential antimicrobial and antifungal properties (Rashed et al., 2014; Abedini et al., 2016). Previous research in the Constabel lab has suggested that high oregonin concentration in alder leaves may be associated with enhanced resistance of alder to western tent caterpillar, a tree-feeding lepidopteran insect (Boateng, 2019). Together with the aforementioned work on the bioactive potential of diarylheptanoids and other polyphenolic secondary metabolites, as well as the medicinal history of red alder as a traditional insecticide, miticide and antibiotic, lead to my hypothesis that oregonin may act as a toxin or deterrent to herbivorous insects and fungal plant pathogens.

Figure 1.3 Staining of red alder bark and phloem surrounding *Alniphagus aspericollis* (alder bark beetle) entrance holes and galleries. The coloration results from oxidation of the diarylheptanoid compound oregonin. Bark has been peeled back next to the entrance hole.

1.5 Research objectives and significance

The main hypothesis underlying this thesis is that oregonin functions to protect red alder leaves and bark from insect herbivores and/or fungal attack. The overarching strategy was to test these biological activities directly using oregonin purified from red alder leaves and bark.

Specific objectives of the research were as follows:

i) To develop an efficient method for purifying oregonin from red alder leaves and bark (*Alnus rubra*)

ii) To test oregonin for antiherbivore and insecticidal activity

iii) To determine if oxidation or hydrolysis of oregonin enhances its antifeedant activity

iv) To characterize the seasonal pattern of oregonin concentrations in red alder bark and leaves in field-grown trees

v) To test purified oregonin for inhibition of plant-associated fungi, including several alder pathogens

In Chapter Two, the development of a novel method of oregonin extraction from red alder foliage and bark, which combines aqueous extraction, spray drying, and flash chromatography is described. Purity of extracted oregonin was validated by HPLC and MS, and structural identity was confirmed using NMR and UV in collaboration with Chakravarthi Simhadri and Jeremy Wulff in the Chemistry department at the University of Victoria. The described extraction and purification methods represent the first examination of the effects of spray drying on diarylheptanoids, as well as the most complete characterization of oregonin to date. In Chapter Three, oregonin was tested for anti-insect activity against a range of generalist tree-feeding lepidopterans, including several red-alder feeding species. In addition, oregonin oxidation and hydrolysis products were generated and their activity as feeding deterrents compared to that of oregonin. As part of this chapter, the seasonal variation in oregonin concentration in various alder tissues and developmental stages is examined. Oregonin did not exhibit any toxic effects against the species tested; however, the results suggested that biological concentrations found alder leaf tissue early in the spring is sufficient to deter leaf-eating herbivores. In Chapter Four, the antifungal activity of oregonin was tested against a variety of plant-associated or plant pathogenic fungi of the phyla Ascomycota and Basidiomycota, as well as against a fungal-like pathogen in the phylum Oomycota. Contrary to several published works that have reported antifungal activity of oregonin against human pathogens (Saxena et al., 1995; Novaković et al., 2015; Abedini et al., 2016), no activity against any of the plant-associated fungal species tested was observed. In summary, this research represents the first examination of the biological activity of purified oregonin, or its potential products, against leaf-eating insects and plant-associated microorganisms.

Chapter 2. Efficient purification of the diarylheptanoid oregonin from red alder (*Alnus rubra*) leaves and bark combining aqueous extraction, spray drying and flash-chromatography

Abstract

The diarylheptanoid xyloside oregonin ((5S)-1,7-bis(3,4-dihydroxyphenyl)-5-(β-D-xylopyranosyloxy)-heptan-3-one) has significant medicinal potential and is found at high concentration in leaves and bark of red alder (*Alnus rubra*). Inexpensive and easily scaled methods for the extraction and purification of oregonin from timber by-products, however, still need to be established. In this chapter, a method combining aqueous extraction with spray drying of red alder extract into a powder was developed, thus reducing the need for organic solvents used in traditional Soxhlet extraction or in solvent partitioning. Crude spray-dried alder extract was comprised of an average of 9% the diarylheptanoid compound oregonin. Less than 10% thermal degradation of oregonin was observed using extraction temperatures between 25-50 ºC, followed by spray drying. Flash chromatography was utilized to purify oregonin from crude spray-dried alder extract. The developed method was robust, repeatable, and yielded purified oregonin of greater than >95% purity (average of 95.8%). The structure of purified oregonin was validated using HPLC, MS, UV, and NMR. This analysis represents the most complete NMR characterization of oregonin reported to date.

2.1 Introduction

Red alder is the most widely managed hardwood tree species in the Pacific Northwest of North America, in part due its ability to fix soil nitrogen and thrive on sites with nutrient-poor soils (Harrington et al., 1994; Hibbs and DeBell, 1994; Harrington, 2006; Edmonds and Tuttle, 2010). The majority of red alder is used for lumber and veneer manufactured from sawlogs greater than six inches in diameter (Niemiec et al., 1995). In a typical rotation, however, a large proportion of alder biomass is lost as residues in the form of bark, twigs, leaves, and smaller stems, which are left in the forest, burned, or used for fuel, compost, and insulation (Niemiec et al., 1995; Harrington, 2006; Pásztory et al., 2016).

Although red alder leaves and bark have little commercial value as forest products, extracts prepared from red alder residues have a long history of traditional medicinal use as a treatment for fever, haemorrhages, and burn injuries (Ren et al., 2017). More recently, studies have demonstrated that numerous bioactive natural phytochemicals isolated from alder bark and leaves have antioxidative, antimicrobial, and anti-cancer properties, suggesting that red alder may have pharmaceutical potential (Lee et al., 2005; Yang et al., 2011; Novaković et al., 2015; Dahija et al., 2016; Dong et al., 2017). The dominant bioactive constituents of alder extracts are the diarylheptanoids, with oregonin ((5S)-1,7-bis(3,4-dihydroxyphenyl)-5-(β-D-xylopyranosyloxy)-heptan-3-one) being most abundant (Figure 2.1) (Sati et al., 2011). Oregonin is found at high concentrations (4-9%) in red alder leaf and bark tissue. Forestry alder waste products are therefore a major abundant potential source of this compound (González-Hernández et al., 2000; Telysheva et al., 2011); however, its commercial extraction and purification would require methods that are easily scaled for the large-scale processing of forestry residues.

To date, most reported extraction and purification methods for oregonin and other diarylheptanoids use Soxhlet extraction under reflux or maceration into organic solvents such as chloroform, dichloromethane, and methanol (Alberti et al., 2018). Extraction is usually followed by an additional defatting step by solvent partitioning into hexane or chloroform due to the solubility of membrane lipids and epicuticular wax into organic solvents. By contrast, direct aqueous extraction eliminates waxes and lipids from extracts, as these are sparingly soluble in water (Myung et al., 2013). Extraction methods involving liquid-liquid partition are expensive because they require large volumes of organic solvent and are not easily scaled to the quantities needed for large-scale processing of timber by-products. Moreover, solvent evaporation requires ventilation devices and specialized respiratory equipment in an industrial setting.

Oregonin is easily extracted by water; however, Klarić et al., (2017) showed that the compound may be prone to thermal degradation at temperatures above 103 °C (Klarić et al., 2017), and thus the removal of water after aqueous extraction is a technical challenge. Desiccation of thermally sensitive aqueous extraction products is typically performed using lyophilization or spray drying (Tan et al., 2014). Lyophilization of aqueous alder extract has been carried out successfully, but production costs are extremely high in comparison to spray drying (Liu et al., 2011; Klarić et al., 2017). Here, we report the application of aqueous extraction and spray drying to oregonin purification as a new alternative method to extraction under reflux. Our method greatly reduces the need for organic solvents. Purification of oregonin and separation from other water-soluble components was accomplished using flash chromatography. We also report a complete NMR characterization of oregonin.

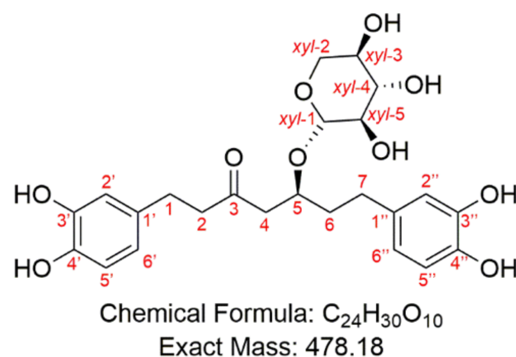


Figure 2.1 Chemical structure, formula and exact mass of oregonin ((5S)-1,7-bis(3,4-dihydroxyphenyl)-5-(β-D-xylopyranosyloxy)-heptan-3-one). The numbering of atoms in oregonin structure (shown in red) are used for spectral assignments (see Table 2.4).

2.2 Materials and Methods

2.2.1 Plant material and reagents

Leaf and bark material were harvested from 7-year-old red alder trees on the University of Victoria Campus (48.4634° N, 123.3117° W), established in 2017 from clonal cuttings of red alder accessions at the Cowichan Lake Research Station, B.C. Ministry of Forests, Lands, Natural Resource Operations and Rural Development. Harvested tissues were stored at 4 °C prior to processing. The analytical oregonin standard (>95%) was purchased from Sigma-Aldrich (Oakville, ON, Canada). HPLC grade solvents used for analysis were obtained from ThermoFisher Scientific (Ottawa, ON, Canada). Water used in HPLC and flash chromatography

experiments was deionized using a ThermoFisher Scientific Barnstead MegaPure MP-1 water distillation system.

2.2.2 Aqueous extraction

Alder leaves, twigs and bark (50 g) were macerated with water (150 mL) using a blender at high speed for 10 min at temperatures ranging from 20-65 °C. The resulting slurry was agitated with moderate stirring at ambient laboratory temperature (22 °C) for an additional 30 min and filtered through cotton batting to remove insoluble plant residues. The filtered extract was centrifuged at 1400 RPM at 20 °C for 12 min. The resulting supernatants were semi-transparent, orange-tan liquids. Supernatants from each extraction were assayed for oregonin concentration by HPLC as described below and set aside for spray drying. Total dissolved solids content of the aqueous extract was measured using a Mettler Toledo HE53 Moisture Balance.

2.2.3 Spray drying experiments

Spray drying experiments were piloted using a benchtop Buchi Mini-Spray Dryer B290 (Buchi, Switzerland), equipped with a 0.7 mm diameter needle and nozzle tip, 1.5 mm diameter nozzle screw cap, and high-performance cyclone. The spray dryer was equilibrated with distilled water for 15 min prior to each experiment. Spray dryer settings were adjusted qualitatively to reduce the adhesion of particles to the spray dryer chamber walls, reduce the outlet temperature, and maximize particle retention in the collection vessel. Final optimized parameters were as follows: inlet air temperature 190 °C, outlet air temperature 90 °C, aspirator 80% of maximum air flow, flow rate of liquid feed solution 20 mL/min. Process yield was defined as the percentage of total dissolved solids in the liquid feed collected as dry powder in the drying chamber. Process yield was calculated by determining the ratio between dissolved solids in the liquid feed after extraction at each temperature and the weight of powder collected in the drying chamber after each spray drying experiment. Since only degradation would change the oregonin composition of the extracted solids after extraction, we assumed that any reduction in oregonin composition of the dissolved solids during the spray drying process was due to thermal degradation. Thermal degradation was calculated by determining the ratio between the oregonin content of the dissolved solids in the liquid feed, and oregonin composition of the spray-dried powder.

2.2.4 Flash chromatography separation

A 10% solution (w/w) of spray-dried alder extract was prepared in distilled water. Batch injections (15 mL) were subjected to reverse phase flash chromatography on C18, Agela Technologies Claricep™ flash columns (120 g, particle size: 20-25 µm, pore size: 100Å), using a 30 min gradient program of solvent A (acetonitrile) and solvent B (distilled water): 15% solvent A was held for two min, linearly increased to 30% over 16 min, linearly increased to 100% over 3 min, held for 4 min, linearly decreased to 15% for 3 min, and held for 2 min. Flow rate was maintained at 40 mL/min. Fractions to be collected were identified by monitoring eluting analytes at 280 nm. Each column was equilibrated with water: acetonitrile (85: 15 % v/v) for at least three column volumes prior to separation. Purity of each flash chromatography fraction was estimated by HPLC analysis (see section 2.2.5 below) and calculating percent total peak area at 280 nm. Fractions containing >85% oregonin by peak area were pooled, and spray-dried under the same conditions as described above (Section 2.2.3) to yield a brown, amorphous solid. Due to the small volume of pooled fractions, to reduce loss to glassware, the spray drying collection vessel was rinsed with a small quantity of methanol and dried under vacuum. Percent recovery was estimated by dividing the peak area (mAU) of oregonin in each fraction over the total peak area of all peaks in each fraction. To validate our estimated purity results by peak area, the fractions containing oregonin at the greatest concentration from each experiment was pooled, dried and assayed by HPLC against analytical standards.

2.2.5 HPLC analysis and quantification

For estimation of purity, fractions obtained from flash chromatography experiments were injected directly onto a Thermo Scientific™ UltiMate 3000 HPLC system equipped with a quaternary pump, auto-sampler, degasser, column oven, and photodiode array detection (DAD). Instrument control, data acquisition, and evaluation were achieved using Chromeleon™ chromatography software version 7.2 SR4. Separation was performed on a Kinetex® reverse phase C18 column (4.6 x 150 mm, particle size: 2.6 µm, pore size: 100Å). Elution was carried out using a 15 min mobile phase gradient of acetonitrile 0.1% formic acid (solvent A) and 0.1% formic acid (solvent B), a flow rate of 1.0 mL/min, injection volume of 5 µL, and oven temperature of 30 °C. The elution gradient was described in Table 2.1. Separation was monitored at 280 nm. The final purified product was quantified against analytical standards and further characterized by UV and NMR as described below. A stock standard solution was prepared by diluting a known quantity of analytical grade oregonin into HPLC grade methanol and stored in

the dark at –20 °C. A standard curve was prepared by diluting the stock standard solution to five concentrations in the range of 92-740 µg/mL.

Table 2.1 Reverse-phase HPLC mobile phase gradient program for oregonin analysis

Time (min)	Solvent A (0.1% Formic Acid Acetonitrile)	Solvent B (0.1% Formic Acid dH_2O)
0.00	15	85
1.0	15	85
9.0	32	68
10.5	100	0
12.5	100	0
14.0	15	85

2.2.6 Repeatability and robustness

The flash chromatography method was evaluated for robustness by increasing the volume of particles in the flash column from 80 g to 120 g and adjusting the injection volume and flow rate proportionately. Injection volume and flow rate for each 80 g column was set to 10 mL and 40 mL/min, whereas injection volume and flow rate for each 120 g column was set to 15 mL and 60 mL/min, respectively. Gradient conditions were programmed as in Section 2.4. The same method was evaluated for repeatability by analyzing the percent relative standard deviation (% RSD) of the oregonin recovery in five separate separations, using five separate pre-packaged C18 columns (Phenomenex). All parameters such as flow rate, gradient, sample volume, and detection wavelength were kept the same. Each fraction was assayed by the HPLC method gradient described in Section 2.5. Recovery was estimated by dividing the peak area (mAU at 280 nm) of oregonin in each fraction, over total peak area of all peaks in each fraction. We assumed that no oregonin was lost to the column, and that all impurities were visible by UV detection at 280 nm. To validate the estimates of purity obtained by analysis of peak area, fractions containing oregonin >95% peak area were dried and quantified by HPLC using the analytical standard.

2.2.7 Analytical instrumentation

Electronic spectra (UV/Vis) were collected by Dr. Chakravarthi Simhadri, and Dr. Jeremy Wulff from the Department of Chemistry at the University of Victoria on a

SpectraMax® M5 multichannel plate reader. Optical rotations were measured at the sodium D line (589 nm) on a Rudolph Research Analytical Autopol® III Automatic Polarimeter. All NMR spectra were recorded at ambient temperature using a Bruker AVANCE Neo 500 spectrometer. Chemical shifts in ^{1}H and ^{13}C NMR spectra were reported in parts per million (ppm) and were referenced to residual protons of NMR solvent used relative to tetramethylsilane. ^{1}H NMR is presented in the format: chemical shift (multiplicity, coupling constant (*J* in Hz), integration). IR spectra were recorded using a Perkin-Elmer ATR spectrometer. IR wave numbers (υ) are reported in cm^{-1}.

2.2.8 Oregonin analytical data

Experimental $[\alpha]_D^{20}$: −17.2º (*c* = 1.0, acetone), reported value $[\alpha]_D^{20}$ −17.5º (*c* = 1.0, acetone) (Lee et al., 2000). Experimental $[\alpha]_D^{20}$: −15.0 º (c = 1.3, methanol). In each case, the sample mass used for the specific rotation measurement was adjusted to account for a residual 0.5 molar equivalents of methanol remaining following vacuum drying.

^{1}H NMR (500.27 MHz, DMSO-*d*$_6$ + 10 μL D_2O) δ 8.70 (s, 4H), 6.60 (d, *J* = 8.0 Hz, 2H), 6.55 (d, *J* = 2.1 Hz, 1H), 6.53 (d, *J* = 2.1 Hz, 1H), 6.40 (m, 2H), 4.98–4.91 (m, 3H), 4.11 (d, *J* = 7.6 Hz, 1H), 3.98 (p, *J* = 6.1 Hz, 1H), 3.69 (dd, *J* = 11.3, 5.4 Hz, 1H), 3.30–3.23 (m, 1H), 3.08 (t, *J* = 8.9 Hz, 1H), 3.01 (t, *J* = 10.8 Hz, 1H), 2.90 (t, *J* = 8.3 Hz, 1H), 2.74 (dd, *J* = 16.4, 6.2 Hz, 1H), 2.67 (t, *J* = 7.5 Hz, 2H), 2.61–2.52 (m, 3H), 2.48–2.41 (m, 1H), 2.40–2.32 (m, 1H), 1.68–1.55 (m, 2H).

^{13}C NMR (125.8 MHz, DMSO-*d*$_6$) δ 209.28, 145.05, 143.32, 143.14, 133.13, 132.19, 119.05, 119.00, 115.83, 115.61, 102.75, 76.72, 74.49, 73.52, 69.74, 65.90, 47.33, 44.98, 37.27, 30.43, 28.57.

IR (diamond-ATR) υ: 3351, 2926, 1700, 1605, 1519, 1444, 1365, 1283,1195, 1039.

MS (ESI+) m/z calculated for $C_{24}H_{30}O_{10}$: 478.18, found: 478.15; (ESI–) [M–H] calculated for $C_{24}H_{29}O_{10}^{-}$ 477.18, found: 477.25.

2.2.9 Statistical analysis

Pearson's correlation coefficient was used to calculate correlations among oregonin concentration in the liquid feed, oregonin degradation, process yield (dependent variables) and extraction temperature (independent variable) and expressed as R^2 and significance level (ρ < 0.05%). All statistical analysis was performed in R version 3.5.2.

2.3 Results and Discussion

2.3.1 Extraction and spray drying of crude spray-dried powder

Oregonin was extracted from red alder bark and leaves by maceration into water. A range of temperatures (20-65 °C) were used to test for optimal extraction efficiency. Each extract was spray-dried to yield a crude oregonin-containing powder concentrate. Thermal degradation of oregonin after spray drying from each extract was compared in order to determine the optimal aqueous extraction temperature.

