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Matthew Mendelsohn March 27, 2017

Anti-bacterial Potential of the Genus *Rubus*

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Abstract

Anti-bacterial Potential of the Genus *Rubus*
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With the decreased efficacy of many antibiotics in the face of rising drug resistance, there is an urgent need for the development of new and alternative therapeutics to combat this looming crisis. A novel approach to killing resistance bacteria utilizes active compounds to inhibit bacterial defense mechanisms, thereby increasing the efficacy of current antibiotics. Previously, an extract prepared from the roots of *Rubus ulmifolius,* was shown to inhibit biofilm formation in *S. aureus*, a major bacterial defense mechanism, and significantly improve biofilm clearance when used concomitantly with an antibiotic. The present study investigates the chemistry, bioactivity and anti-biofilm properties against *S. aureus* of eight different *Rubus* species and their various plant parts to see if the activity and chemistry are conserved across the genus.

Liquid extraction and partitioning techniques were employed to prepare 4 refined partitions and 1 crude extract for each of the 8 *Rubus* species and 11 plant parts tested. Broth dilution methods were employed to determine the minimum inhibitory concentrations (MIC) after 18 hours of growth using an optical density reading. Anti-biofilm effects were assessed by growing biofilms for 24 hours, then fixing and staining with crystal violet. After washing, the biofilms were eluted, optical density readings were taken, and minimum biofilm inhibitory concentrations (MBIC) were calculated. The presence of ellagic acid and two of its glycosidic derivatives was assessed using HPLC, matching retention times and UV spectrums of our extracts to the three standards prepared.

Ellagic acid was found to be present within every *Rubus* species; however, none of the species contained either of the two derivatives examined. Extracts prepared from each plant species exhibited an MIC_{50} at concentrations ranging from 32 – 256 μg/ml. Each species tested inhibited biofilm formation at a concentration below where you see growth inhibition. Two extracts in particular, 730 and 735D, potently inhibited biofilm formation at 8 μg/ml without inhibiting growth significantly and therefore represent promising candidates for the development of novel natural product inhibitors of biofilm formation. We recommend further studies and bioassay-guided fractionation be done to develop these compositions into antibiotic adjuvant therapeutics against *S. aureus.*

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Table of Contents

List of Tables and Figures

CHAPTER 1: INTRODUCTION

My Inspiration

Before I delve into the basis of my research, I present this personal anecdote as an introduction to my field of study and to illustrate the pervasive influence of the problem at hand: antimicrobial resistance.

In Spring 2016, I noticed a significant abscess growing on my inner right thigh. This had happened to me before so I was not too concerned. I immediately applied topical antibiotics (Mupirocin) that I had used in the past; however, the abscess and the pain only grew larger with each day. Finally, after sustaining the abscess for four days without any progress I decided to go to the local urgent care clinic in order to have the abscess incised and drained. I left the clinic that day thinking that my problem was over. I was given a regiment of Bactrim DS and I knew that this "magic bullet" would cure me fully. Within two days, I was admitted into the hospital for methicillin resistant *Staphylococcus aureus* bacteremia. My liver enzyme levels were ten times higher than the normal range and my white blood cell count was severely elevated. I was given a different antibiotic (vancomycin) intravenously in order to rid my body of the bacteria due to the ineffectiveness of the Bactrim DS. Unfortunately, I had an allergic reaction to this new antibiotic only exacerbating my current condition and limiting my options for treatment. Each day I felt worse experiencing pain I had never felt before. With few alternatives left, I was given the last line of defense antibiotic known as linezolid. Fortunately, the linezolid was very effective and my bacteremia was resolved. Over the next month I regained my health and my liver levels returned to the normal range.

Although I did not face any further complications or any permanent liver damage, I can't help but wonder, what would have happened if the linezolid did not work or the *S. aureus* was resistant to the linezolid? Would I still be standing here today?

Looming crisis of antibiotic resistance in *S. aureus*

Each year in the United States, there are at least 2 million people that acquire severe infections from bacteria that are resistant to at least one class of antibiotics utilized to treat those infections (Control and Prevention 2015). The CDC estimates that there are at least 23,000 deaths caused by antibiotic resistance yearly (Control and Prevention 2015). We are rapidly regressing towards the pre-antibiotic era where a simple flesh wound could cause severe harm and even kill a patient.