Spray drying settings were adjusted to improve retention of particles in the collection vessel and reduce particle deposition on the drying chamber, while preventing the outlet temperature from exceeding 90 °C. This is the critical parameter in the spray drying process because it is the maximum temperature to which the almost-dry powder is exposed (BÜCHI Labortechnik AG, 2002). We observed less than 10% degradation of oregonin following extraction and concentration by spray drying at extraction temperatures between 10-50 °C (Table 2.2). Degradation more than doubled to 21% at 65 °C. There was a significant positive relationship between oregonin concentration in the aqueous extract and extraction temperature ($R^2 = 0.95$, $\rho = 0.046$); however, this relationship was not observed for process yield ($R^2 = -0.095$, $\rho = 0.9$), or for oregonin concentration in the actual spray-dried powder ($R^2 = 0.4$, $\rho = 0.6$).

Table 2.2 Spray drying process yield and thermal degradation of oregonin from red alder leaves and bark using aqueous extraction temperatures from 20-65 °C.

Extraction Temperature[a] (°C)	Total dissolved solids in aqueous extract[b] (g/100 mL)	Oregonin content of total dissolved solids[c] (%)	Weight of spray-dried powder[d] (g/100mL)	Oregonin content of spray-dried powder[e] (%)	Oregonin thermal degradation[f] (%)	Process yield[g] (%)
20	1.00	9.87	0.68	8.91	9.75	68
35	0.99	10.6	0.74	9.68	8.85	75
50	1.00	10.9	0.69	9.90	8.82	69
65	1.00	12.3	0.69	9.76	20.8	69
Average	1.00	10.9	0.70	9.56	12.1	70

[a]Temperature used to prepare aqueous extract (AE) (N=1)

[b]Total dissolved solids in AE (n=4 measurements)

[c]Oregonin content of total dissolved solids, calculated using HPLC (n=4 injections) and correcting for amount of total dissolved solids in aqueous extract

[d]Weight of spray-dried powder in collection chamber after drying 100 mL aqueous extract (N=1)

[e]Oregonin content of the spray-dried powder measured using HPLC (n=4 injections)

[f]Oregonin thermal degradation throughout the spray drying process calculated as the difference between oregonin content of total dissolved solids, and oregonin content of the spray-dried powder

[g]Spray drying process yield calculated as the difference between weight of spray-dried powder from 100 mL aqueous extract, and total dissolved solids in 100 mL aqueous extract

The crude spray-dried powder was a water soluble, brown, amorphous solid. Particles were slightly tacky and hygroscopic, characteristic of spray-dried powders from plant extracts containing low molecular weight sugars or organic acids (Tan et al., 2011; Muzaffar et al., 2015). The particles tended to agglomerate and become increasingly sticky when stored in an unsealed container. At extraction temperatures above 35 °C, we found that the powder became increasingly stickier and difficult to clean from the glassware. This may have been the result of waxes and sugars being extracted into aqueous solution at higher temperatures. Loss to the glassware due to an increase in stickiness may have caused the reduction in process yield and increase in thermal degradation. The spray-dried powder was stored at –20 °C in an airtight container; however, this hygroscopicity may limit long-term stability, based on the tendency of oregonin to degrade in the presence of water (Chapter 4, Figure 4.3). In further studies, oregonin powder shelf-life will be optimized under various storage conditions to limit degradation. The

process yield for our experiments averaged 70%, which is typical for Buchi bench-top spray dryers (average 50-70%) (BÜCHI Labortechnik AG, 2010). Process yield, tackiness, and hygroscopicity could potentially be improved by addition of encapsulation agents such as maltodextrin, gum arabic or gelatin (Mishra et al., 2014).

Although the liquid feed passes the inlet at a temperature of 190 °C, the evaporation of water is a highly endothermic process and has a significant cooling effect. Once the liquid feed has passed the drying chamber and reaches the outlet, almost all water has evaporated. The relationship between outlet temperature and thermal degradation is also related to aspirator speed and liquid feed rate. Therefore, reducing aspirator speed increases the amount of time particles are exposed to heat, and also increases outlet temperature. Conversely, increasing the feed rate increases drying time and reduces outlet temperature; under such conditions, dry particles are exposed to heat for a shorter time. We found that at aspirator speeds above 80% total instrument capacity, much of the powder was forced out of the collection vessel and lost to the filter. At liquid feed rates above 20 mL/min, liquid droplets began to deposit on the drying chamber and were lost. By maximizing aspirator speed and liquid feed rate, we were able to reduce the outlet temperature as much as possible, the critical parameter in the spray-drying process most likely to cause degradation of thermal-labile ingredients (BÜCHI Labortechnik AG, 2002).

We observed the greatest process yield (75%) and least thermal degradation at an extraction temperature of 35 °C; therefore, this temperature was used to prepare liquid extract for the production of spray-dried alder extract purified in flash chromatography experiments.

2.3.2 Flash chromatography separation and HPLC analysis

Oregonin was further purified from aqueous solution (10%) of spray-dried alder extract using reverse phase flash chromatography, which allows for straightforward scale-up to industrial-scale (kilogram size) flash cartridges. The compound consistently eluted as a single peak between 10 and 15 min (fractions 24-33) over a series of five replicate separations (Supplementary Table 1). The single peak corresponding with oregonin consistently separated into two fractions of high purity: 85.35% (3.11% RSD), and 95.83% (1.00% RSD) respectively. When both fractions were pooled, the average recovery was 90.25% (2.33% RSD). If only the second fraction was used, the recovery was reduced to 43.46% (4.21% RSD) (Table 2.3). The low RSD (less than 5%) indicates excellent method repeatability for both purity and recovery. Our results suggest that using a single column, if purity above 95% is required, recovery would

be reduced by half; however, recoveries greater than 90% can be achieved for a final product of greater than 90% purity.

Table 2.3 Oregonin percent recovery and percent peak area in five separate flash chromatography separations

	Run Number							
	1	2	3	4	5	**Mean**	**STDev**	**% RSD**
Fraction *	**Oregonin Percent Recovery**							
1	48.6	47.2	47.9	44.3	45.9	**46.8**	1.7	3.7
2	43.0	45.0	42.9	45.5	41.0	**43.5**	1.8	4.2
Total	91.57	92.2	90.8	89.9	86.8	**90.3**	2.1	2.3
	Oregonin Purity (Percent Peak Area)							
1	87.2	86.0	86.5	80.7	86.5	**85.4**	2.7	3.1
2	96.1	94.1	96.2	96.5	96.2	**95.8**	1.0	1.0

*Fractions with the greatest oregonin concentration for each separation run

Robustness of the method was examined by increasing the particle mass of the pre-packaged C18 column from 80 to 120 g and proportionately adjusting the injection volume and flow rate. Because oregonin eluted after 10 min from both flash columns, an 8 min delay in fraction collection was used in further experiments. The resulting chromatograms obtained from both separations were similar (Figure 2.2). Oregonin eluted slightly later from the 110 g column than the 80 g column (fractions 12.5-14.0 min vs. 11.5-13.0 min respectively). The slight difference in oregonin retention time may be due to differences in packing of the columns. These results suggest the described method is robust and could be scaled-up to industrial or preparative scales. Together with the efficient aqueous extraction and spray drying steps, these experiments provide a practical method for the large-scale purification of oregonin.

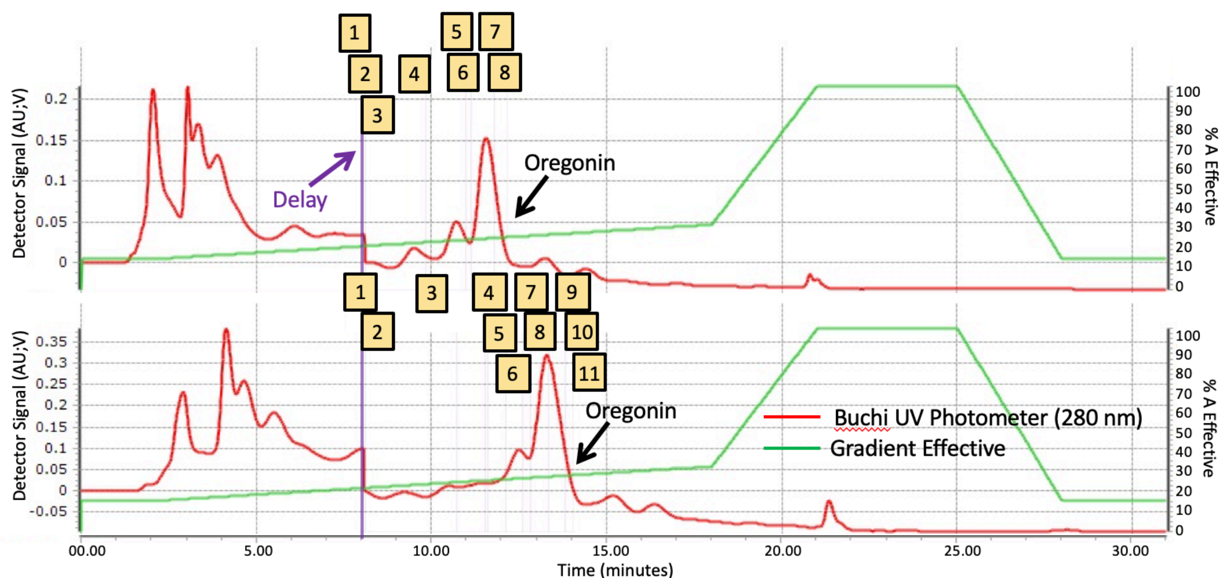


Figure 2.2 Flash chromatogram of spray-dried red alder leaf and bark extract separated on 80 g C18 (top) or 120 g C18 columns (bottom). Yellow numbers correspond to fractions collected by monitoring at 280 nm (red trace). Mobile phase gradient (green trace) corresponds to percent mobile phase A (acetonitrile). Delay time (purple) indicates start of fraction collection at 8 min.

2.3.3 Spectral characterization of oregonin

The structure of the purified compound was confirmed by Dr. Chakravarthi Simhadri, and Dr. Jeremy Wulff from the Department of Chemistry at the University of Victoria using NMR spectroscopy (including both 1D and 2D NMR experiments; refer to the Supplementary Information for original spectra, and Table 2.4 for assignments), as well as optical rotation, infrared and UV/Vis spectroscopy, and mass spectrometry. All data were consistent with spectral details from previous isolations (Suga et al., 1982; Ohta et al., 1984; Lee et al., 2000).

1D NMR analysis (^{1}H and ^{13}C), together with correlations observed in the COSY (^{1}H–^{1}H correlation) and HSQC (Heteronuclear Single-Quantum Coherence experiment, to determine ^{1}H–^{13}C connectivity) spectra, indicated the presence of six methylene groups, together with one oxymethine (C-5), one ketone (C-3), and two 3,4-dihydroxyphenyl moieties (Figure S1–S4). The characteristic glycoside signals were also clearly observed, and the coupling pattern for the *xyl*-1 proton (d, $J = 7.6$ Hz, 1H), together with the chemical shift for the corresponding carbon atom (102.75), confirm the β-linkage configuration of the glycoside unit (refer to Figure 1 for atom numbering). The key correlation in the HMBC (Heteronuclear Multiple-Bond Correlation) spectrum between the *xyl*-1 proton and C-5 (as well as a corresponding coupling between H-5

and the *xyl*-1 carbon) confirmed the direct attachment of the glycoside unit to the oxymethine carbon (C-5). Other HMBC correlations (H-5/C-3, C-3/H-1, H-2'/C-1 and H-5/C-7, H-6/C-1'', C-7/H-2'') support the remaining structural assignments (Figure S3). The UV spectrum of the purified oregonin sample showed maximum absorbances at 230 and 284 nm, together with a λ_{min} at 254 nm (Figure S5). These data likewise agree with the previous report (Karchesy et al., 1974).

To confirm the stereochemistry at C-5, we turned our attention to measurement of the specific rotation ($[\alpha]_D^{20}$). In one seminal previous report reference (Suga et al., 1982), specific rotation was apparently measured using an extremely high concentration of sample (*c* = 13 g/100 mL), but subsequent literature measurements have employed more standard concentrations (*c* = 1.0 g/mL) and have reported a similar specific rotation (Lee et al., 2000). We measured the specific rotation of our isolated oregonin in both acetone (*c* = 1.0 g/mol) and methanol (*c* = 1.3 g/mol), obtaining values of –17.2° and –15.0°, respectively. The former value agrees closely with the literature report from Lee and co-workers ($[\alpha]_D^{20}$ –17.5º (*c* = 1.0 g/mol in acetone)) (Lee et al., 2000), confirming that the configuration at C-5 is *S*.

Table 2.4 ^{13}C and ^{1}H NMR Assignments for oregonin

Carbon number	^{13}C chemical shift ppm	^{1}H Chemical shift ppm (number of protons attached)
C-1	28.57	2.61–2.52 (2)[c]
C-2	44.98	2.67 (2)
C-3	209.28	–
C-4	47.33	2.74 (1), 2.61–2.52 (1)[c]
C-5	74.49	3.98 (1)
C-6	37.27	1.68–1.55 (2)
C-7	30.43	2.48–2.41 (1), 2.40–2.32(1)
C-1’	132.19[a]	–
C-1’’	133.13[a]	–
C-2’/C-2’’	115.83 (coincident)	6.55 (1; C-2’), 6.53 (1; C-2’’)
C-3’/C-3’’	145.05 (coincident)	–
C-4’/C-4’’	143.32 and 143.14[b]	–
C-5’/C-5’’	115.61 (coincident)	6.60 (2)
C-6’/C-6’’	119.05 and 119.00[b]	6.40 (2)
xyl-1	102.75	4.11 (1)
xyl-2	65.90	3.01 (1), 3.69 (1)
xyl-3	69.74	3.30–3.23 (1)
xyl-4	76.72	3.08 (1)
xyl-5	73.52	2.90 (1)

[a]C-1’ and C-1’’ chemical shifts were assigned as they were resolved in HSQC and HMBC correlations. [b]Precise chemical shift assignments could not be made for these pairs of signals, due to poor resolution of the peaks in the HSQC and HMBC spectra. [c]Number of protons associated with each signal were deconvoluted/simplified from the 3-proton multiplet at 2.61–2.52.

2.4 Conclusion

In this study, an efficient method of oregonin extraction and purification that could be easily scaled to processing of timber by-products was developed. Using this method, oregonin of >95% purity can be readily purified using aqueous extraction, spray drying, and flash chromatography, thus minimizing the use of organic solvents. Less than 10% thermal degradation of oregonin was observed at extraction temperatures from 25-50 ºC, suggesting that spray drying is an acceptable means for drying crude oregonin following aqueous extraction. The optimized flash chromatography method was robust, repeatable, and yielded oregonin of greater than >95% purity. Purified oregonin was further characterized using HPLC, MS, UV, and NMR. This study represents the most complete NMR characterization of oregonin to date. Future studies should investigate if the new purification methods are similarly applicable to other diarylheptanoids.

Chapter 3. Anti-herbivore activity of oregonin, a diarylheptanoid found in leaves and bark of red alder *(Alnus rubra)*

Abstract

Plants synthesize a wide range of bioactive secondary metabolites to defend against pests and pathogens. Red alder (*Alnus rubra*) bark, root, and leaf extract have a long history of use in traditional medicine and hygiene. Diarylheptanoids, especially oregonin ((5S)-1,7-bis(3,4-dihydroxyphenyl)-5-(β-D-xylopyranosyloxy)-heptan-3-one) have been identified as major bioactive constituents. Diarylheptanoids have become a focus of research following reports of their antioxidant, antifungal, and anti-cancer activities. Our recent discovery that high oregonin concentration is associated with resistance of red alder leaves to western tent caterpillar (*Malacosoma californicum*) defoliation was the motivation to test the effects of this compound directly on leaf-eating insects. Purified oregonin was examined in a series of insect choice and toxicity tests using lepidopteran caterpillars. The compound exhibited significant antiherbivore activity against cabbage looper (*Trichoplusia ni)*, white-marked tussock moth (*Orgyia leucostigma*), fall webworm (*Hyphantria cunea*), and *M. californicum* at concentrations corresponding to oregonin content of the most resistant alder clones in previous experiments. Our results suggest that concentrations of oregonin found in red alder leaves early in the growing season are sufficient to protect the plant from leaf-eating insects.

3.1 Introduction

Red alder (*Alnus rubra*) is the most abundant broadleaf tree species in the Pacific Northwest, ranging from southern California to southern Alaska (Hibbs and DeBell, 1994; Harrington, 2006). Its common name comes from the rapid staining of alder sapwood, which changes from pale yellow to reddish-brown within 30 min of exposure to air (Irving, 1910). Original phytochemical studies of red alder bark identified the diarylheptanoid xyloside oregonin ((5S)-1,7-bis(3,4-dihydroxyphenyl)-5-(β-D-xylopyranosyloxy)-heptan-3-one); Figure 3.1) as the presumptive chemical causing red alder staining, based on its ability to rapidly form orange-red pigments *in vitro* following hydrolysis of the xylose residue and/or oxidation (Karchesy, 1974). Since its discovery, oregonin has attracted significant biomedical interest due to its wide range of properties, in particular its antimicrobial activity. Several studies have shown that oregonin is active against a variety of fungal and bacterial human pathogens such as *Staphylococcus aureus* and *Candida albicans* (Saxena et al., 1995; Novaković et al., 2015; Abedini et al., 2016). Further studies have highlighted oregonin's significant antioxidant and cytoprotective properties including hepatoprotective and anti-cancer effects (Lee et al., 2005; Park et al., 2010; Telysheva et al., 2011; Lee et al., 2013; Ponomarenko et al., 2014). Red alder accumulates oregonin at high concentration in both bark and leaf tissue (4-9%), suggesting it could also have an important biological role in plant defense (González-Hernández et al., 2000; Telysheva et al., 2011).

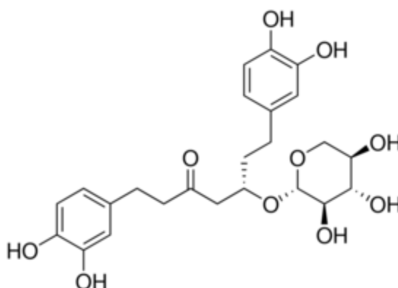


Figure 3.1 Structure of the diarylheptanoid xyloside oregonin ((5S)-1,7-bis(3,4-dihydroxyphenyl)-5-(β-D-xylopyranosyloxy)-heptan-3-one)

The diarylheptanoids are a family of polyphenolic secondary metabolites comprised of two aromatic rings joined by a heptane chain (Lv and She, 2012). Curcumin, an abundant diarylheptanoid found in the rhizomes of turmeric, was first isolated over 200 years ago (Kunnumakkara et al., 2017). To date, almost 500 additional diarylheptanoids have been identified, mostly from a small group of plants of the genera *Zingiber*, *Alpinia*, *Alnus*, *Betula*, *Curcuma*, and *Myrica* (Lv and She, 2012; Alberti et al., 2018); however, their adaptive role in

the plant remains largely unknown (Hu and Wang, 2011; Sati et al., 2011; Yang et al., 2011; Dong et al., 2017). The diarylheptanoid platyphylloside and its product centrolobol act as inhibitors of digestion in ruminants, and may have broad roles as anti-nutritive defenses (Sunnerheim and Bratt, 2004). Although platyphylloside and other phenolic constituents have been discussed as potential resistance factors of paper birch against bronze birch borer (Muilenburg et al., 2011), purified diarylheptanoids have never been directly tested against tree-feeding insects.

Phenolic plant secondary metabolites including tannins, diverse phenolic esters, and glycosides have all been implicated in plant insect defense, and their efficacy seems to be highly concentration-dependent and species-specific (Hemming and Lindroth, 2000; Barbehenn and Peter Constabel, 2011; Boeckler et al., 2014). Furthermore, there is substantial variation in the ability of insects to tolerate the often very high levels of phenolics found in many trees. For example, hydrolysable tannins are toxic to non-adapted graminivorous grasshoppers such as *Aulocara ellioti*, while polyphagous tree-feeding species such as *Melanoplus sanguinipes*, which typically encounter high levels of tannins in their diet, are more tolerant (Barbehenn, 2002). In transgenic hybrid aspen, a strong upregulation of the condensed tannin pathway did not appear to deter feeding by the forest tent caterpillar (*Malacosoma disstria*) or gypsy moth (*Lymantria dispar*), suggesting that leaf palatability may depend on other metabolic pathways (Boeckler et al., 2014). In willow and poplar, salicinoids are the major antiherbivore defense chemicals (Boeckler et al., 2011); however, adapted insects such as the willow sawfly have evolved strategies to metabolize or sequester the compounds, and may even perform better on salicinoid-containing trees (Matsuki and MacLean Jr., 1994). Likewise, the lepidopteran Salicaecae specialist *Cerura vinula* detoxifies salicortin at least in part by metabolic conjugation with quinic acid (Feistel et al., 2018). Therefore, the potency of phenolic phytochemicals in plant defense should be evaluated on a case-by-case basis.

The efficacy of phenolics in herbivore defense is further complicated by interactions with enzymes causing oxidation, which can lead to more toxic reaction products. For example, enzymes such as polyphenol oxidase (PPO) may be induced in the plant on insect herbivory. PPOs use molecular oxygen to oxidize *ortho*-diphenolic compounds, such as caffeic acid or catechol to reactive quinones: potent electrophiles able to form covalent bonds with proteins (Constabel and Barbehenn, 2008). The adverse effects associated with ingested polyphenols may, therefore, be linked to their oxidation products such as quinones and reactive oxygen species (ROS), including hydroxyl radicals and superoxide anion radicals, which are known to

disrupt insect gut membrane integrity, digestion, and metabolism (Appel and Martin, 1990; Appel, 1993; Barbehenn et al., 1996). Such reactions are critically dependent on the physiochemical conditions of the insect herbivore foregut, which may result in differential tolerance to oxidation (Appel and Martin, 1990; Appel, 1993). Compounds that readily oxidize are thought to be effective defenses against lepidopteran herbivores, which have an alkaline midgut (> pH 9), thus promoting PPO-generated quinones, and therefore the alkylation of amino acids and other oxidative reactions (Barbehenn et al., 2006; Constabel and Barbehenn, 2008).

Similarly, glycosylated compounds may be activated by enzymatic removal of the stabilizing glucose residue by foliar or insect hydrolases, forming reactive quinones (Boeckler et al., 2011; Vassão et al., 2018). In poplar, it is likely that the toxicity of phenolic glycosides, such as salicortin and tremulacin, is activated by deglycosylation to the reactive aglycone form. Breakage of the glycosidic bond may result from the activity of β-glucosidases enzyme, spontaneous hydrolyzation, or other enzymes (Feistel et al., 2018). In trembling aspen, the phenolic glycoside salicortin decomposes on herbivory in the alkaline foregut of forest tent caterpillars to form the reaction product catechol, and is thought to be further oxidized by PPO into reactive quinones (Haruta et al., 2001). Therefore, both oxidation and hydrolysis are common reactions with phenolic compounds resulting in the production of toxic products. Oregonin comprises a readily hydrolyzable 5-xylopyranosyl group, a C-3 carbonyl, and catechol moieties at C-1' and C-1", and has been shown to readily oxidize and generate colored products. It is thus plausible that during insect feeding the catechol of oregonin moieties are oxidized, or the xylose residue is hydrolyzed, leading to further downstream reactions in a manner similar to other phenolic acids and glycosides, which are known to form reactive quinones and ROS.

Few studies on the chemical nature of red alder defense have been carried out. Previous work investigated the correlation between diarylheptanoids, phenolics, tannins and other phytochemicals with a reduction in herbivory by vertebrates (Radwan et al., 1978; Robbins et al., 1987; McArthur et al., 1993; González-Hernández et al., 2000). The general conclusion of these early reports found that as the foliar concentration of oregonin, tannins and other phenolics decreases during the growing season, the preference of vertebrate herbivores to red alder foliage increases, suggesting a deterrent effect. These studies collectively propose that a seasonal change in chemical composition results in an increase in consumption of red alder by cervids in the fall. Previous work in our laboratory had found that western tent caterpillar resistance in red alder breeding accessions was similarly correlated with high oregonin concentration early in the spring

(Boateng, 2019). Together with the known bioactive potential of diarylheptanoids, these data suggested that oregonin could act as a chemical defense of alder against insect herbivory.

Here, we test the potential of oregonin as a defense against insect herbivores directly by conducting choice and toxicity bioassays with purified oregonin on four species of lepidopteran caterpillars. We tested western tent caterpillar (*Malacosoma californicum*) and fall web worm (*Hyphantria cunea*), both red alder-feeding species found throughout red alder's native range. In addition, we examined effects on white-marked tussock moth (*Orgyia leucostigma*), a tree-feeding lepidopteran from Eastern North America that does not encounter oregonin or red alder naturally, and the *Brassica* specialist cabbage looper (*Trichoplusia ni*), a non-tree-feeding lepidopteran. Based on the potential of oregonin to oxidize or hydrolyze after tissue disruption, we also tested *in vitro*-generated oregonin reaction products for toxic effects. Finally, to provide the ecological context for these studies, we also determined changes in oregonin content in alder leaves and bark throughout the growing season.

3.2 Methods and materials

3.2.1 Chemicals and reagents

Oregonin used in insect bioassay experiments was isolated and purified (96.14 % dry wt.) from red alder leaves and bark using methods described earlier (Chapter 2). An analytical standard (>95%) of oregonin was obtained from Sigma-Aldrich (Mississauga, ON, Canada). Neem powder (41.16% azadirachtin) was obtained from Ecosafe Natural Products Inc. (Saanichton, BC, Canada). Solvents used for high performance liquid chromatography (HPLC), and ultra high performance liquid chromatography mass spectroscopy (UHPLC-MS) analysis were from Fisher Scientific (Ottawa, ON, Canada). Water was deionised using a ThermoScientific Barnstead Mega Pure MP-1 water distillation system.

3.2.2 Insects

T. ni (cabbage looper) and *O. leucostigma* (white-marked tussock moth) eggs and artificial diet were obtained from Insect Production Services (IPS) of the Canadian Forest Service (CFS) at NRCan's Great Lakes Forestry Centre, Sault Ste. Marie, Canada (https://www.nrcan.gc.ca/science-data/research-centres-labs/forestry-research-centres/great-lakes-forestry-centre/insect-production-services/13467). *T. ni* larvae were hatched and maintained on McMorran artificial diet (agar, alphacel, ascorbic acid, aureomycin, casein, formaldehyde, linseed oil, methyl paraben, potassium hydroxide, sugar, vitamins, water, wheat

germ, wesson salt) at 27 ± 2 °C, with a 16:8 LD diurnal cycle at 60 ± 10% relative humidity. *O. leucostigma* larvae were hatched and maintained on Bell artificial diet (agar, ascorbic acid, casein, choline chloride, linseed oil, methyl paraben, sorbic acid, vitamins, water, wheat germ, wesson salt) at 22 ± 2 °C, with a 16:8 LD diurnal cycle at 50 ± 10% relative humidity. *H. cunea* (fall webworm) and *M. californicum* (western tent caterpillar) nests were collected in the Victoria area (48.4284° N, 123.3656° W) from the branches of Pacific madrone (*Arbutus menziesii*) and Pacific willow (*Salix lucida*), respectively. Whole nests and branches were held in a screened bucket at ambient laboratory conditions 22 ± 2 °C and supplemented with additional foliage if necessary.

3.2.3 Plant material

Cabbage plants (*B. oleraceae* var. Danish ballhead) used in *T. ni* choice bioassays were grown to the 10-12 leaf stage (8-10 weeks) in the Glover Greenhouse, University of Victoria, in Sunshine potting mix (Type 4, Sun Gro®Horticulture, Vancouver, BC, Canada), and Osmocote slow-release fertilizer (14–14–14, Scotts, Marysville, OH, USA). Trembling aspen (*Populus tremuloides*) foliage used in *M. californicum* and *H. cunea* choice bioassays was collected from young shoots of trees in forested areas on the University of Victoria campus. Leaves were rinsed with distilled water and excess water removed before use. For the *O. leucostigma* choice bioassays, leaves from greenhouse grown hybrid aspen saplings (*Populus tremula x tremuloides*) maintained in 4-litre pots with Sunshine potting mix (Type 4) and Osmocote slow-release fertilizer (14–14–14) were used. Saplings were roughly 1 metre in height before use in insect bioassays. Mature, fully unfurled leaves with no insect damage were selected and randomized. No synthetic agrochemicals are used in wooded areas on the university campus, or in the university's Glover Greenhouse facility.

3.2.4 Insect bioassays

Contact toxicity was determined by topical application to fourth instar *T. ni.* larvae (N=20) using modified methods by Akhtar et al. (2012). A methanolic solution of each compound (1 μL) was applied to the dorsal surface of each larvae (20, 200 or 500 μg/larvae). Methanol was used as a solvent control. After application of the test solution, individual larvae were transferred into new cups containing artificial diet and held for 48 h in a growth chamber (27 ± 2 °C, 16:8 h LD diurnal cycle, 60 ± 10% relative humidity). Mortality of larvae was determined after 24 and 48 h. Larvae were considered dead if they did not respond to prodding

with forceps. Experiments were performed three times. Median lethal toxicity (LD_{50}) of each compound was calculated using regression analysis of the log of each concentration vs. average percent mortality.

Ingested toxicity assays (no-choice bioassays) were performed using fourth instar *T. ni.* larvae (N=20) using modified methods by Akhtar et al. (2012). Larvae were starved for 4 h in a Petri dish containing only a moist cotton ball to prevent desiccation. Starved larvae were offered a small piece (est. 3 x 3 mm) of artificial diet to which 1 µL of a methanolic solution of each compound (20, 200 or 500 µg/larvae) had been applied. When larvae had completely ingested the artificial diet (3-5 h), they were transferred into new cups containing fresh artificial diet and held for 48 h in a growth chamber (27 ± 2 °C, 16:8 h LD diurnal cycle at 60 ± 10% relative humidity). If the larvae had not consumed all of the diet within five h, these replicates were excluded from the analysis. Mortality of larvae was determined after 24 and 48 h. Larvae were considered dead if they did not respond to prodding with forceps. Experiments were performed three times. Median lethal toxicity (LD_{50}) of each compound was calculated using regression analysis of the logarithm of each concentration vs. average percent mortality.

Feeding deterrence assays (choice bioassays) were conducted using methods adapted from. (Akhtar et al., 2012). Fourth instar larvae were used in the bioassays (N=10 *T. ni*; N=20 *O. leucostigma*, *H. cunea* and *M. californicum*). Larvae were starved 3-4 h prior to the bioassay in a Petri dish containing only a moist cotton ball to prevent desiccation. After starvation, larvae were placed in a Petri dish arena and offered two test leaf discs (1.5 cm diameter, freshly cut with a cork borer). Cabbage leaf discs for were used for *T. ni*, quaking aspen for *M. californicum* and *H. cunea,* and hybrid aspen for *O. leucostigma.* Average water content in the leaf discs from each plant species was measured using a Mettler Toledo HE53 Moisture Balance (Fisher Scientific, Ottawa, ON, Canada) and used to calculate the concentration of each compound painted on the leaf disc in dry weight (DW). One leaf disc per arena was coated with 10 µL of a methanolic solution of the test substance, applied to both sides using a micropipette. Four concentrations of oregonin (50, 100, 200 or 300 mg/g DW) were applied. A second disc (solvent control) was treated on both sides with 10 uL methanol. Leaf discs painted with a methanolic solution of neem powder (41.16% azadirachtin) were used as a positive control. The solvent was allowed to evaporate, and discs were placed 0.7 cm apart in the Petri dish arena. A single larvae was introduced into the centre of the dish, equidistant from the two leaf discs. Petri dishes comprising discs and larvae were placed into the insect growth chamber at the end of the day cycle in complete darkness as larvae are expected to feed more actively at dusk, and to prevent shadows,

which could confound insect choice bioassays (per. com.). Larvae were removed from each arena when approximately 50% of either leaf disc had been eaten (3-5 h). If neither disc had been eaten within 5 h, the replicate was excluded from analysis. To test the effects of oregonin oxidation or hydrolysis on feeding deterrence, experiments were repeated with fourth instar *T. ni* larvae (N=20) using oxidation and acid hydrolysis products. For these experiments, leaf discs were painted on both sides with a methanolic solution of oregonin to achieve a final concentration of 112.9 mg/g DW, the FD_{50} (concentration causing 50% feeding inhibition) of oregonin determined in previous experiments. The second leaf disc was painted on both sides with methanol. The entire set of feeding deterrence experiments was repeated twice using new larvae for each experiment.

The extent of feeding was calculated by determining the area of leaf discs consumed during the experiment. Discs were placed on transparency film and scanned using a desktop scanner, and disc areas calculated using Scion Image software (O'Neal et al., 2009). Feeding Deterrence Index (FDI) was determined using the formula $FDI = [(C - T)/(C + T)] \times 100$, where C and T are the control and treated leaf areas consumed by the larvae (Akhtar et al., 2010). The data for feeding deterrence and toxicity could not be transformed to meet the assumptions of ANOVA; therefore, treatment effects were analyzed with non-parametric Kruskal-Wallis tests (significance level $\alpha=0.05$, 95% CI) (Kruskal and Wallis, 1952). Where significant differences were found between groups, post-hoc analysis was performed using Dunn's multiple comparisons test and ρ-value rank adjustment using the Benjamini-Hochberg method (Dunn, 1964; Benjamini and Hochberg, 1995). Statistical analysis and data transformations was performed in R (version 3.5.2). FD_{50} was calculated in MS Excel software using regression analysis of the log of oregonin concentration (four concentrations from 50-300 mg/g DW), vs. FDI.

3.2.5 Measurement of oregonin in alder leaves and bark

Red alder leaf and bark material was collected monthly from 7-year-old red alder trees (N=6) on the University of Victoria Campus (48.4634° N, 123.3117° W) from May-Oct. 2019. Plants were originally obtained from the red alder clone bank at the Cowichan Lake Research Station operated by the B.C. Ministry of Forests, Lands, Natural Resource Operations, and Rural Development. For consistency, all samples were taken from the southern, sun-facing side of the trees. Juvenile leaves were defined as fully unfurled leaves taken from the apex of branches. Mature leaves were defined as fully unfurled leaves taken three leaf nodes below the shoot tip.

Juvenile bark was defined as bark scraped from previous year's growth. Mature bark was defined as bark scraped from the trunk at breast height. Four samples were taken for each tissue type at each time point, except for mature bark samples, where only one sample was taken in order to avoid permanent damage to the tree. Leaves or twigs with any visible damage were excluded from sampling. Following collection, samples were immediately ground into a fine powder under liquid nitrogen, lyophilized, and stored at -20 °C until analysis of oregonin by HPLC.

For oregonin quantification, lyophilized and ground tissue samples were extracted into methanol, sonicated for 10 min, and then passed through 0.45 μm syringe filters. Filtered samples (N=4 per sample, dilution factor: 10) were injected directly onto a Thermo Scientific™ UltiMate 3000 HPLC system equipped with a quaternary pump, auto-sampler, degasser, column oven, and diode array detector (DAD). Instrument control, data acquisition, and evaluation were achieved using Chromeleon™ chromatography software version 7.2 SR4. A Kinetex® reverse-phase C18 column (Phenomenex; diameter: 4.6 mm, length: 150mm, particle size: 2.6 μm, pore size: 100Å) was used for separation. Elution was carried out with a 15 min mobile phase gradient of acetonitrile 0.1% formic acid (solvent A) and 0.1% formic acid (solvent B) with a flow rate of 1.0 mL/min, injection volume of 5 μL, and oven temperature of 30 °C. The gradient began at 15% solvent A and was held for one min, linearly increased to 32% for 8 min, linearly increased to 100% for 1.5 min, held for 2 min, linearly decreased to 15% over 1.5 min and held at 15% for 1 min. Separation was monitored at 280 nm. Standards were prepared from commercial analytical grade oregonin in HPLC grade methanol at five concentrations in the range of 92-740 μg/mL, and stored in the dark at –20 °C.

For concentration data, square root transformation was used to achieve approximate normality of the residuals and equal variances using Levene’s test (Levene, 1960). Samples taken from each tree were considered independent. Effects were analyzed using a two-way ANOVA with sampling date, and tissue type as categorical independent variables, and oregonin concentration as the continuous dependent variable. When significant F-values were found, post hoc analysis was performed using Tukey’s multiple comparisons tests (Tukey, 1997). Statistical analysis was performed in R version 3.5.2.

3.2.6 Oxidation and hydrolysis of oregonin

Oregonin aqueous oxidation and hydrolysis products were prepared from pure oregonin. For oxidation tests, oregonin (91.80% dry wt.) was dissolved in deionised water and maintained at 54 °C for 7 days, allowing the purified compound to oxidize and decompose. At the end of the

7-day period, the solution had changed from light orange to brownish-red in color. Much of the solute had precipitated as a brownish-red, insoluble polymer. The water-soluble fraction was passed through a 0.45 µm syringe filter, and lyophilized. The resulting product was a brownish-red amorphous power.

For hydrolysis, oregonin was dissolved in 1M HCl and heated to 90 °C for 10 min. Upon heating, the solution rapidly changed to a vibrant red color, and again a brownish-red insoluble precipitate was formed. The water-soluble fraction was passed through a 0.45 µm syringe filter and lyophilized. The resulting acid-hydrolysis product was a bright red, amorphous powder.

Oregonin and its water-soluble hydrolysis and oxidation products were taken up in water and normalized to the same mass to volume ratio for analysis. Samples were sonicated for 10 min and passed through 0.45 µm syringe filters prior to analysis by UHPLC-MS. Purified oregonin was dissolved and filtered in parallel to be used as a control. Filtered samples were injected directly onto a Waters Acquity UHPLC equipped with an auto-sampler, degasser, column oven, QDa mass detector and photodiode array detector. A Waters Acquity UHPLC BEH C18 Column (diameter: 2.1 mm, length: 50 mm, particle size: 1.7 µm, pore size: 130Å) was used for separation. Instrument control, data acquisition, and evaluation were performed using MassLynx software version V4.1. Elution was carried out with an 11 min gradient of acetonitrile 0.1% formic acid (solvent A) and dH_2O 0.1% formic acid (solvent B), a flow rate of 0.5 mL/min, injection volume of 1 µL, and oven temperature of 40 °C. The gradient began with 5% solvent A, was held for 1 min, linearly increased to 50% over 7 min, linearly increased to 95% for 1 min, held for 1 min, and linearly decreased to 5% over 1 min. Separation was monitored at wavelengths from 210-800 nm at a sampling rate of 20 points/second. Negative ion electrospray mass (ES-) spectrometric data was collected at a unit resolution between 50-500 m/z and a sampling frequency of 20 hz. Each sample was injected once at each of three cone voltages: 12V, 15V, and 18V. Mass data were collected for all major peaks.