One of the most prominent pathogens causing this problem is *Staphylococcus aureus,* which is "responsible for 1% of all hospital admissions and leading to an estimated cost of $9.5 billion per year in the United States alone" (Pollitt, West et al. 2014). The strength of this pathogen can be explained by its persistent ability to acquire resistance to a multitude of antibiotics, most famously: methicillin. MRSA (methicillin resistant *Staphylococcus aureus*) kills more Americans each year than HIV/AIDS, Parkinson's disease, emphysema, and homicide combined (Gross 2013, Golkar, Bagasra et al. 2014).

Natural products as a source of new chemical entities: an ethnobotanical approach to drug discovery

To help combat this looing crisis of antibiotic resistance, an ethnobotanical approach to drug discovery should be employed. Ethnobotany is the study of human interaction with plants (Quave and Pieroni 2015). Ethnobotany can be employed to learn

and apply the far-reaching wealth of knowledge about the uses of plant and animal products, accumulated by indigenous peoples over centuries, to maintain human health (Cowan 1999). Throughout history, humans have relied on plants and nature for their basic needs including food, shelter, clothing, fertilizers, flavors and fragrances, and importantly, medicines (Gurib-Fakim 2006). Plants have formed the basis of traditional and alternative medicines that have worked for thousands of years. Over the past century, the study of ethnobotany has played a vital role in the development of new drugs and therapeutics. In fact, "natural products and their derivatives represent more than 50% of all the drugs in clinical use in the world" (Gurib-Fakim 2006). Additionally, 25% of the drugs prescribed by physicians in the developed world contain the chemicals produced by flowering pants (Houghton 2001).

Plants possess a rich and unique chemistry thought to have originated as a defense mechanism against predation by microorganisms, insects, and herbivores (Cowan 1999). They often contain hundreds of secondary metabolites, many of which have been found to have antimicrobial properties (Cowan 1999). These secondary metabolites are stereospecific molecules with very complex skeletons (Houghton 2001). The extensive molecular complexity and diversity of secondary metabolites throughout the plant kingdom provides a rich source for the discovery of novel drugs (Houghton 2001). In some cases, certain chemical compounds from plants may not be therapeutic and can actually cause serious harm; however, the natural compound can still be used as a guide to synthesize related compounds that are therapeutic and cause no harm. This approach led to the introduction of several major groups of drugs, including one of the most well-known drugs in the world, aspirin (Houghton 2001). Directing research toward the study

of plants is beneficial because plants as a raw material may possess useful compounds or therapeutics and the molecular biology and biochemistry of the plant provides guidelines for rational drug development (Gurib-Fakim 2006).

With the looming crisis of antibiotic resistance, there is an urgent need for the development of new antimicrobials. Until the 1980s, all of the major pharmaceutical companies ran antibacterial research and development programs (Leeb 2004). Today, many of these programs have been fully dropped or severely reduced, and there are many reasons for this. If a pharmaceutical company develops a new antimicrobial to which there is no resistance, which would be the goal, this antibiotic would rarely be used. This is due to the nature of antibiotic resistance because with increased use there is increased resistance. Therefore, this new drug would be saved as a last line of defense, utilized very little, and would not be a very profitable product (Leeb 2004). Furthermore, antibiotics are not very profitable because they cure disease rather quickly. Companies favor focusing on chronic diseases such as hypercholesterolemia and arthritis, for which patients will be purchasing therapeutics over a lifetime (Leeb 2004). With antibiotics, a patient may use the drug for one week and then never encounter it again. Lastly, the cost of drug development itself is so high, which often pressures companies to develop high return products (Leeb 2004). Directing our focus towards natural products is a much cheaper and sustainable method for developing new antimicrobials.

Project Aims and Research Question:

The aim of this research is to investigate the bioactivity and chemical makeup of eight distinct *Rubus* species and their various plant parts. Previously, an extract prepared from the roots of Elmleaf blackberry, or *Rubus ulmifolius*, exhibited dose-dependent inhibition of biofilm formation that was conserved across all *S. aureus* clonal lineages, including clinical MRSA isolates (Quave, Estévez-Carmona et al. 2012). When this extract was used concomitantly with antibiotics from varying functional classes to treat an infective device, significant improvement in biofilm clearance was observed over treatment with antibiotic alone (Quave, Estévez-Carmona et al. 2012). This study aims to build off these results to discover whether differing *Rubus* species share the same bioactivity and chemical makeup to that of *Rubus ulmifolius.* Additionally, the study aims to discover if the anti-biofilm activity of *Rubus ulmifolius* against *Staphylococcus aureus* is conserved across the genus.