3.3 Results

3.3.1 Oregonin shows no contact and ingested toxicity

Oregonin contact (topical) and ingested toxicity was examined on 4th instar *T. ni* (cabbage looper) larvae at three concentrations ranging from 20-500 µg/larvae as described under Methods. No significant differences in mortality in both 24- and 48-h contact and ingested toxicity tests were observed between the oregonin treatment and the solvent controls (Dunn's

Test, ρ-adjusted > 0.05). By comparison, the natural insecticide azadirachtin positive control exhibited strong topical as well as ingested toxicity at both 24 h and 48 h (Table 3.1; Figure 3.2). Oregonin concentrations above 200 µg/larvae were not included in the analysis of ingested toxicity because less that 30% of the larvae from each experimental treatment had consumed the entire amount of test compound within the 5 h experimental time period. Significant feeding inhibition observed at high concentrations suggested that oregonin may have feeding deterrent rather than toxic effects for lepidopteran insects.

Table 3.1 Percent mortality of 4th instar cabbage looper larvae after topically applied or ingested oregonin (96.14% purity) treatments (24 h and 48 h)

Test Condition	Treatment	% Mortality* (24 h)	24 h LC_{50}[1] (µg) (R^2)[2]	% Mortality (48 h)	48 h LC_{50} (ug) (R^2)
	Oregonin	1.67 ± 2.9 a	-	8.33 ± 5.8 a	-
Topical	Neem powder[3]	25.0 ± 5.0 b	815 (0.93)	55.0 ± 5.0 c	110 (0.96)
	MeOH[4]	0.00 ± 0.0 a	-	1.67 ± 2.9 a	-
	Oregonin	0.00 ± 0.0 a	-	4.42 ± 4.0 a	-
Ingested	Neem powder	27.5 ± 3.3 b	-	52.6 ± 4.6 c	-
	MeOH	0.00 ± 0.0 a	-	1.67 ± 2.9 a	-

*Average larval percent mortality ± SD after 24 h and 48 h (200 µg/larvae). Letters indicate a statistically significant difference in mortality after each treatment (Dunn's Test, ρ-adjusted < 0.05). N=20 larvae*3 experiments

[1]LC_{50} values (concentrations resulting in 50% mortality) calculated based on logarithmic regression analysis of three concentrations/compound (20, 200 and 500 µg/larvae) vs. FDI.

[2]R^2 represent the coefficient of determination (not tested)

[3]Positive control (41.16% azadirachtin)

[4]Methanol solvent control

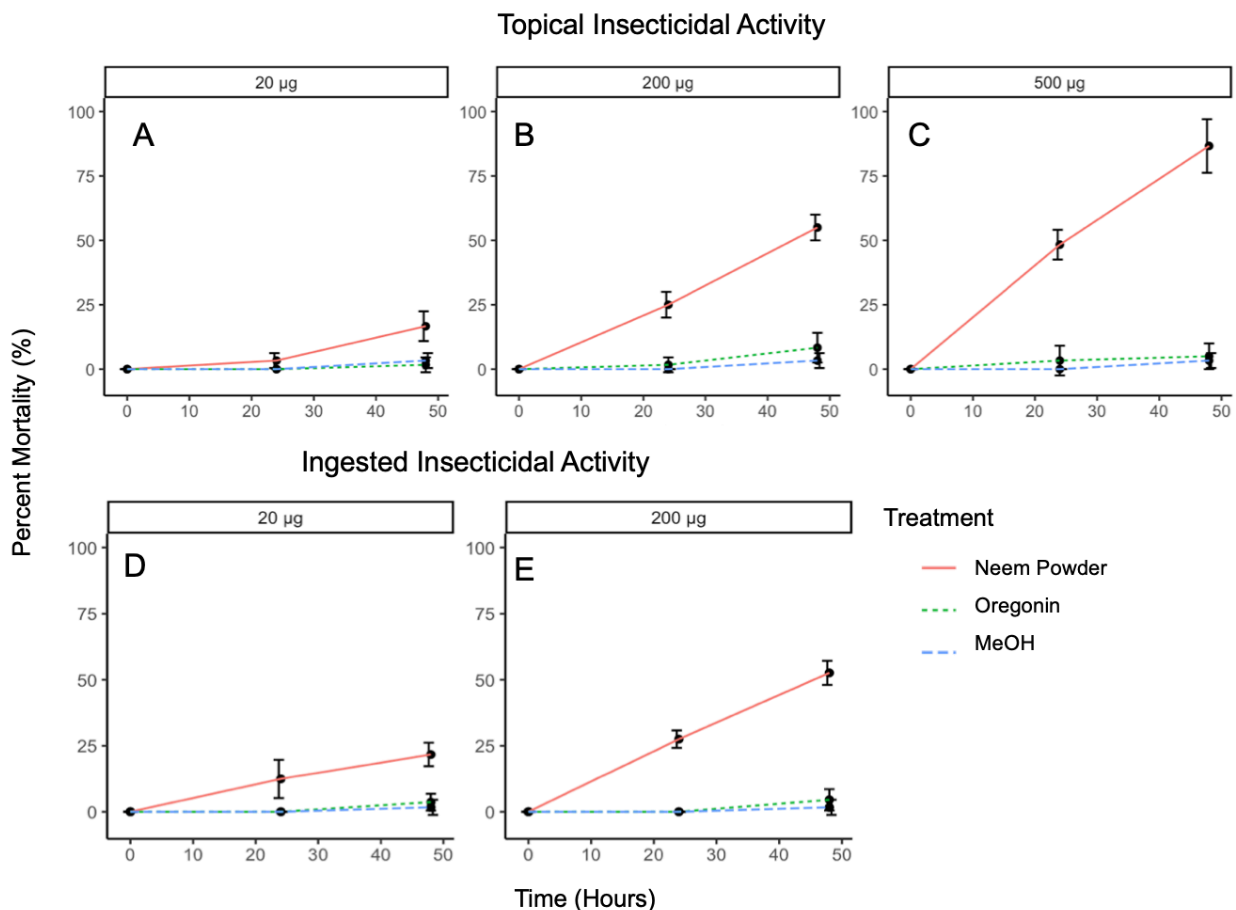


Figure 3.2 Percent mortality of 4th instar *Trichoplusia ni* (*T. ni*) larvae at 24 h and 48 h after topical application (A-B, N=20 larvae per treatment*3 experimental replicates) and ingestion (D-E, N=13-20 larvae per treatment*3 experimental replicates) of oregonin. Insects received 20, 200, or 500 µg of each treatment. Error bars represent the standard deviation (SD) between percent mortality of each experimental replicate. Neem powder (41.16% azadirachtin) was used as a positive control. Pure methanol (MeOH) was used as a solvent control.

3.3.2 Oregonin acts as a feeding deterrent against leaf-eating caterpillars.

In order to test for potential feeding deterrence by oregonin, leaf disc choice bioassays were conducted using Petri dish arenas and 4th instar lepidopteran larvae of all four test species (*H. cunea, O. leucostigma, M. californicum* and *T. ni*). The mean feeding deterrence index (FDI) at each assay concentration was determined and the FD_{50} (concentrations resulting in 50% feeding deterrence compared to negative control) calculated for the four test insects. All four species were deterred by oregonin in the range of 100-200 mg/g DW (Table 3.2, Figure 3.3). Species naturally feeding on alder (*M. californicum* and O. *leucostigma*) appeared to be the least deterred by oregonin, whereas the alder-naïve species (*T. ni* and *H. cunea*) tended to be the most affected. At the lowest concentrations tested, *O. leucostigma* larvae were the most deterred (25.3 ± 17.6%), whereas *M. californicum* larvae were slightly attracted (14.2 ± 16.8%). Similarly, at the highest concentrations (300 mg/g DW), *M. californicum* larvae appeared to be less deterred (64.5 ± 16.6%) than the other species (all had greater than 90% repellence) (Table 3.2, Figure 3.3). While these were not significantly different (Kruskal-Wallis, ρ-value > 0.05), the tendency was for *M. californicum* to be the least deterred (Table 3.2). The measurement of small changes in feeding deterrence using leaf disc choice bioassays is technically challenging because reductions in deterrence or attractance result in an increase in standard deviation between measurements. Although our observations have biological relevance is will be necessary to increase the statistical power of our measurements in future experiments.

Table 3.2 Feeding deterrence by oregonin applied to leaf discs to 4th instar lepidopteran larvae

Insect	Treatment	FD (%)[1] 50 mg/g DW	FD (%) 300 mg/g DW	FD_{50} (mg/g DW)[2] (R^2)[3]
Fall webworm	Oregonin	0.17 ± 19.1 a	90.08 ± 9.51 b	151 (0.88)
White-marked tussock moth	Oregonin	25.3 ± 17.6 a	96.19 ± 1.66 b	104 (0.97)
Western tent caterpillar	Oregonin	**-14.2 ± 16.8 a**	64.45 ± 16.6 b	204 (0.94)
Cabbage looper	Oregonin	3.82 ± 21.8 a	94.14 ± 2.65 b	113 (0.98)
Cabbage looper	Neem powder[4]	-	-	21.9 (0.84)

[1]Mean feeding deterrence (FD) of oregonin applied to leaf discs (50-300 mg/g DW) to 4th instar lepidopteran larvae ± Standard Error. N = 6-9 *T. ni* (cabbage looper), N = 7-13 *M. californicum* (western tent caterpillar), N = 9-14 *O. leucostigma* (white-marked tussock moth), N = 8-13 *H. cunea* (fall webworm) larvae per treatment*2 experiments. Means followed by the same letter are not significantly different (Dunn's Test, ρ-adjusted < 0.05). Negative values (bold) indicate attraction

[2]FD_{50} values (concentrations resulting in 50% feeding deterrence compared to negative control) calculated based on the logarithmic regression analysis of four concentrations/compound vs. FDI, using MS Excel

[3]R^2 represent the coefficient of determination (not tested)

[4]Positive control (41.16% azadirachtin)

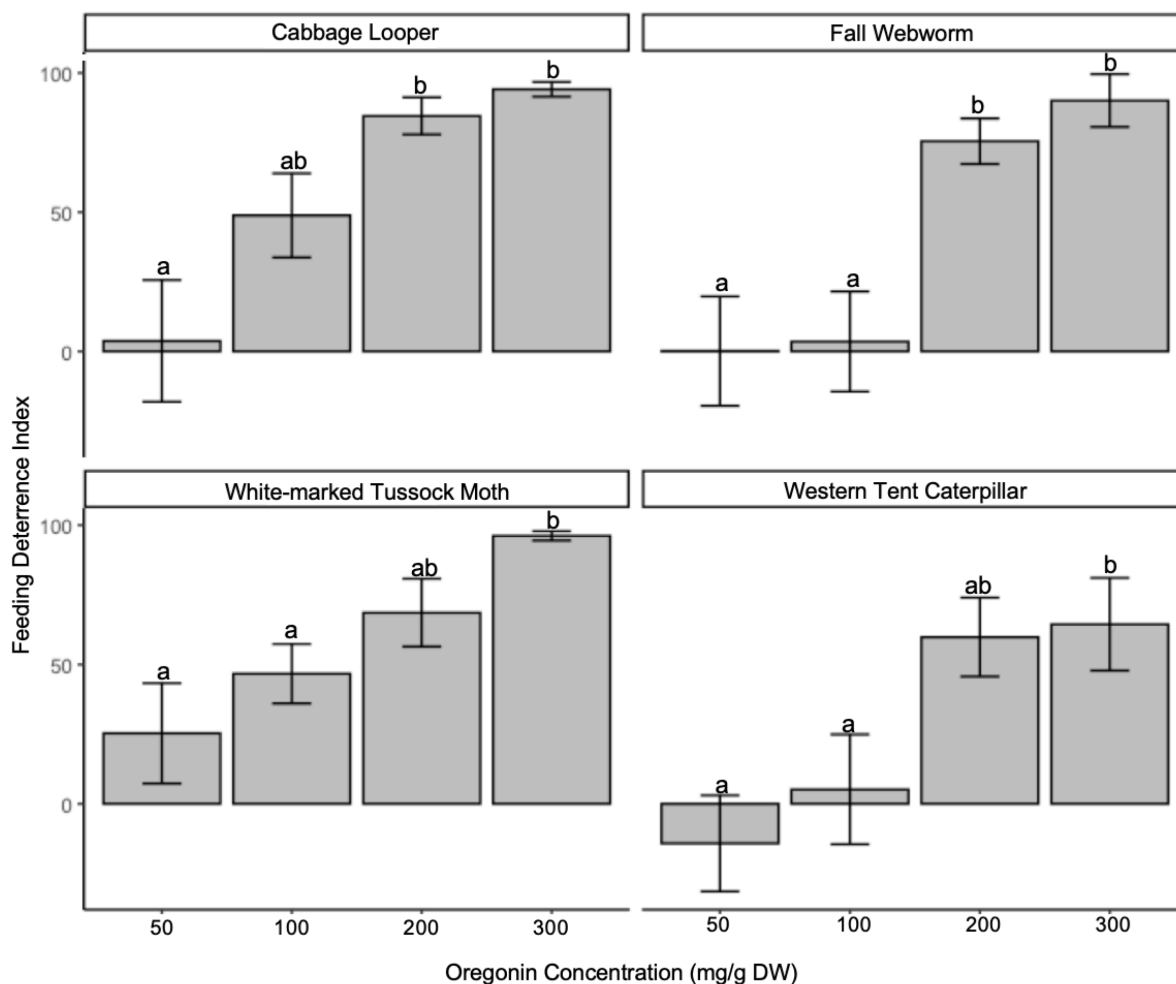


Figure 3.3 Mean feeding deterrence index (FD) of oregonin applied to leaf discs (50-300 mg/g DW) to 4th instar lepidopteran larvae. N = 6-9 *T. ni* (cabbage looper), N = 7-13 *M. californicum* (western tent caterpillar), N = 9-14 *O. leucostigma* (white-marked tussock moth), N = 8-13 *H. cunea* (fall web worm) larvae per treatment*2 experiments. Error bars represent standard error (SE). Letters indicate statistical significance (Dunn's Test, ρ-adjusted < 0.05).

3.3.3 Oregonin oxidation and acid hydrolysis leads to red coloration but no increase in insect feeding deterrence.

To determine if oregonin deterrent effects might be activated via enzymatic or spontaneous reactions, we let oregonin oxidize and decompose in aqueous solution, a process that gives rise to the red coloration of damaged alder bark. Likewise, we tested if removal of the xylose by acid hydrolysis could produce products with altered bioactivity. A brownish-red insoluble polymer was produced after both types of reactions, potentially resulting from oxidative reactions (Karchesy, 1974). Choice bioassays using soluble reaction products were repeated with painted cabbage leaf discs at 113 mg/g DW (*T. ni* oregonin FD_{50}), in order to test if *T. ni* larvae were more or less deterred by oregonin hydrolysis and breakdown products. Feeding deterrence of 4th instar *T. ni* larvae to the soluble oregonin hydrolysis and breakdown products were not statistically different from that with purified oregonin (Kruskal-Wallis, ρ-value > 0.05; Table 3.3).

Table 3.3 Mean Feeding Deterrence (FD) of 4th instar *Trichoplusia ni* larvae to oregonin acid hydrolysis and oxidative decomposition products applied to leaf discs

Compound	FD (%)*
Oregonin (96.14%)	46.59 ± 15.5 a
Acid Hydrolyzed Oregonin	51.65 ± 12.8 a
Oxidized/Decomposed Oregonin	60.85 ± 14.5 a

*Average Feeding deterrence (FD) ± Standard Error (SE). N = 10-16 larvae per treatment*2 experiments. Means followed by the same letter are not significantly different (Dunn's Test, ρ-adjusted < 0.05).

The water-soluble oregonin reaction products produced above were analyzed by UHPLC-UV-MS to assess potential changes and to quantify reduction in oregonin concentration. Oregonin eluted at 3.90 min (Figure 3.4A, and Figure 3.5), together with an unknown peak which consistently eluted at 3.48 min and appeared to be co-purified with oregonin in our purification. This apparent impurity may correspond to rubranoside A, another diarylheptanoid found in red alder (Ren et al., 2017). Rubranoside A is very similar in structure to oregonin but missing the C-3 carbonyl (Figure 3.5, compound 5). Due to its structural similarity, this compound likely co-eluted with oregonin during purification.

A reduction in oregonin peak area after acid hydrolysis and the appearance of new products at 5.37, 6.24, and 7.59 min indicated a loss of oregonin (Figure 3.4B). The two new peaks at 5.37 and 6.24 min (corresponding to m/z of predicted structures 3 and 4), are hypothesized to result from acid catalyzed E1 elimination and subsequent substitution of the chlorine halide (Figure 3.5, structures 3 and 4). The additional product eluting at 7.59 min remained unidentified (m/z 293.12). Two of the same peaks were observed in the samples allowed to oxidize and decompose at 5.37 and 7.59 min (Figure 3.4C); however, oregonin appeared to degrade less rapidly during the oxidation experiment than after acid hydrolysis, and the intact compound was still present at significant concentrations (Figure 3.4C). Both reaction products suggest the transient production of the aglycone, and subsequent aqueous elimination of the hydroxyl group to form a double bond along the diarylheptanoid 7-carbon skeleton; however, a peak with mass corresponding to the genuine aglycone was not observed (Figure 3.5, structure 2). More detailed experiments will need to be performed to further characterize these products in the future.

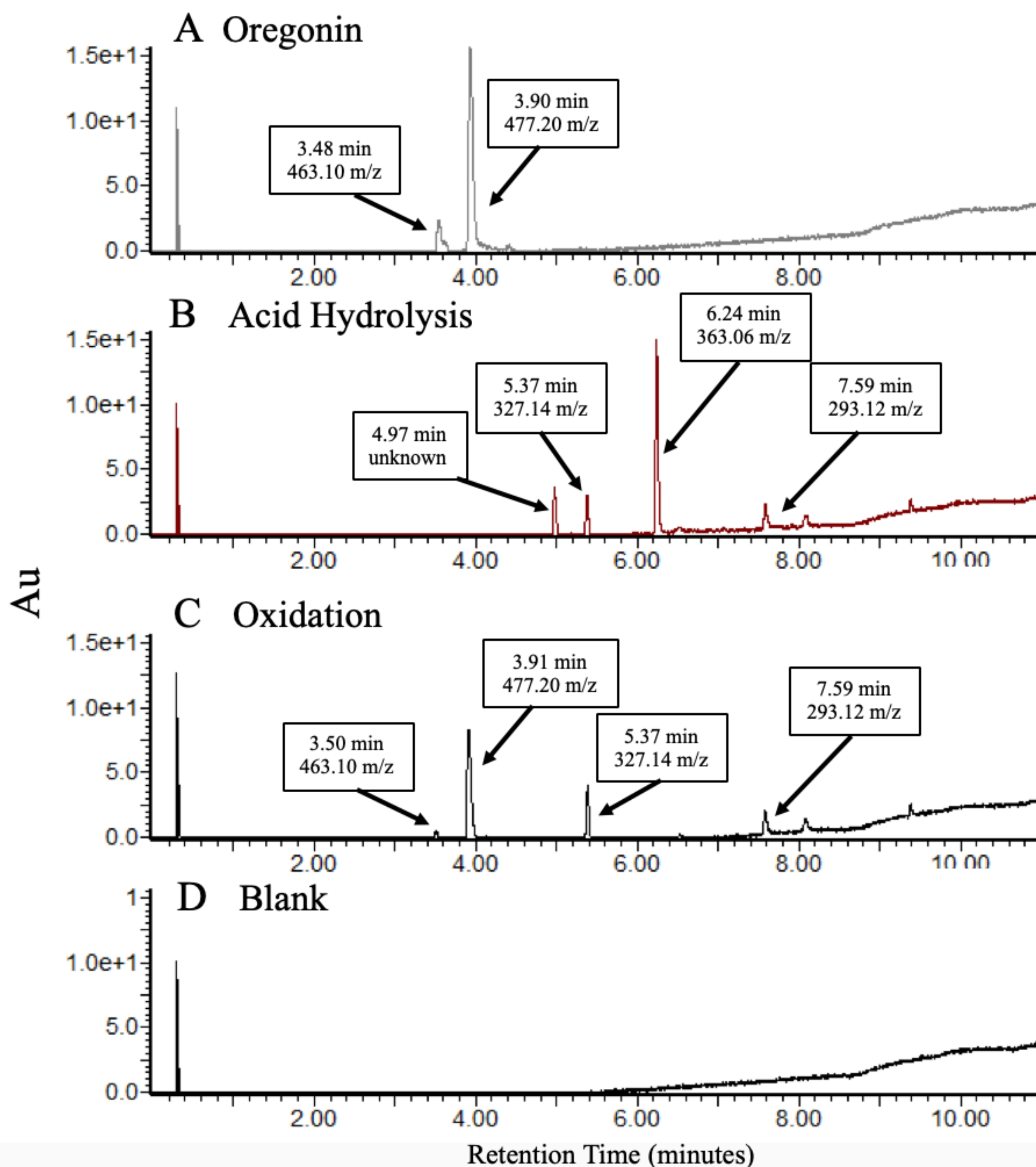
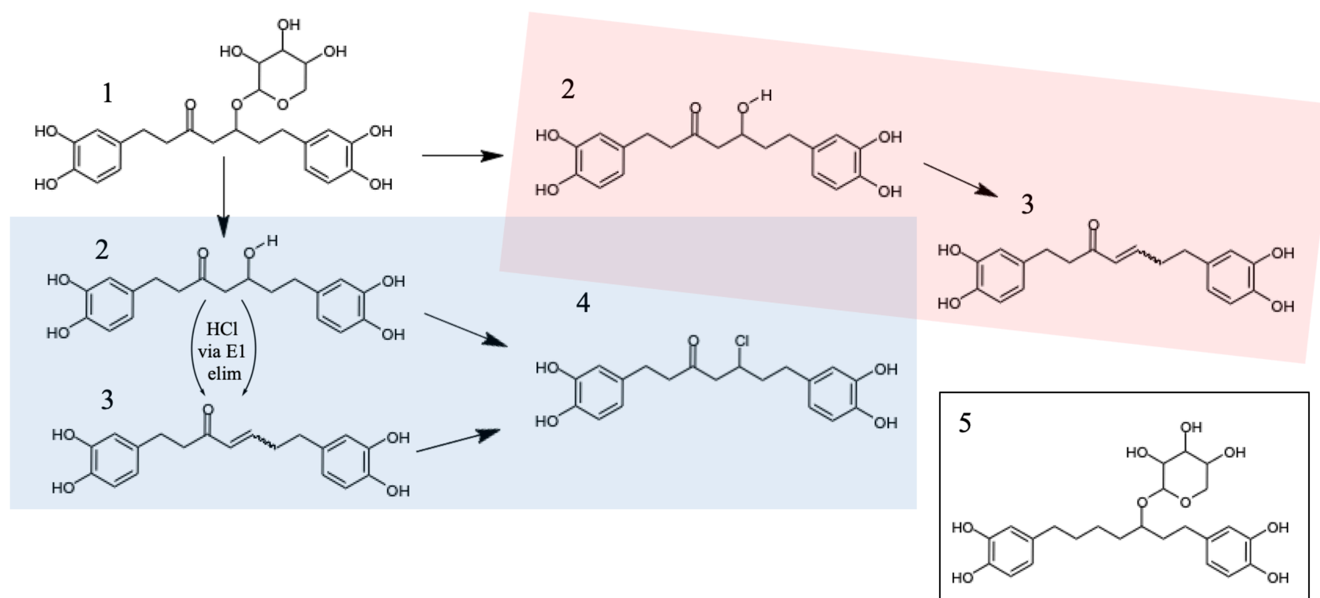


Figure 3.4 Representative HPLC-DAD (210-800 nm) chromatograms of (A) oregonin, (B) oregonin acid hydrolysis products, (C) oregonin oxidation/decomposition products, and (D) a method blank. Mass to charge ratio (m/z) in ESI negative mode, and retention times (min) are identified for each major peak.



Structure ID	Chemical Formula	Retention Time (min)	m/z- Expected	m/z- Observed
1 (oregonin)	$C_{24}H_{30}O_{10}$	3.90	477.18	477.20
2 (aglycone)	$C_{19}H_{22}O_6$	not observed	345.14	not observed
3	$C_{19}H_{20}O_5$	5.37	327.13	327.14
4	$C_{19}H_{21}ClO_5$	6.24	363.11	363.06
5 (rubranoside A)	$C_{24}H_{30}O_9$	3.48	462.19	463.10

Figure 3.5 Hypothesized transformations of oregonin observed after spontaneous oxidation and decomposition (pink) and acid hydrolysis (blue). Structure 5 (rubranoside A) is a diarylheptanoid that likely coeluted during purification. Numbers correspond to hypothesized structures. m/z- expected represents the theoretical mass-to-charge ratio for each structure in negative ESI- mode. m/z- observed corresponds to LC-MS peaks with retention times as indicated (Figure 3.4).

3.3.4 Seasonal changes in red alder oregonin content

We measured oregonin content in leaves and bark over one growing season in order to determine if oregonin concentration is variable in time. Both mature and juvenile tissues were harvested and analyzed monthly, beginning with bud break in the spring (May 8, 2018) until leaf senescence (Oct. 04, 2019) (Figure 3.6). An analysis of variance showed that both the date of sampling and tissue type had a significant effect on oregonin concentration; however, the interaction was not significant (Table 3.4). Oregonin concentrations were greatest from May through July and began to decrease significantly from August to October in all tissues, except for mature bark. Oregonin concentrations were significantly greater in mature bark tissue than juvenile bark tissue (Tukey's multiple comparisons, ρ-value<0.05). The opposite trend was observed for leaf tissue, where concentrations were greater in juvenile tissues than mature tissues (Tukey's multiple comparisons, ρ-value<0.05). In general, oregonin concentration was almost 2-fold greater in bark tissue than in leaf tissue.

Table 3.4 F-value and respective significance level (ρ-value) of sampling date over the growing season (month), and tissue type on oregonin concentration using two-way analysis of variance (ANOVA).

Variable	**F-value**	**ρ-value**
Sampling date	90.75	<2e-16
Tissue	565.84	<2e-16
Sampling date:Tissue	1.55	0.0834

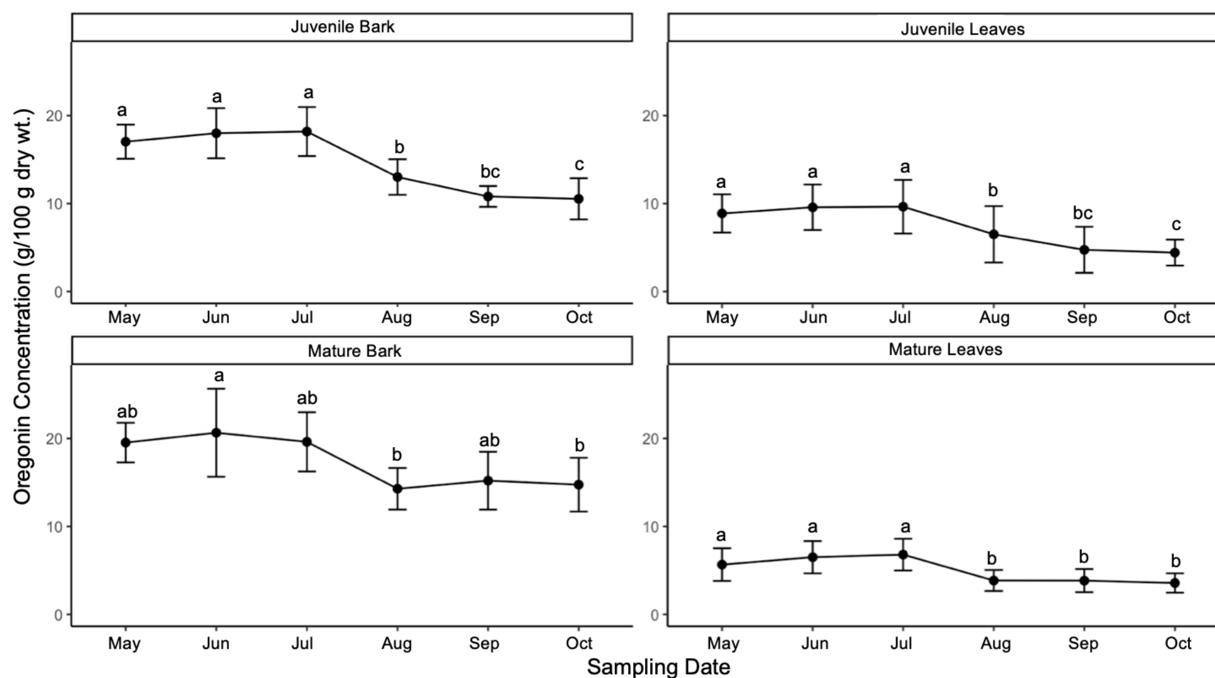


Figure 3.6 Seasonal changes in oregonin concentration in 7-year old alder trees across various tissue types (N=6). Tissue types collected included: juvenile leaves (n=4 samples*6 trees), mature leaves (n=4 samples*6 trees), juvenile bark (n=4 samples*6 trees), and mature bark (n=1 sample*6 trees). Error bars represent standard deviation of means (SD). Letters indicate statistical significance between sampling dates for each tissue type (Tukey's multiple comparisons, ρ-value < 0.05).

3.4 Discussion

3.4.1 Insecticidal and insect deterrence activity

At high concentrations, oregonin deterred feeding of all four lepidopteran test species. We observed a general trend that alder-feeding species such as *H. cunea* and *M. californicum* were less deterred at high concentrations. Although these species are not considered to be alder specialists, they are two of a small number of lepidopteran species known to feed on red alder (Muthigani, 1971). At low concentrations, oregonin was somewhat attractive to *M. californicum,* suggesting the species may be adapted to feed on plant phenolics, and that these adaptations are potentially offset by a nutritional benefit. At high concentrations, such adaptations may be overwhelmed, resulting in feeding deterrence. This pattern has been observed in forest tent caterpillars, which have been reported to show a preference for high tannin plants, often considered to be deterrent to generalist insects (Boeckler et al., 2014). The feeding deterrence of *M. californicum* (FD_{50} 204 mg/g DW) was similar to the threshold of 200 mg/g DW oregonin in *M. californicum* alder leaf bioassays, above which little larval feeding was observed (Boateng, 2019). In that study, larvae were offered whole alder branches and the oregonin content analyzed, as opposed to leaf discs painted with oregonin. The consistent result between two separate experimental procedures further corroborates to the key finding of this report: that oregonin acts as a feeding deterrent at high concentrations. There was, however, no evidence of oregonin toxicity to *T. ni.* larvae. *T. ni* larvae are not natural pests of red alder, and are most highly specialized to feed on plants of the mustard family (Brassicaceae), thus the lack of toxicity is unlikely due to feeding adaptation (Lingren and Green, 1984). We concluded that high foliar concentration found in red alder most likely function to deter insect feeding, rather than as an insect toxin.

Polyphenolic secondary plant metabolites can be potentiated by enzymes or oxidation leading to an increase in toxicity (Appel, 1993). For example, when leaf tissues are damaged, polyphenol oxidase (PPO) and peroxidase activity is known to be released and oxidize phenolic compounds and produce reactive quinones and ROS (Barbehenn et al., 2007; Constabel and Barbehenn, 2008). Previous reports have observed PPO activity in alder tissue (Tscharntke et al., 2001). We hypothesized that foliar PPO may potentiate oregonin and confirmed that oregonin is an excellent substrate for a commercial fungal polyphenol oxidase enzyme (tyrosinase) (data not shown). However, feeding deterrence to *T. ni* of either oxidation/decomposition or hydrolysis reaction products was not significantly different from the deterrent effects of oregonin. The

results of LC-MS analysis suggest that both spontaneous oxidation and decomposition of oregonin, and hydrolysis can result in production of the aglycone, with subsequent elimination of the hydroxyl group to form a double bond along the 7-carbon diarylheptanoid chain (Figure 3.5); however, a molecular mass corresponding with the aglycone was never observed, and it is likely rapidly transformed to downstream products. In *Betula*, anti-nutritive activity of the diarylheptanoid platyphylloside was shown to be correlated with hydrolysis to the aglycone form, and subsequent reduction into the active product centrolobol, a compound highly similar in structure to the oregonin aglycone (Sunnerheim-Sjöberg and Knutsson, 1995; Sunnerheim and Bratt, 2004) (For structures, see Figure 3.7). It is thus likely that similar transformations occur with oregonin.

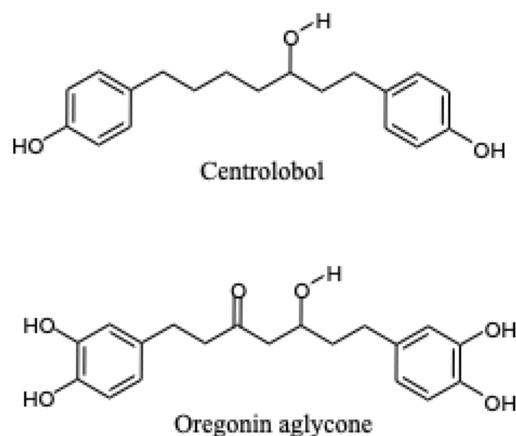


Figure 3.7 Structure of described diarylheptanoids

Earlier reports by Karchesy suggest that the oregonin aglycone is present only transiently and may rapidly polymerize, and thus the compound may have fallen out of solution prior to lyophilization (Karchesy, 1974). Our approach was thus limited by the potential production of products that are insoluble, unstable, or otherwise transient, such as the aglycone. Therefore, we likely could not detect any transiently enhanced biological activity of oregonin conferred by production of the aglycone and the rapid formation of reactive quinones formed by oxidation of catechol moieties at C-1' and C-1". To test this, one could feed oregonin to larvae simultaneously with a xylosidase or PPO, such that the formation of the aglycone and oxidation products could occur during insect feeding or digestion. It will also be important to definitively determine the structures observed in the LC-MS spectra (Figure 3.4) using accurate mass spectroscopy and NMR, and to purify individual oregonin products to test for activity in additional choice bioassays.

The redox conditions within the gut of herbivores range from highly reducing to highly oxidizing, suggesting that herbivores have different strategies to deal with plant phenolics. This likely influences the anti-herbivore activity of oregonin and should be taken into consideration. For example, in herbivores with an acidic to neutral gut pH, such as beetles and grasshoppers, tannins are thought to form hydrogen bonds with proteins in digestive tract, which thereby precipitate and cannot be digested. In the alkaline gut environment of lepidopterans, however, hydrogen bond formation is unlikely to occur (Appel, 1993; Mansfield et al., 1999; Barbehenn and Peter Constabel, 2011). The biological activity of oregonin on lepidopterans should therefore be compared with alder insect pests with different gut physiology, such as bark-boring insects. This will be addressed in future work.

3.4.2 Dynamic nature of oregonin content in alder tissues.

We examined the seasonal patterns of oregonin accumulation in *Alnus rubra* in leaves and bark. This would help to assess if variation aligned with the life history of alder insect herbivores native to the Pacific Northwest. We discovered that oregonin decreased in concentration from May-October in all tissue types. In North America, both *O. leucostigma* and *M. californicum* undergo a single generation per year; however, larval populations occur at high density at opposite times (Williams and Myers, 1984). *M. californicum* overwinter as egg masses, and hatch as larvae early in the spring (Myers, 2000). Conversely, *O. leucostigma* overwinters as cocooned pupa in the soil, and emerges as an adult in the spring. Adults begin to lay eggs in June, and larvae emerge over the summer and into the fall (Schowalter and Ring, 2017). Therefore, they feed on alder at very distinct times in the growing season. Based on our results, red alder adaptation to *M. californicum* life history aligns with the observed seasonal variation in foliar oregonin concentration.

Our results are consistent with previous reports suggesting that oregonin concentration is highest in leaf tissue in the spring (González-Hernández et al., 2000); however, it was previously unknown if this finding was also true for bark. Interestingly, our conclusions support earlier observations that alder logs harvested in the spring stain redder and darker than those harvested in the summer and winter months (Thompson et al., 2008), given that the staining phenomenon is thought to result from the oxidation of oregonin (Karchesy, 1974).

In leaves, we observed that oregonin concentrations tended to decrease both over the growing season and as they matured. The enhanced allocation of carbon to potential defense compounds early in the spring has been observed in other deciduous trees in the Pacific

Northwest, such as quaking aspen and paper birch, and is thought to correlate with heightened herbivore pressure (Lindroth, 2001; Riipi et al., 2002). This pattern also agrees with recent models of plant defense which suggest that the allocation of carbon to defense is best explained by a combination of competing physiological trade-offs, such as resource availability, and selective pressure from herbivores (Stamp, 2003). As tissues mature and expand, defense chemicals may be diluted, and their protective function compensated with physical attributes such as leaf toughness and trichome density (Ochoa-López et al., 2015).

In May-July, when foliar oregonin content peaked, its concentration in juvenile leaf tissue averaged between 88.7-96.4 mg/g DW, consistent with a previous report that reported spring foliar oregonin concentrations at a mean of 96.4 mg/g DW (González-Hernández et al., 2000). Leaf oregonin content in spring was similar to the FD_{50} (concentrations resulting in 50% feeding deterrence) for *O. leucostigma,* and *T. ni* (104, and 113 mg/g DW respectively), suggesting these species are unlikely to feed on juvenile alder leaf tissue. These foliar oregonin concentrations are unlikely to deter the alder feeding species *O. leucostigma* and *M. californicum,* with a FD_{50} of 151 and 204 mg/g DW, respectively. We observed a tendency of alder-feeding lepidopterans to be less deterred by oregonin at high concentrations, highlighting that constitutive oregonin concentrations may be sufficient to prevent defoliation from exotic and introduced lepidopteran species in the future. This trend should be investigated further in future work. Such experiments could provide a possible explanation as to why so few insect species are known to feed on red alder when compared to other broadleaf trees (Hibbs and DeBell, 1994).

In contrast to the seasonal pattern observed in leaves, juvenile bark tended to be lower in oregonin concentration than mature bark, and a reduction in seasonal concentration was only significant in juvenile tissue. A similar ontological increase in phenolic content with bark tissue maturity, and little variation from spring to fall has been observed in conifer species (Brennan et al., 2020). Our study, however, only measured bark oregonin concentration from May-October. If we had continued our study, we might have seen an increase in bark oregonin concentration at the end of the growing season, when allocation of resources shifts from leaves to permanent tissues (Schultz et al., 2013). The bark-boring alder specialist *Alniphagus aspericollis* has been observed to attack early in May, and again later in the summer (Borden, 1969). Relatively little is known about the resistance of angiosperms to wood-boring insects; however, constitutive diarylheptanoids have been implicated as defenses against bronze birch borer in paper birch (Muilenburg et al., 2011). In general, bark oregonin concentrations were higher than leaf oregonin concentrations. Future studies should examine genetic variation of oregonin in alder

bark and determine if there are any correlations between oregonin bark concentration and seasonal patterns of insect attack.

3.5 Conclusion

The results of this study suggest that at concentrations found in the natural environment, oregonin could reduce damage by herbivory of red alder by lepidopteran herbivores. Oregonin exhibited no direct toxicity to test insects, but deterred insect feeding. Comparisons of deterrence to *O. leucostigma, M. californicum, H. cunea*, and *T. ni* suggest that alder-feeding insects may be slightly better adapted to oregonin than generalist pests.