This process involves a thorough literature review on the historical and traditional uses of *Rubus*. In addition to historical and traditional use, the review addresses the current state of research on the *Rubus* genus and its anti-biofilm properties, active compounds, and cytotoxicity in order to guide laboratory research and evaluate results. These *S. aureus* biofilm inhibitors isolated from the various *Rubus* species could ultimately be used as alternative or adjuvant therapies to improve the efficacy of existing antimicrobials.

CHAPTER 2: LITERATURE REVIEW

Why blackberries?

History of Rubus Pharmacology

While both blackberries and raspberries are commonly thought of as a food source or as a simple shrub, the diverse genus *Rubus* has been used for centuries due to its medicinal properties. This history dates back to the Ice Age when *Rubus* species were a food source and medicinal plant for native peoples (Husted and Connolly 2003). In the New World, archeologists found evidence of *Rubus* as a food source in Newberrt Crater near Bend, OR. Radiocarbon dating puts the artifacts and food remnants at ~8000 BCE (Husted and Connolly 2003). The next documentation of *Rubus* was in the writings of both Aeschylus (Hummer and Janick 2006) and Hippocrates around 500-370 BCE (Hummer 2010). Within these documents, Hippocrates advocated blackberry (*batos)* stems and leaves soaked in white wine to be used as an acerbic poultice on certain wounds and in difficulties of childbirth (Littré 1999). A poultice is a type of bandage or cloth typically filled with plant material applied directly to the body in order to relieve any soreness or inflammation.

Following Hippocrates and Aeschylus, the first published description and image of *Rubus* written as *Batos* (blackberries) and *batos idaia* (raspberries) are referenced within the *Juiliana Anicia Codex*: a medicinal manuscript written around 500 CE that is largely based upon the *De Materia Medica* written by Dioscorides around 65 CE (Figure 2.1. A)(Hummer and Janick 2006). *De Materia Medica* has been translated into several different languages and reproduced countless times; however, the most famous of these manuscripts to survive is the *Juliana Anicia Codex* (Hummer 2010*)*. The English

translation of the reference to both *Rubus* spp. and *Rubus ideaus* in *De Materia Medica* states, “The decoction of its branches (*batos)* contracts, desiccates, dyes hair, and stops diarrhea when drunk, keeps in control, leucorrhea, and is suitable for the bit of the *prester* (A kind of serpent whose bite is poisonous). When chewed, the leaves strengthen the hums and health the thrush, plastered on, they keep in control shingles, treat head scurf, prolapses of the eyes, callous lumps, and hemorrhoids, and they are suitable to apply ground up on those with stomach and heart ailments. But its juice, extracted from the stems and leaves and condensed in the sun, will accomplish everything better. The juice of its fully ripened fruit is suitable for mouth ailments, its half-ripe fruit stops diarrhea when eaten, and its flower check diarrhea when drunk with wine” (Figure 2.1. A) (Beck 2005). Dioscorides continues on the next page of the manuscript to discuss the additional use of a different *Rubus* species, the raspberry, or *batos idaia*. He writes, “it can treat the same conditions as the one before it, but its flower helps far more for eye inflammations when triturated with oil and smeared over them; it also cools erysipelas and it is given in a drink with water to those with stomach problems” (Beck 2005).

During medieval times, much of the standard information regarding herbal medicine was similar to the ancient Latin and Greek texts; however, the works were additionally instilled with “pagan superstition and Christian ritual” (Hummer 2010). More specifically, in 920 CE, there was an Anglo-Saxon physician’s manual titled, The *Læchbook of Bald* which detailed which herbal remedies were needed for certain ailments (Hummer 2010). An image of a blackberry is detailed within the text along with a caption that suggests the use of a bramble for dysentery (Figure 2.1. B) (Rohde 1922). Raspberries and blackberries continued to be detailed in numerous texts after the

Læchbook of Bald; however, most writings did not reference any medicinal uses of these fruits until around the middle of the 16th century with the advent of the work of John Gerard, a London botanist and herbalist (Hummer 2010). John Gerard published Gerard's Herbal, a book that details the use of red raspberries to heal the eyes that "hang out" as well as the use of the same decoction previously mentioned to fasten teeth (Hummer 2010). By the mid 1600s in Europe, the use of brambles (*Rubus* species) was very well known and a new manual or guidebook was not considered needed. *Rubus* was used in the same manner as before: to dye hair, as an antidiarrheal, and soothe the mouth. In addition to those common uses, a decoction of the leaves was used as a mouthwash for throat cankers as well as a wash for wounds and the bark was used for diarrhea (Hummer 2010).