Chapter 4. Antimicrobial activity of the diarylheptanoid oregonin against plant pathogens and parasites

Abstract

Oregonin is a naturally occurring diarylheptanoid found in the leaves and bark of *Alnus rubra* (red alder), an important deciduous tree native to coastal forests in western North America. Red alder bark has a long history of traditional use in medicine and hygiene. Previous results suggest that oregonin has antimycotic potential against several human pathogens; however, oregonin has never been tested against fungal plant pathogens and parasites. This study aimed to test oregonin against a selection of plant-associated fungi of the phyla Basidiomycota, Ascomycota, and against fungal-like protists of the phylum Oomycota. In contrast to earlier studies, we were not able to detect any antifungal activity of oregonin with any of the species tested. Our results suggest that fungal inhibition of oregonin is more species-specific than expected, and that certain fungal groups, especially plant-associated fungi, may be more resistant. The molecular basis of this resistance will need to be investigated further by directly comparing susceptible and non-susceptible fungal species.

4.1 Introduction

Diarylheptanoids are a group of plant secondary metabolites characterized by two aromatic rings joined by a 7-carbon bridge. Almost all diarylheptanoids reported have been found in a small group of plants, belonging to the genera *Curcuma, Zingiber*, *Betula*, *Alpinia*, and *Alnus*. The diarylheptanoid xyloside oregonin ((5S)-1,7-bis(3,4-dihydroxyphenyl)-5-(β-D-xylopyranosyloxy)-heptan-3-one) was first isolated and characterized in *Alnus rubra* in 1974 (Karchesy et al., 1974), but has become a focus of study after its wide range of biological properties, including reports of antioxidant, fungicidal, and insecticidal activity, were discovered (Lee et al., 2005; Novaković et al., 2015; De Souza Tavares et al., 2016; Dong et al., 2017). Despite being present at high concentration (4-9%) in red alder bark and leaves, the biological function of oregonin remains unknown (González-Hernández et al., 2000; Telysheva et al., 2011).

The antimicrobial properties of oregonin were first reported after a number of medicinal plants native to British Columbia, Canada were assessed for bioactivity (Saxena et al., 1995). Fractionation of compounds from *Alnus glutinosa* bark and subsequent antimicrobial testing revealed that oregonin exhibits inhibitory activity against several opportunistic human pathogens, including *Staphylococcus aureus* and *Candida albicans,* with an effective concentration of 15.6 to 125 µg/mL (Abedini et al., 2016). Inhibition of human-associated yeasts and bacterial pathogens by oregonin has been shown in other studies, including against *Aspergillus*, and *Penicillium* (Rashed et al., 2014). Structure-activity analysis has suggested that the C-3, Δ^4 double bond, and 5-xylopyranosyl group (enone) are the major contributors to oregonin's antimicrobial activity (Novaković et al., 2015; Abedini et al., 2016). Screening of metabolites from black and green alder revealed that conidial germination of several plant-associated *Fusarium* species (F. *semitectum, F. tricinctum, F. equiseti, F. proliferatum*) was also inhibited by oregonin with MIC values ranging from 9.76 – 313 µg/mL. This suggested that plant-associated fungi may also be susceptible to oregonin (Novaković et al., 2015).

While the aforementioned studies suggest that oregonin possesses antimicrobial activity against a wide range of medically important bacteria and fungi, there have been no reports of its antimicrobial activity against alder-associated plant pathogens or parasites, an important step for determination of the biological function of oregonin. Further, plant-associated microbes of the genus Basidiomycota or Oomycota have never been examined for susceptibility to oregonin. While all pathogenic microorganisms must overcome host defense mechanisms during colonization, the defense responses of host plants and animals are considerably different

(Casadevall, 2007). Many plant-associated fungi contain virulence factors that target plant cell walls, lignin, or plant defense metabolites, allowing them to invade plants, which may differ from virulence factors associated with microbial species adapted to invade animal hosts. They may also manipulate pH of the infection site, leading to rapid cell death of host tissue. Virulence factors are not conserved among species, and may differ considerably depending on the host organism and ecological niche (Rodriguez-Moreno et al., 2018). Thus, microorganisms exhibit a great deal of species-specific variation and adaptations concerning resistance to antimicrobial plant metabolites. We therefore asked if oregonin is as effective against plant-associated pathogens or parasites as it is to the medically relevant pathogens previously reported (Rashed et al., 2014; Novaković et al., 2015; Abedini et al., 2016).

To address these questions, we evaluated the inhibitory activity of oregonin against a selection of plant-associated microbial species of the phyla Ascomycota and Basidiomycota, including the alder pathogens *Chondrostereum purpureum,* and *Ganoderma applanatum*, and alder-associated *Botrytis cinerea.* We also tested *Saprolegnia parasitica,* a filamentous protist of the phylum Oomycota. A number of soil-borne Oomycota cause infectious disease in plants of considerable economic importance (Vettraino et al., 2005; Kamoun et al., 2015). Other tested fungal taxa, such as *Fusarium spp.*, *Stemphylium vesicarium,* and *Truncatella angustata* cause disease in many important agricultural products, resulting in extensive and costly annual crop losses (Swain and Ray, 2009; Misawa and Yasuoka, 2012; Fisher et al., 2013). By testing several alder-associated fungi, we aimed to determine if oregonin could contribute to resistance of alder to fungal diseases. However, in contrast to previous studies that have reported antimicrobial activity, in our experiments none of the plant-associated microbes tested were inhibited or affected by oregonin.

4.2 Methods

4.2.1 Chemicals and reagents

Oregonin was purified (96.14 % dry wt.) from red alder bark and leaves using methods described previously (Chapter 2). An analytical standard (>95%) of oregonin was purchased from Millipore Sigma (Oakville, ON, Canada). HPLC grade solvents used for analysis were obtained from Fisher Scientific (Ottawa, ON, Canada). Water used in HPLC experiments was dionized using a ThermoScientific Barnstead Mega Pure MP-1 water distillation system.

4.2.3 Microorganisms and growth media

Plant pathogenic and parasitic species of the phyla Basidiomycota (*Chondrostereum purpureum and Ganoderma applanatum)*, Ascomycota (*Fusarium spp.*, *Botrytis cinerea, Stemphylium vesicarium, Truncatella angustata, and Boeremia exigua)*, and Oomycota (*Saprolegnia parasitica*) were provided by Dr. Paul de la Bastide and Dr. Will Hintz, Centre for Forest Biology, University of Victoria. *Ganoderma applanatum* was originally isolated from a hardwood tree stump on the University of Victoria campus. *S. parasitica* was isolated from the tissue of Atlantic salmon (*Salmo salar*). All other microbial taxa had been previously isolated from the plant species Long's Braya (*Braya longii*) and the Fernald's Braya (*Braya fernaldii*). Taxonomic identification of fungal isolates was confirmed by Dr. Paul de la Bastide using sequence analysis of the ITS-rDNA region and comparison to reference sequences available in the NCBI GenBank DNA database (https://blast.ncbi.nlm.nih.gov/Blast.cgi). All taxa were identified to species except for the *Fusarium* isolate, as differentiation among *Fusarium acuminate, F. lateritium,* and *F. avenacea* was not possible using only the ITS-rDNA region. All selected species were filamentous and grew well on agar plates and in liquid culture. *C. purpureum* and *G. applanatum* were grown on malt extract agar (MEA, 20 g/L malt extract, 15 g/L bacto agar), *Fusarium sp.*, *B. cinerea, S. vesicarium* and *T. angustata* on potato dextrose agar (PDA, 36 g/L potato dextrose agar), and *S. parasitica* on glucose peptone agar (GPA, 3 g/L D-glucose, 1.25 g/L bacto peptone, 15 g/L bacto agar), (Spiers et al., 1998; Trabelsi et al., 2017; Latorre et al., 2015; Diéguez-Uribeondo et al., 1994). *Staphylococcus aureus* was grown on tryptic soy agar (TSA, 15.0 g/L trypticase peptone, 5.0 g/L phytone peptone, 5.0 g/L sodium chloride,15.0 g/L agar). Cultures were maintained by weekly transfer of an agar plug (approximately 0.5 cm^2) onto fresh agar plates.

4.2.4 Hyphal inhibition assay

Hyphal inhibition by oregonin was tested using a disc diffusion method (Foss et al., 2014). Agar plates were centrally inoculated with a mycelial plug from each culture (0.5 cm^2) and incubated at 22 °C until actively growing (approximately 24-72 h). Discs (1 cm^2) were cut from Whatman™ grade 1 qualitative filter paper (Fisher Scientific, ON, Canada) and sterilized. Solutions of oregonin in methanol (125 µg/mL and 500 µg/mL oregonin) were applied to each disc (6.25, 24.0, 75.0 or 125 µg oregonin/disc), and the methanol allowed to evaporate. *Fusarium sp.* isolates were tested at up to 10 mg oregonin/disc. Positive controls consisted of discs treated with hygromycin, a known antifungal agent applied in MeOH (25 µg/disc) (González et al.,

1978). Methanol-treated discs were used as a solvent control. Filter paper discs were placed 0.5 cm from each actively growing culture and incubated at 22 °C. Hyphal inhibition was assessed by measuring the zone of inhibition surrounding each disc after 24 and 48 h (n=4 agar plates for each oregonin concentration and fungal isolate).

4.2.5 Conidial germination inhibition assay

Conidia production of *Botrytis cinerea* and *Fusarium spp.* was induced by incubating actively growing fungal cultures on agar plates for approximately three weeks at 22 °C. Spore suspensions were prepared by washing the sporulating fungal cultures with sterilized Triton-X solution (0.1 g/L). The fungal culture plate wash solution, containing spores and hyphal debris, was filtered over glass wool into sterile saline (8.5 g/L NaCl). Filtered spore suspensions were centrifuged for 20 min at 3600 revolutions per minute (RPM) at 15 °C. The supernatant was discarded, and the spore pellet resuspended in sterile saline to obtain a concentration range of 1.0 x 10^6 – 5.0 x 10^6 spores/mL. Aliquots (100 uL) of each suspension were evenly distributed onto agar plates using a glass cell spreader. A methanolic solution of oregonin (10 mg oregonin/disc) was applied to sterilized filter paper discs (1 cm^2) and allowed to evaporate. Filter paper discs treated with methanol were used as a negative solvent control. Treatment discs were arranged 2 cm apart on agar plates (N=4 agar plates per fungal isolate). The inhibition of spore germination was determined by measuring the zone of inhibition around each disc after a 24 h incubation period at 22 °C.

Oregonin was also tested for fungicidal activity using a *Fusarium graminearum* conidial germination assay. This experiment was conducted by Dr. Rajagopal Subramaniam (Ottawa Research and Development Centre, at Agriculture and Agri-Food Canada) using a plate broth microdilution assay with *F. graminearum* spores at 1.0 x 10^3 cells/mL (Mogg et al., 2019). The solution in each well contained oregonin in methanol at 500-1000 µM (239 – 478 µg/mL) in potato dextrose broth (N=4 replicates per concentration). Wells containing oregonin with no spores were used as a chemical control. Wells containing spores, and no oregonin were used as a solvent control. Change in optical density (620 nm) was monitored for 42 h.

4.2.6 Bacterial inhibition assay

Colonies of *S. aureus* (4 -5) were suspended in sterile deionized water (500 uL) and a 100 uL volume of the suspension was evenly distributed onto TSA plates using a glass cell spreader (N=4 agar plates). Filter paper discs (1 cm^2) were autoclaved and treated with a methanolic

solution of oregonin (10 mg µg oregonin/disc), and the methanol was allowed to evaporate. Discs treated with methanol only were used as a negative solvent control. Treatment discs were arranged near the centre of each agar plate 2 cm apart. Microbial inhibition was determined by measuring the zone of inhibition around each disc after a 24 h incubation at 37 °C.

4.2.7 Oregonin stability

Oregonin was monitored for aqueous degradation by first dissolving a known quantity into distilled water. Aliquots of this solution (4 mL) were then maintained in glass vials (N=1) at 4 °C, 22 °C, 37 °C or 54 °C, and sampled at time zero, 24, 48, and 72 h. At each storage time point, 100 µL samples (n=4) were collected, passed through 0.45 µm nylon syringe filters and assayed by High Performance Liquid Chromatography (HPLC). Samples (5 uL) were injected onto a Thermo Scientific™ UltiMate 3000 HPLC system equipped with a quaternary pump, auto-sampler, degasser, column oven, and photodiode array detection (DAD). Chromeleon™ chromatography software version 7.2 SR4 was used to achieve instrument control, data acquisition, and evaluation. A Kinetex® reverse-phased C18 column (diameter: 4.6 mm, length: 150mm, particle size: 2.6 µm, pore size: 100Å) was used for separation. Elution was carried out with a 15 min mobile phase gradient of acetonitrile 0.1% formic acid (solvent A) and 0.1% formic acid (solvent B), a flow rate of 1.0 mL/min, and oven temperature of 30 °C. Elution gradient was held at 15% Solvent A for 1 min, linearly increased to 32% over 8 min, linearly increased to 100% over 1.5 min, held for 2 min, and linearly decreased to 15% over 1.5 min. Degradation was monitored by comparing total oregonin peak area (mAU at 280 nm) at each storage time point.

4.3 Results

4.3.1 Hyphal inhibition assay

A preliminary test of oregonin on hyphal growth inhibition was performed using in-house cultures of *C. purpureum, Fusarium spp*., *B. cinerea, S. vesicarium, T. angustata, G. applanatum., B. exigua*, and *S. parasitica* with filter paper discs treated with 6.25 - 25.0 µg oregonin/disc. No hyphal inhibition was observed for any of the species tested during the 24 h test period, yet fungal inhibition was observed for the positive control hygromycin. When the procedure was repeated with 75.0 - 125 µg oregonin/disc, similarly no inhibition was observed (Figure 4.1). We decided to further investigate activity of oregonin against the in-house *Fusarium* isolate tested previously (species not known), because susceptibility of oregonin had

been reported against *Fusarium semitectum, F. tricinctum, F. equiseti, and F. proliferatum* (MIC values 9.76 – 313 µg/mL) (Novaković et al., 2015) . Using the same hyphal inhibition procedure, we tested susceptibility of *Fusarium spp.* using filter paper discs treated with 10 mg oregonin/disc, yet again no antifungal activity was observed following incubation for 24 h at 22 °C.

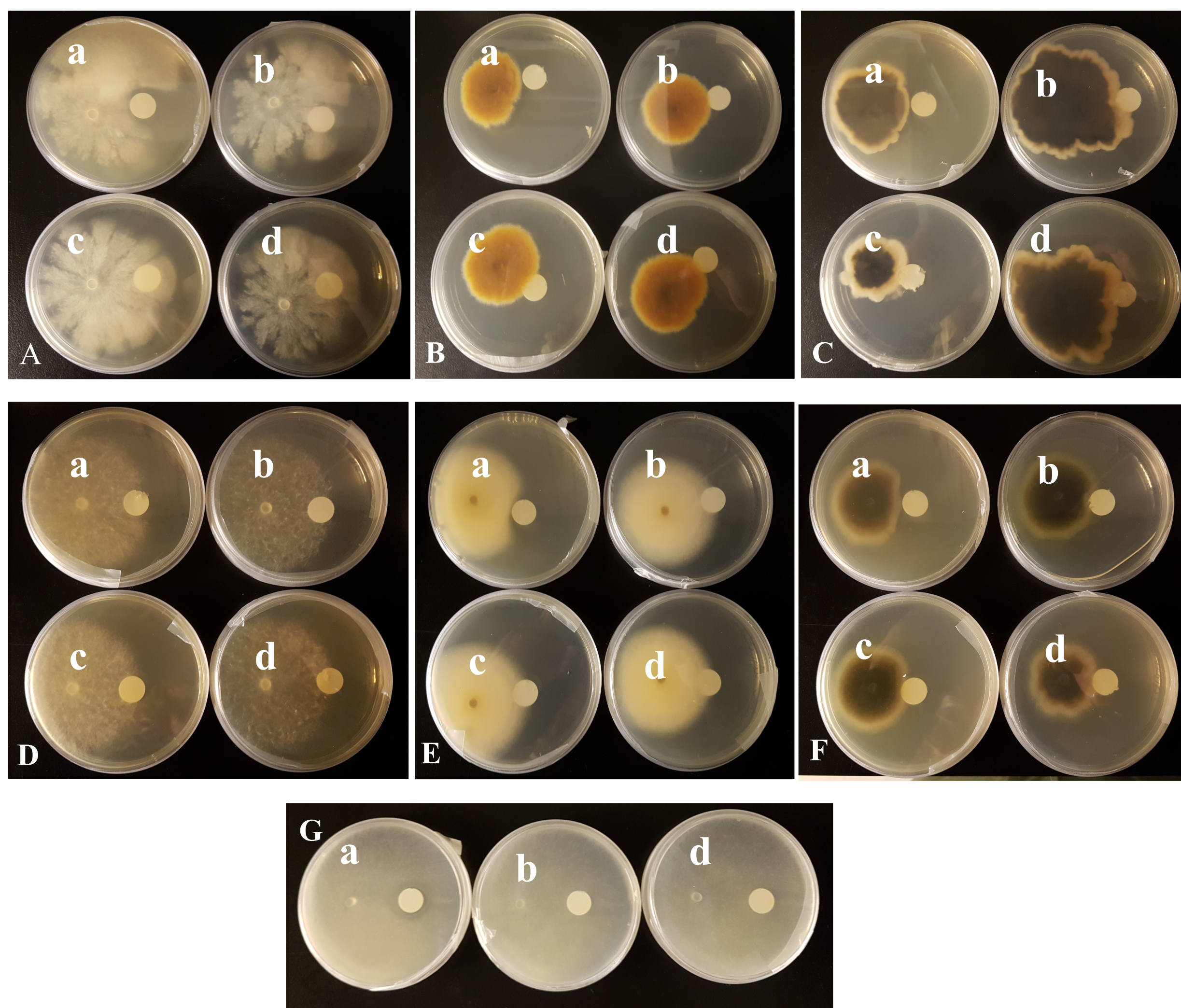


Figure 4.1 Agar plate assay to evaluate hyphal growth inhibition of selected plant-associated microorganisms by oregonin. Isolates shown: (A) *Ganoderma applanatum* (B) *Fusarium spp.* (C) *Boremia exigua* (D) *Chondrostereum purpureum* (E) *Truncatella angustatta* (F) *Stemphylium vesicarium* (G) *Saprolegnia parastica*. Each plant contained a disc with (a) 25 µg hygromycin (b) Methanol only. (c) 75 µg oregonin in MeOH (d) 125 µg oregonin in MeOH

4.3.2 Conidial germination inhibition assay

Next, we examined the effect of the compound on *B. exigua* and *Fusarium spp.* conidia, after considering that conidial germination may be more sensitive to oregonin than hyphal growth. No inhibition was observed for either species after 24 h. Oregonin was also tested against *F. graminearum* conidial germination using a broth microdilution procedure, by collaborators at Agriculture and Agri-Food Canada Ottawa Research and Development Centre. No inhibition at oregonin concentrations ranging from 500-1000 µM was observed (data not shown). Finally, we checked for growth inhibition of *S. aureus,* a bacterial pathogen previously reported to be sensitive to oregonin (Choi et al., 2012). After 24 h, we observed an average 2 mm zone of inhibition (Figure 4.2).