A **B**

Figure 2.1. Ancient texts referencing *Rubus.*
(A) Blackberry *Batos* image *Juliana Anicia Codex* Vienna, Osterreichische Nationalbibliothek, Cod. Med.gr.1. fol. 83r. 512 CE. Vienna (Hummer 2010). (B) Saxon

image of blackberry at the time of the Læchbook of Bald, about 920 CE. Sloane 1975, folio 37a, in Rhone, 1922 (Hummer 2010)

Traditional and Folk Medicine

As mentioned earlier, *Rubus* is a very diverse genus that enjoyed widespread use in traditional medicine all across the world. In traditional Indian medicine (Ayurveda) the teachings and tradition are based upon Sanskrit texts from around 200 CE (Hummer 2010). In Ayurveda, *Rubus* bark and leaves are used as a diuretic and an infusion of the *Rubus* leaves are said to aid in childbirth, stomach issues, and menopause (Hummer 2010). Furthermore, in traditional Chinese medicine, the Shen Nung Ben Tsao (25 to 220 CE) is known to be the oldest book on oriental herbal medicine. Within its text, *Fructus rubi,* one of the Chinese raspberry species, is detailed and its medicinal functions include: "preventing frequent urination, enuresis, premature ejaculation, impotence; reducing sore lower back; improving eyesight or blurry vision; and preventing uterine, cervical, and colon cancer" (Hummer 2010).

Medicinal uses of *Rubus* are also seen within a variety of different folk medicines. For example, aboriginal Australians use a decoction of raspberry leaves to treat diarrhea, painful menstruations, childbirth, flu and morning sickness (Symons and Symons 1994). In Hawaii, the ash originating from dried *Rubus hawaiiensis* stems is used for dandruff, to relieve chest burning, and as a stomach ailment for vomiting (Chock 1968). In the Pacific Northwest, the Quileutes, a native tribe from western Washington, chewed the leaves or bark and would then spit that mixture onto infections and burns to sooth the pain and heal the wound (Hummer 2010). *Rubus ulmifolius* is used along the Tuscan archipelago on the islands of Elba, Giglio, and Capraia and is also used within Chilean folk medicine. While both cultures utilize the same species of *Rubus, Rubus ulmifolius* is

used for its hypoglycemic activity in Chilean folk medicine (Lemus, Garcia et al. 1999) while in the Tuscan region it is used for its anti-inflammatory properties (Manganelli and Tomei 1999). In Bulgarian traditional medicine, the fruits, leaves, and roots of *Rubus idaeus* are used as an astringent and anti-inflammatory (Leporatti and Ivancheva 2003). Lastly, within Traditional Turkish medicine, three different species of *Rubus* are commonly used for a variety of different ailments such as hemorrhoids, stomachaches, and wound healing (Yeşilada, Sezik et al. 1999).

Chemistry and bioactivity of the Genus *Rubus*

The Genus *Rubus* and Select Species

The genus *Rubus L.*, a member of the Rosaceae family, is native to six different continents and can be found in a multitude of different geographical locations. *Rubus* species "are found on all arable continents, from the lowland tropics to subarctic regions" (Thompson 1995). There are around 740 different species of *Rubus,* with the South Pacific Islands having more species indigenous to their region than any other region (Hummer 2010). Most of these species are "perennial shrubs with biennial canes above" (Hummer 2010) meaning that their roots live on for many growing seasons yet their stems require two years to fully complete their lifecycle. This genus is also of high economic importance as a crop. Across the world there is production of two species of *Rubus* for consumption: *Rubus idaeus* and *Rubus allegheniensis* (Hummer 1996). The former is the common raspberry and the latter is the blackberry. Both of these crops are cultivated throughout Central America, Mexico, the United States, South America, Asia, Oceania, and Africa (Strik, Clark et al. 2008).

Chemistry of *Rubus*

The *Rubus* genus has been extensively investigated phytochemically and many of the constituents found exhibit various bioactivities. The main classes of chemicals isolated from this genus include terpenes (Li, Fu et al. 2009), flavonoids, steroids (Li, Du et al. 2015), alkaloids (Ding 2011), phenylpropanoids, and phenols (Li, Du et al. 2015).

Terpenes are typically found within the essential oil of plants and are the largest group of natural products. Terpenes have potent antibacterial (Rastogi, Abaul et al. 1998) and antifungal activity (Lunde and Kubo 2000) and are also toxic to insects (Justicia, Oltra et al. 2005), nematodes (Lorimer, Perry et al. 1996), mollusks, and fish (Ito, Muranaka et al. 1997).