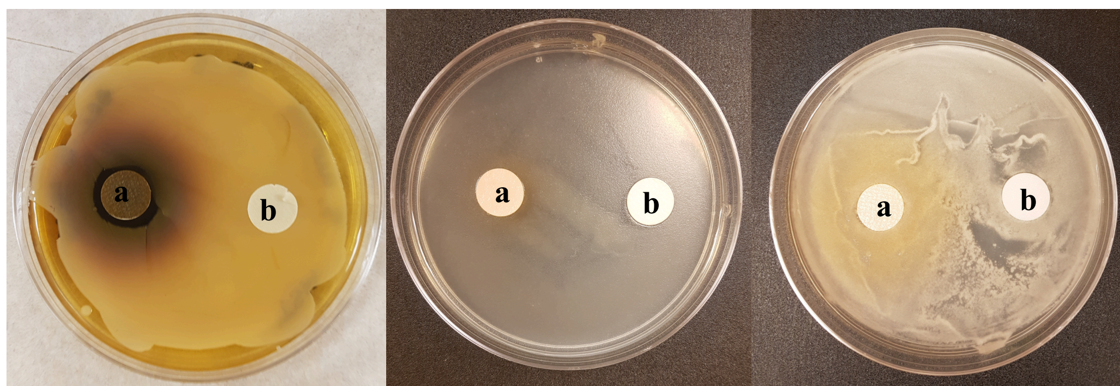


Figure 4.2 Agar plate assay for inhibition of conidial germination by oregonin. Isolates shown include: (A) *Staphylococcus aureus*, positive control. (B) *Botrytis cinerea* conidia (C) *Fusarium spp*. conidia. Each plate assay test contained a disc with: (a) 10 mg oregonin (b) MeOH only (solvent control)

4.3.3 Oregonin storage stability

To rule out the possibility that the lack of anti-fungal activity observed was due to oregonin degradation in aqueous agar media, we tested the stability of oregonin in aqueous media directly. Oregonin was dissolved in aqueous solution at temperatures ranging from 4 – 54 °C. As determined by HPLC, aqueous oregonin solutions degraded approximately 13 % every 24 h at 22 °C. Aqueous degradation of oregonin occurred slowly over time at low temperatures, and more rapidly at high temperatures (Figure 4.3A). Degradation was visible as a change in color of the solution from colorless to orange (Figure 4.3 B-C), which has been shown to be associated with oregonin oxidative or hydrolytic degradation (Karchesy, 1974). Our results suggest that oregonin degradation was too slow to have substantially affected our bioassays, which ran for a 24 h period.

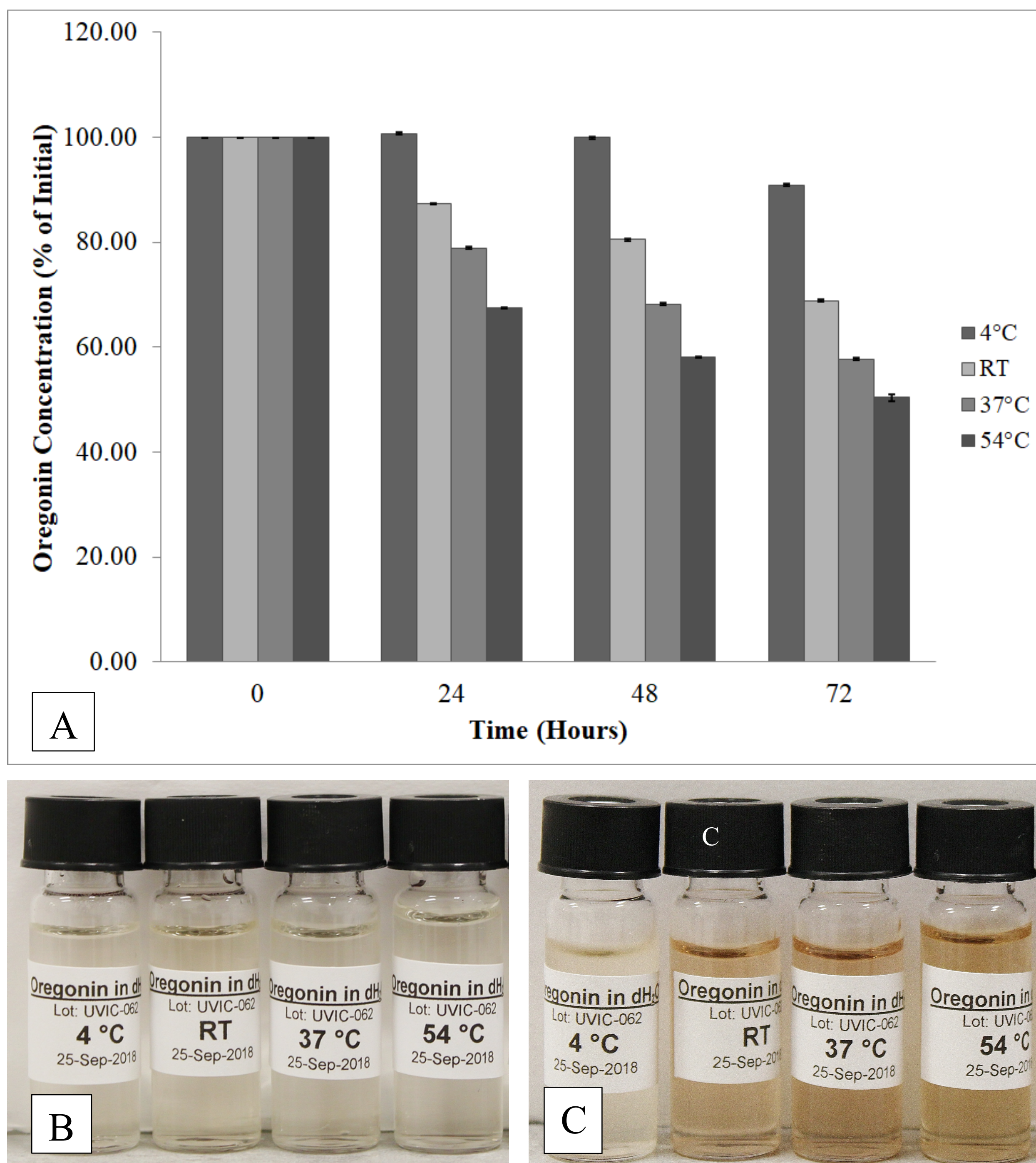


Figure 4.3 (A) Oregonin stability in aqueous solution at different storage temperatures over 72 h expressed as a percentage of initial concentration. Error bars represent standard deviation (SD) between HPLC replicates. (B) Oregonin in solution at T=0 h. (C) Oregonin in solution at T=72 h.

4.4 Discussion

In contrast to several previous studies that have reported antifungal activity of oregonin against various yeasts and human pathogens, we observed no inhibition for any of the plant pathogenic fungal species tested. We considered that the discrepancy may be due to the purity of our compound, differences in activity between selected species, or due to differences in our methods from previous reports. To validate our results, we tested for both hyphal and conidial growth inhibition. In addition, an independent study was performed by Dr. Rajagopal Subramaniam at Agriculture and Agri-Food Canada using *F. graminearum* spores. We also confirmed that under our experimental conditions, oregonin degradation is too slow to account for our results. These controls confirmed that the discrepancy between our results and those previously reported was not due to an error in methodology in the current study, or due to degradation of the compound in agar media. Furthermore, in chapter 2, the structure and purity of oregonin used in our study was exhaustively validated by NMR, UV, HPLC and MS, demonstrating the purity of our compound (96.14 %). In previous studies by Saxena et al. (1995), Novaković et al. (2015), and Abedini et al. (2016), oregonin was isolated using thin layer chromatography, semi-preparative HPLC and centrifugal partition chromatography, respectively. In all cases, preliminary extraction was performed using organic solvent. The extraction method in Chapter 2 used a novel aqueous extraction technique, thus any co-eluting minor impurities were potentially dissimilar to those in previous reports. One possible explanation for the discrepancy, therefore, is that a minor oregonin impurity in the previous literature enzymatically activated oregonin activity or had a synergistic effect.

We examined species specificity of oregonin bioactivity further by performing more rigorous testing on the in-house *Fusarium spp*. A study by Novaković et al. (2015), had reported oregonin activity against several *Fusarium* species (F. *semitectum, F tricinctum, equiseti, proliferatum*); however, we did not observe any oregonin inhibition against our *Fusarium spp.* at considerably greater concentrations (10 mg oregonin/disc) than the MIC values detailed in the report by Novaković et al (9.76 – 313 µg/mL) (Novaković et al., 2015). However, when using disc diffusion assays to test for oregonin inhibition of *S. aureus* growth, a bacterial species known to be susceptible (Choi et al., 2012), a small zone of inhibition (2 mm) was observed. Our results suggest that oregonin inhibition may be highly species-specific for fungal taxa, and the

plant-associated taxa selected for testing may be more resistant than the strains of fungi evaluated in previous reports.

The functional groups thought to contribute activity to oregonin, such as the C-3, Δ^4 double bond, and 5-xylopyranosyl group (enone), may be targets for hydroxylases, peroxidases or other cellulolytic enzymes that are secreted by plant-associated fungi (Novaković et al., 2015). Furthermore, since oregonin is labile at acidic pH, it may be subject to structural modification from acidifying fungal secretions (Karchesy, 1974). The microorganisms examined in this study possess numerous virulence factors as well as a complex array of substrate-degrading enzymes that may contribute to oregonin degradation. For example, *Botrytis cinerea*, a non-specific necrotrophic plant pathogen, releases a number of peroxidases and hydrolytic enzymes. Such enzymes may break oregonin down into products with reduced activity (Gil-ad et al., 2000; Kapat et al., 1998). *Chondrostereum purpureum*, a lignin degrading basidiomycete and frequent colonizer of alder, produces enzymes that function to degrade host cell walls (e.g., pectinases, laccases, peroxidases) (Vartiamäki et al., 2008), but could potentially also target and oxidize oregonin functional groups. The water mold *S. parasitica* expresses the tyrosinase gene as part of the melanin biosynthetic pathway, an oxidase enzyme we previously determined can use oregonin as a substrate (Saraiva et al., 2014) (tyrosinase data not shown). The genus *Fusarium* encompasses both human and plant pathogens (Dignani and Anaissie, 2004), and possesses species dependent virulence factors including acid production (oxalic acid, fusaric acid), hydrolytic enzymes, and peroxidases (Marín et al., 1998; Swain and Ray, 2009; Ding et al., 2018; Lee et al., 2018). As a wood decay species, *G. applanatum* similarly produces peroxidases and other cellulolytic enzymes (Maganhotto De Souza Silva et al., 2005). Although little is known about the factors contributing to *T. angustata*, and *S. vesicarium* virulence, they may similarly produce or secrete cellulolytic enzymes or virulence factors to infect plant hosts. Therefore, many plant-associated fungi secrete enzymes that may oxidize and degrade compounds subject to oxidative or hydrolytic degradation, including oregonin (Karchesy, 1974).

Our results suggest oregonin may not be active as a general anti-fungal plant metabolite, and that red alder produces oregonin for another biological purpose. The data presented in Chapter 3 demonstrated that foliar oregonin can deter lepidopteran insects, which may explain why red alder produces the compound in such large quantities. More work will need to be done to explain the discrepancy between the lack of fungal activity observed in our work and high levels of bioactivity observed in previous reports. It would be interesting to investigate oregonin degradation products in agar media after fungal inoculation to determine if fungi tested are able to degrade

oregonin. Future work should investigate additional fungal species so that the observed trends can be confirmed, or the discrepancy with previously published research resolved. One could further compare the secretome of susceptible and non-susceptible groups of fungi to determine the virulence factors or secreted enzymes, or metabolic pathways that are contributing to a lack of fungal sensitivity to this inhibitory compound. Future work should also investigate whether or not the antimicrobial activity of oregonin is activated by enzymes and synergists that may have been removed during purification of oregonin in our study.

Chapter 5. General conclusions and future directions

This thesis represents the first systematic evaluation of the biological activity of the diarylheptanoid xyloside oregonin, a phytochemical found at significant concentrations in red alder leaf and bark material, on plant pathogenic fungi and insects. My studies were motivated by recent findings suggesting that alder accessions with high foliar oregonin content showed reduced feeding by *Malacosoma californicum* (western tent caterpillar) (Boateng, 2019). The literature also suggested that oregonin exhibits significant antimicrobial activity against various medically relevant microbial pathogens (Choi et al., 2012; Novaković et al., 2015; Dahija et al., 2016). To evaluate oregonin, I first developed and optimized an efficient method of oregonin purification. After purifying sufficient quantities of the compound from red alder leaves and bark, I tested oregonin's effect on hyphal and spore germination inhibition against a variety of important plant-associated fungi, including several alder pathogens. Using insect choice and toxicity tests, I evaluated oregonin's activity on four species of lepidopteran larvae: *Orgyia leucostigma* (white-marked tussock moth), *Hyphantria cunea* (fall webworm), western tent caterpillar (*Malacosoma californicum*), and *Trichoplusia ni* (cabbage looper). The activity of phenolic phytochemicals is sometimes potentiated by foliar or insect oxidases or hydrolytic enzymes; therefore, I assessed the level of insect feeding deterrence after subjecting purified oregonin to oxidation and acid hydrolysis (Appel, 1993; Hemming and Lindroth, 2000). Finally, I evaluated the seasonal variation of oregonin content in various tissue types and ontogenic stages of red alder, to indirectly assess how well alder might be defended from insects and fungal plant pathogens across the growing season. Major findings and future research recommendations are as follows.

5.1 An easily-scaled method was developed for the extraction and purification of oregonin from red alder leaves and bark

In order to directly test oregonin's biological activity, I needed to obtain sufficient quantities for biological testing. Methods established in Chapter 2 were developed with potential scale-up in mind and are easily augmented to handle volumes necessary for the processing of red alder forestry residues. As we learn more about the potential therapeutic value of oregonin and other diarylheptanoids, the ability to easily process red alder forestry residues may prove to be valuable, as they typically contain oregonin at high concentrations and are otherwise of little commercial value. The method developed in this thesis was the first to examine the robustness of

oregonin to spray drying, a useful and frequently used industrial tool used to dry large quantities of water from thermally sensitive molecules. My results suggest that spray drying may be a useful method to dry aqueous extracts of similar compounds without significant degradation. These techniques may serve as a guide in future studies that extract and purify other heat labile phytochemicals from plant material. The purified oregonin was characterized by HPLC, MS, UV, and NMR, and represents the most complete characterization of oregonin to date. My results, therefore, will be a beneficial reference in forthcoming studies requiring oregonin identification.

5.2 Oregonin exhibits feeding deterrent activity against several leaf eating lepidopteran insects

Although oregonin is known to possess a wide range of important pharmaceutical properties, its biological role has thus far been largely undetermined (Ren et al., 2017). A few studies have suggested that oregonin, platyphylloside and other diarylheptanoids may act as anti-nutritive defenses, and correlate with a decrease in preference to browsing ruminants (Sunnerheim-Sjöberg and Knutsson, 1995; González-Hernández et al., 2000). Further, platyphylloside, oregonin and other phenolics have been discussed as potential contributors of insect resistance in red alder and paper birch (Muilenburg et al., 2011; Boateng, 2019); however the compounds have never been directly tested on insects. This thesis, therefore, was the first study to directly test a purified diarylheptanoid against tree-feeding caterpillars. In Chapter 3, I examined oregonin's biological activity on a variety of leaf-eating lepidopterans, including against *H. cunia* and *M. californicum*, frequent alder pests in the Pacific Northwest of North America (Harrington, 2006). I concluded that oregonin has low insecticidal activity, but may act as an insect feeding deterrent at concentrations similar to the threshold observed in western tent caterpillar leaf-feeding bioassays (Boateng, 2019).

The biological activity of certain phenolics is often potentiated by enzymes such as hydroxylases or oxidative reactions, which may generate toxic superoxide anion radicals, hydroxyl radicals, peroxidases and quinones. In Chapter 3, I observed no difference in oregonin feeding deterrence after oxidation or hydrolysis, suggesting that the observed feeding deterrent activity may be a general phenolic response, and is not hydrolytically or oxidatively activated. Our approach, however, was limited by oregonin reaction products that may have been transient, and unstable for testing. Additional work will need to be done to determine if oregonin activity is associated with short-lived downstream reaction products, such as the oregonin aglycone. Further, I only examined tree-feeding lepidopteran larvae, and did not consider other groups or

feeding guilds of insects. Although lepidopterans represent one of the largest and most important group of red alder pests in Pacific Northwest riparian ecosystems, the order is thought to be adapted to feed on a diet rich in phenolic compounds due to an alkaline midgut which prevents oxidative reactions (Appel and Martin, 1990).

5.3 Oregonin exhibits seasonal variation in alder leaves and bark

The results of Chapter 3 highlight that oregonin concentration in red alder leaf and bark tissue varies significantly throughout the growing season. Oregonin decreased from May-October in both mature and juvenile leaves, as well as in juvenile bark. Oregonin concentrations in mature bark, however, remained relatively fixed across the growing season. My results were consistent with previous studies that have explored seasonal changes in oregonin concentration in leaves (González-Hernández et al., 2000); however, the tendency for oregonin to decrease with leaf age and increase with bark tissue age was a novel finding. My results suggested that juvenile leaves may be better protected against lepidopteran insect attack early in the spring. Analysis of oregonin in bark tissue will be useful in future studies that investigate how well alder is protected from bark-boring insects.

5.4 Oregonin antimicrobial activity is more species-specific than previously reported

Research in the biomedical sector has indicated that oregonin and other diarylheptanoids possess considerable antimicrobial activity against a variety of important human pathogens (Saxena et al., 1995; Choi et al., 2012; Rashed et al., 2014; Novaković et al., 2015). In contrast, my assays in Chapter 4 did not demonstrate that oregonin possesses activity against any of the diverse plant-associated microorganisms selected for study. My results imply that oregonin antimicrobial activity may be more species-specific than studies have previously suggested. I hypothesized that the plant-associated microorganisms selected for study were better adapted to defend themselves against the repertoire of phytochemicals they encounter frequently in a plant host environment, whereas the human pathogens examined in previous reports may been more susceptible. My results suggest it is unlikely that oregonin found in red alder leaves and bark is sufficient to protect the tree from the red alder fungal plant pathogens tested in my study. I therefore concluded that oregonin may be serving an alternative biological function *in planta,* or in the surrounding ecosystem. This finding was consistent with the results of Chapter 3, which implied that high levels of oregonin may be acting in alder as an insect feeding deterrent.

5.5 Recommendations for future work

Although oregonin is known to possess a wide range of important pharmaceutical properties, its biological role has thus far been largely undetermined (Ren et al., 2017). Based on my findings in Chapter 3 and 4, it seems most probable that high biological concentrations of oregonin found in red alder leaves and bark functions to deter insects, rather than to inhibit red alder-associated microorganisms. This finding suggests that oregonin could be used as a selection criterion in future red alder breeding programs. The observed differential feeding deterrence between alder-feeding and generalist insects implied the possibility that non-adapted, introduced insects may be more sensitive to oregonin than alder-adapted species. This finding needs to be confirmed in further experiments, considering that novel insects may survive in the Pacific Northwest under future climactic conditions, and alder breeding programs aim to plant alder outside of its native range.

Although our work highlighted that oregonin is deterrent to a variety of leaf-eating lepidopteran insects, these pests may be adapted to a diet rich in phenolics. Future work on hymenopteran alder woolly sawfly, coleopteran alder flea beetle, and alder bark beetle would help to determine if there is variation in oregonin activity between insect genera, or alder-feeding guilds of insects, which may be less adapted (Harrington, 2006). There is also significant oregonin concentrations in red alder bark, which may have an alternative biological explanation. Future work should investigate oregonin's activity against bark boring insect pests such as alder bark beetle (Borden, 1969). Later studies should continue seasonal analysis of oregonin over multiple seasons to determine if bark concentrations continue to increase with tissue maturity over time. Additional work on bark oregonin concentrations over time, tissue maturity, and across alder accessions may elucidate interesting ecological implications.

Despite the high levels of oregonin antimicrobial activity reported on biomedically relevant pathogens in previous studies (Lv and She, 2012; Ganapathy et al., 2019), my results suggested that the activity of oregonin may be reduced on plant-associated microorganisms. I recommend that future work validates these findings by comparing both plant and animal pathogens in parallel. It would also be interesting to determine if the microorganisms selected in my study are similarly tolerant of diarylheptanoids from other plants such as turmeric or paper birch. Although oregonin antimicrobial activity may not be effective on alder fungal pathogens, alder leaf litter has been shown to impact the surrounding soil microbial community (Selmants et al., 2005). It would also be interesting to consider the role of oregonin in shaping the soil microbiome and alder rhizosphere, such as in the development of root symbioses with nitrogen

fixing *Frankia* bacteria. Future studies could assess the impact of oregonin on microbes involved with soil decomposition and regeneration. Although oregonin oxidative and hydrolytic chemical modifications were not shown to affect insect feeding deterrence, it would also be interesting to test if they increase or decrease antimicrobial activity, or for other purposes, such as in agricultural and pharmaceutical applications.

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Appendix A – Supplementary Tables

Table S1 Oregonin absorbance (mAU), percent recovery and percent peak area from five separate flash chromatography separations after each fraction was assayed by high performance liquid chromatography

Separation ID	**1**			**2**			**3**			**4**		
Fraction	Oregonin mAU	% Recovery*	% Peak Area	Oregonin mAU	% Recovery*	% Peak Area	Oregonin mAU	% Recovery*	% Peak Area	Oregonin mAU	% Recovery*	% Peak A
24	0.16	0.12	0.69	0.45	0.41	3.24	0.00	0.00	0.00	0.00	0.00	0.00
25	0.55	0.43	3.71	1.03	0.93	4.33	0.00	0.00	0.00	0.00	0.00	0.00
26	1.13	0.86	4.04	1.80	1.63	3.67	0.00	0.00	0.00	0.00	0.00	0.00
27	2.02	1.55	3.68	0.19	0.17	0.51	0.82	0.88	4.59	0.96	1.00	5.06
28	0.20	0.15	0.46	**52.22**	**47.20**	**86.00**	1.08	1.15	3.29	1.26	1.32	4.08
29	**63.37**	**48.62**	**87.18**	**49.79**	**45.00**	**94.14**	3.18	3.37	9.98	3.60	3.77	10.51
30	**55.99**	**42.96**	**96.08**	4.80	4.34	29.76	**45.17**	**47.94**	**86.46**	**42.41**	**44.32**	**80.66**
31	6.42	4.93	42.37	0.24	0.22	2.22	**40.37**	**42.85**	**96.20**	**43.56**	**45.53**	**96.53**
32	0.50	0.38	3.80	0.13	0.11	0.74	3.60	3.82	40.08	3.89	4.06	59.23
33	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00

*Percent recovery was estimated as the percent of total oregonin absorbance by HPLC in each fraction

The majority of oregonin eluted in two fractions for each separation (**bolded**)

Appendix B – Supplementary Figures

All supplementary figures prepared by Dr. Chakravarthi Simhadri and Dr. Jeremy Wulff in the Department of Chemistry at the University of Victoria.

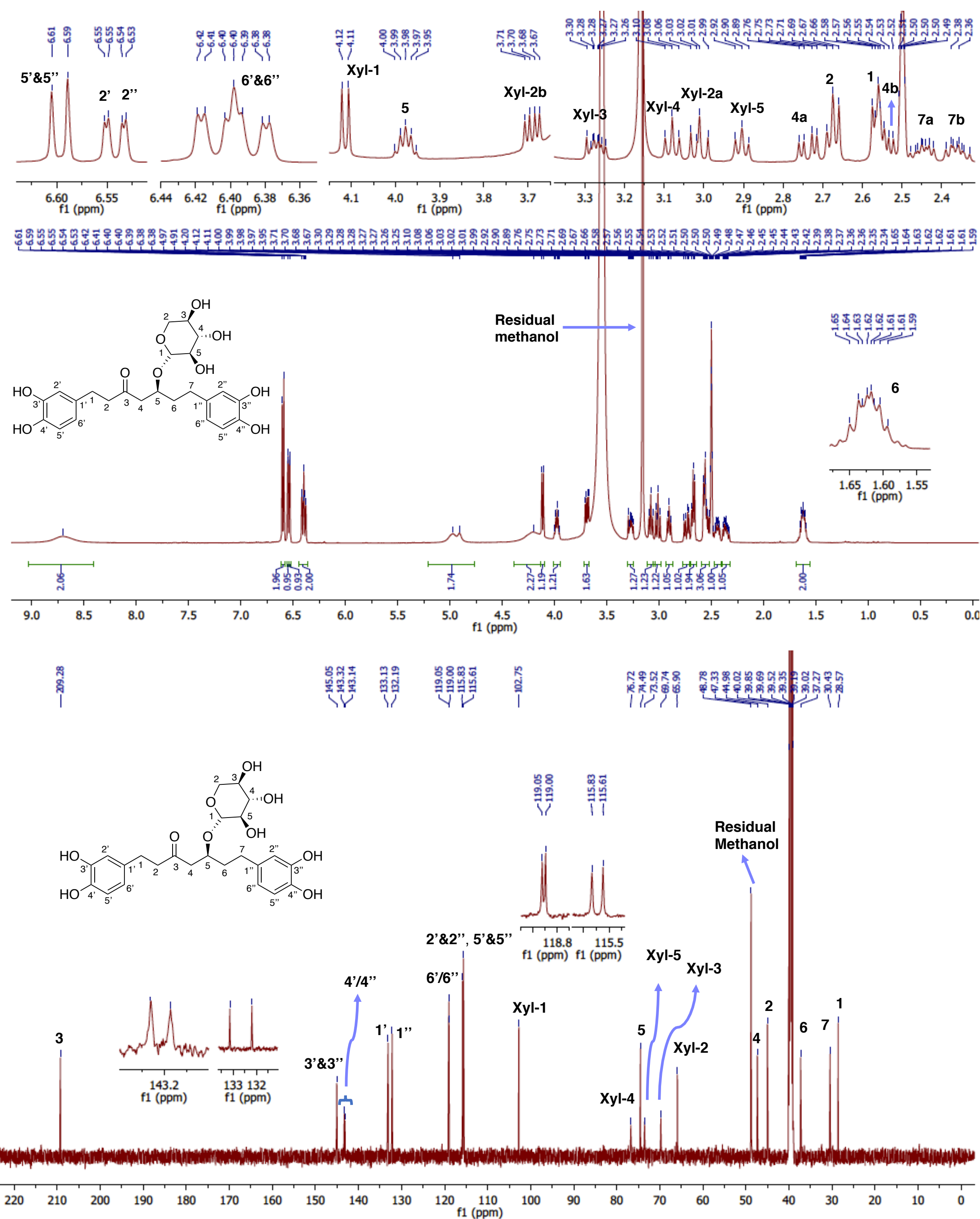

Figure S1 ^{1}H NMR spectrum (top) and ^{13}C NMR spectrum (bottom) of oregonin. Spectra were collected in DMSO-d_6 containing 10 µL D_2O. Proton/deuterium exchange on the phenolic and hydroxyl OH groups led to artificially low integration values; see Figure S5 for a spectrum collected after further evaporation, which shows reduced exchange and more accurate integrations for these signals.

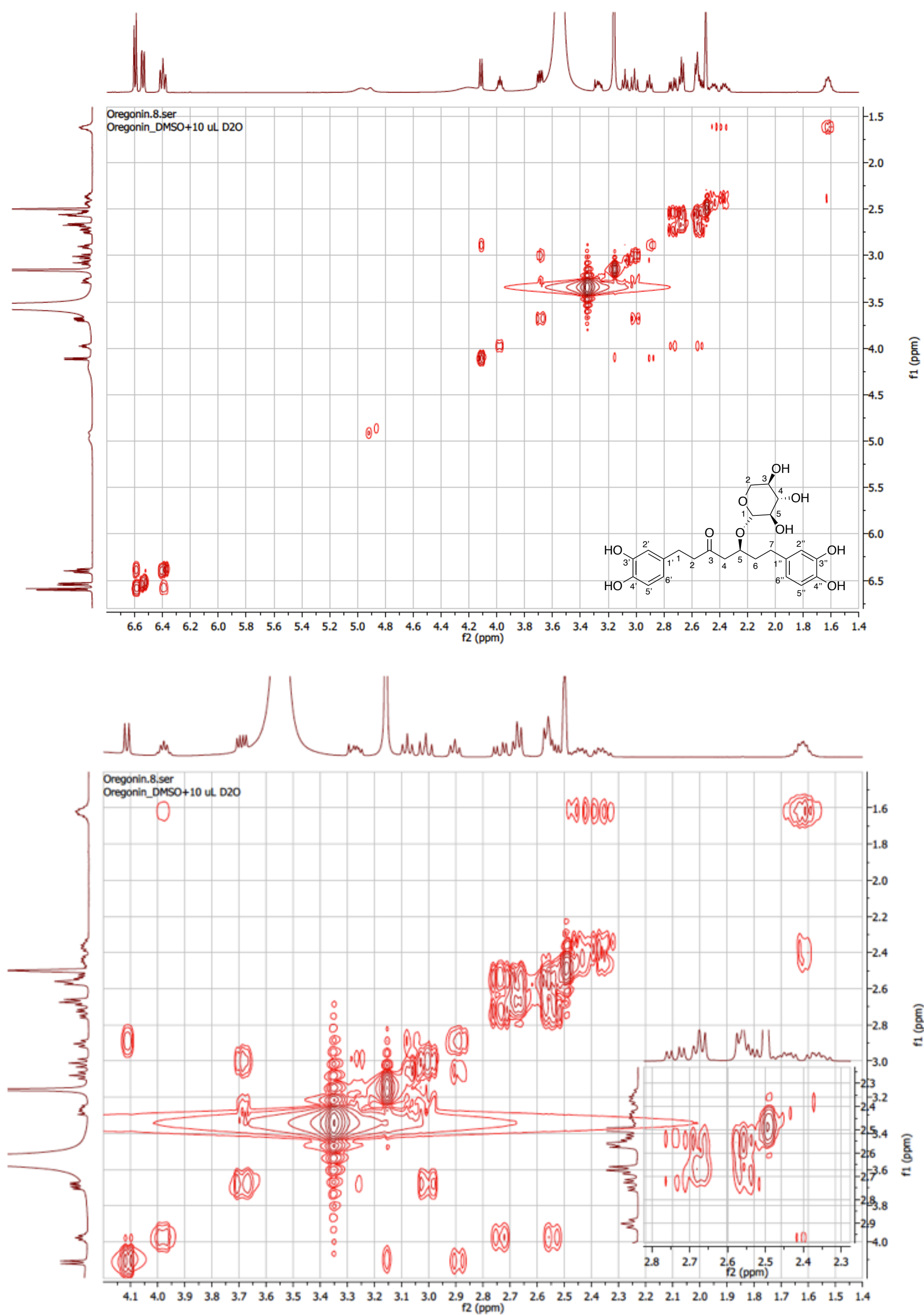

Figure S2 ^{1}H-^{1}H COSY spectrum of oregonin (top), expansion of 1.4–4.2 ppm region (bottom).

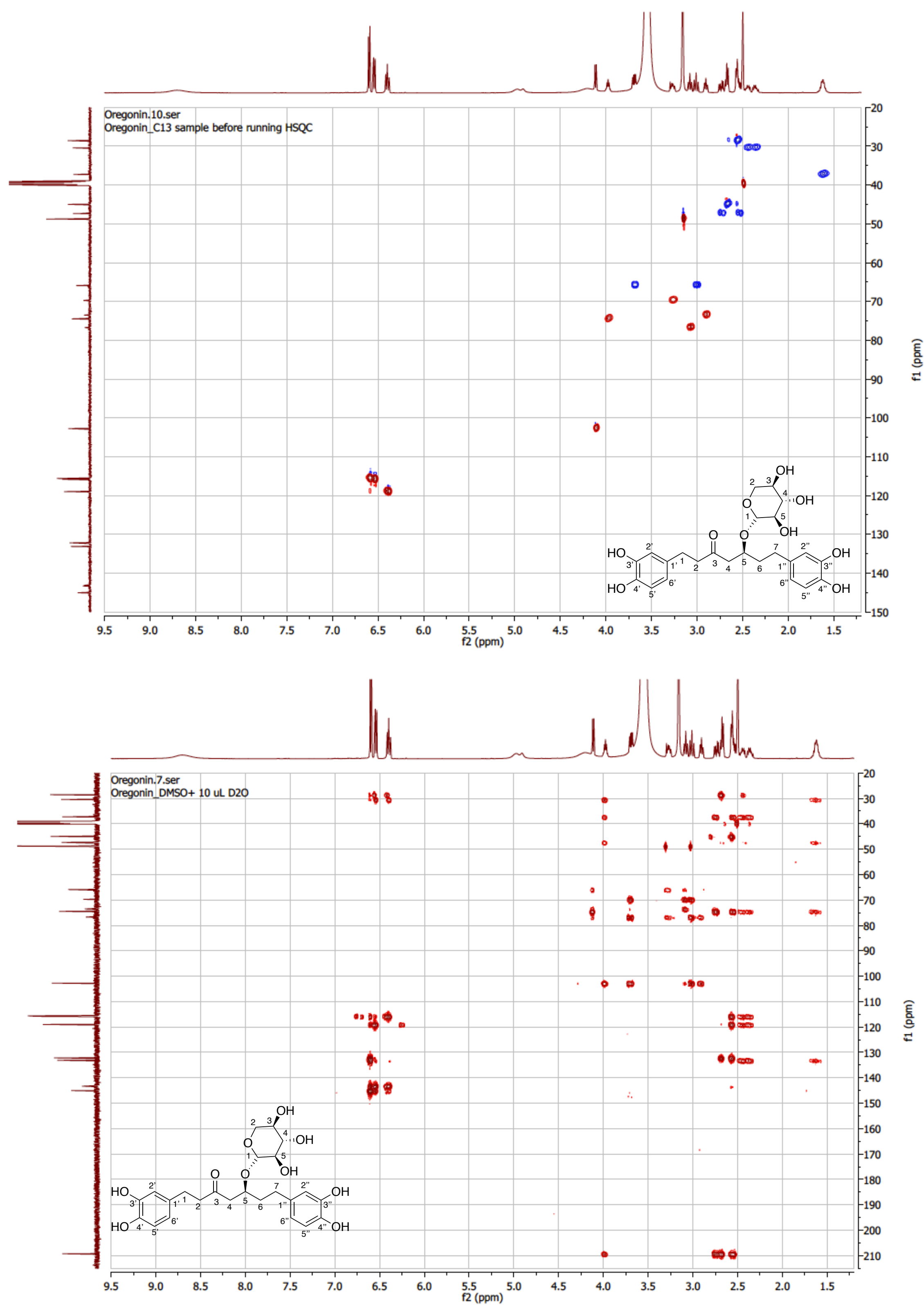

Figure S3 HSQC spectrum (top) and HMBC spectrum (bottom) of oregonin.

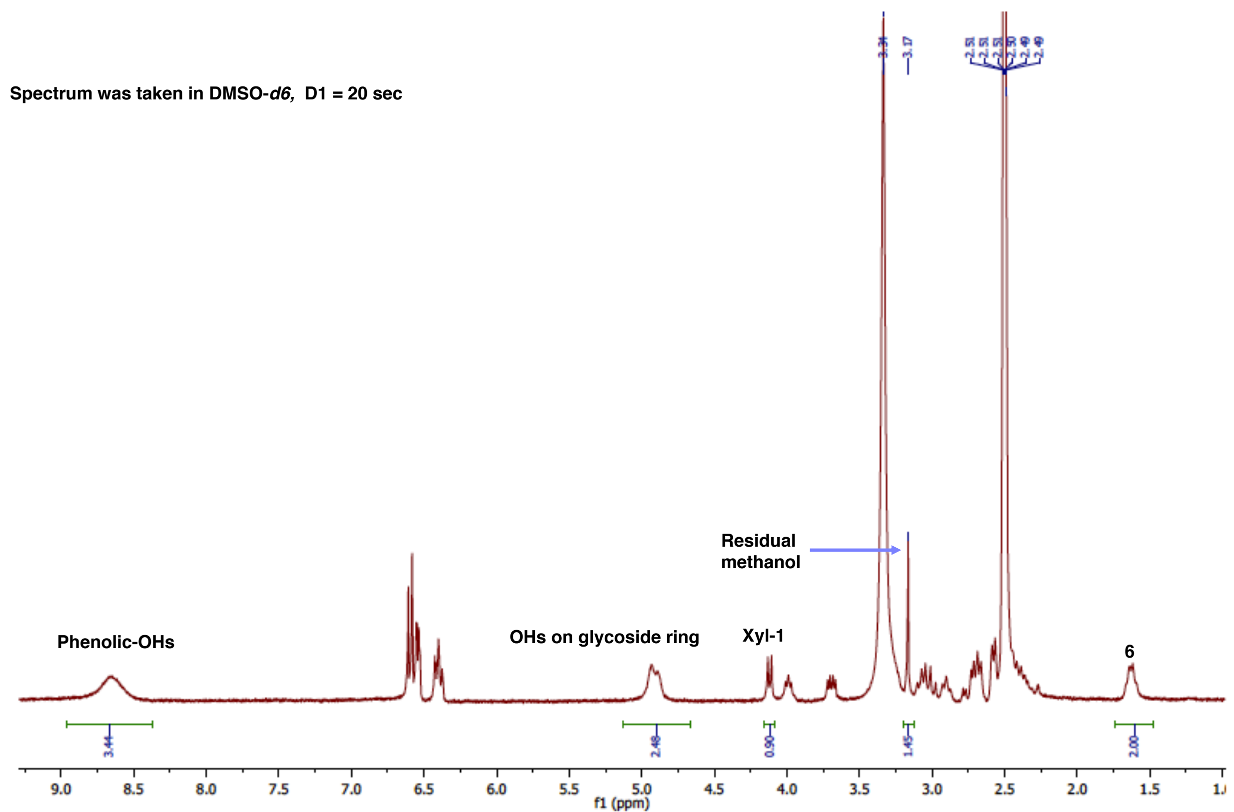

Figure S4 Estimation by ^{1}H NMR analysis of methanol content in the oregonin sample after further vacuum drying, prior to specific rotation measurement.

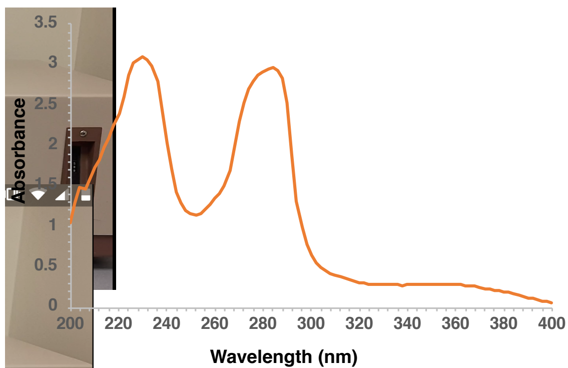

Figure S5 UV spectrum of oregonin in ethanol.