Phenylpropanoids are the second largest group of secondary metabolites found in plants. These compounds are produced typically in response to biotic or abiotic stress such as infections, wounds, UV irradiations, exposure to ozone, pollutants, and other hostile environmental conditions (Korkina 2007). One class of phenylpropanoids that is commonly found in *Rubus* species is the flavonoids. Flavonoids are composed of two benzene rings separated by a propyl unit and are classified based on the presence of oxygen-containing functional groups. Different classifications of flavonoids include flavones, flavanones, flavinols, and anthocyanins (Cseke, Kirakosyan et al. 2016). These compounds help protect plants from UV damage while also pigmenting the flowers and fruits of the plant. Similarly, flavonoids have demonstrated a wide range of bioactivity including anti-inflammatory, analgesic, anti-tumor, anti-HIV, anti-infective, antioxidant, anti-ulcerogenic, and vasodilator activity (Gurib-Fakim 2006).

One of the main focuses of this project is to see whether or not these eight different *Rubus* species and eleven different plant parts (Table 2.2.) display the same bioactivity and chemistry as *Rubus ulmifolius* roots as reported in a prior study that demonstrated the antibacterial capacity of this species. Based upon this study by Quave et al, the main phytochemical responsible for the observed bioactivity and antioxidant capacity was ellagic acid (Quave, Estévez-Carmona et al. 2012). Ellagic acid is a polyphenol found at high concentrations in various different fruits including strawberries, raspberries, blackberries, and black currants. Ellagic acid is derived from two gallic acid molecules linked by ester bonds. Recently, this compound has been a key focus of many studies, primarily due to its antioxidant (Zafrilla, Ferreres et al. 2001, Lee and Talcott 2004), anti-proliferative (Losso, Bansode et al. 2004), anti-estrogenic (Papoutsi, Kassi et al. 2005), anti-inflammatory (Umesalma and Sudhandiran 2010), anti-bacterial (Landete 2011), and protein kinase CK-2 inhibiting effects (Cozza, Bonvini et al. 2006). There have also been reports that display the anti-biofilm properties of this compound (Quave, Estévez-Carmona et al. 2012). Additionally, ellagic acid derivatives (EADs) such as ellagic acid-rhamnoside and ellagic acid-xyloside were present within the roots of *Rubus ulmifolius* and also exhibited anti-biofilm activity (Quave, Estévez-Carmona et al. 2012). EADs have been found to have anti-plasmodial (Simões-Pires, Vargas et al. 2009), anti-babesial (Elkhateeb, Takahashi et al. 2005), antibacterial (Ngounou, Choudhary et al. 2001), and antioxidant effects (Ngounou, Choudhary et al. 2001, Matthew, Kao et al. 2007). Due to the high concentration of these compounds within *Rubus ulmifolius* and the impressive therapeutic capacity of ellagic acid and EADs, the chemical analysis in this

thesis focuses on identifying whether or not these three compounds are present within the eight different species under study.

Biological Activity of *Rubus*

Before discussing more specifically the biological activity of these specific *Rubus* species, it is important to note the wide range of biological activity the genus *Rubus* has been shown to exert in various laboratory studies. This genus has shown antioxidant, anti-inflammatory, antibacterial, anticancer, anti-hyperglycemic, anti-hyperlipidemic, hepatoprotective, cardiovascular, and anti-obesity effects, along with various other activities (Li, Du et al. 2015). After discussing the overall bioactivity of the genus, the specific *Rubus* species that were utilized in this experiment will be discussed. Many of the activities reported herein have been explored in this research for the very first time.

Antioxidant activity

One of the main characteristics of the genus *Rubus* is its antioxidant capacity. Various different *Rubus* species have been reported to possess this ability (Vulić, Tumbas T et al. 2011). For example, the Korean study by Lee et al investigated the antioxidant activity of *Rubus coreanus* and found that supplementation with this species increased glutathione peroxidase concentrations in the plasma of healthy Korean men (Lee, Park et al. 2011). Glutathione peroxidase is an enzyme family with peroxidase activity, and its main role is to protect organisms from oxidative damage. Glutathione peroxidases reduce lipid hydroperoxides to their corresponding alcohols and reduce hydrogen peroxide to water, protecting the body from damage caused by excessive free radical concentrations (Blankenberg, Rupprecht et al. 2003). Another study showed that ethanol, ethyl acetate, and butanol partitions of the dried fruits of *Rubus chingii* possessed strong DPPH free

radical scavenging activity (Ding 2011). Furthermore, one study demonstrated in an animal model that long-term intake of anthocyanins and ellagitannins extracted from blackberries increases the positive effects of the antioxidant enzyme activity and expression and enhances oxidative markers in healthy rats (Hassimotto and Lajolo 2011). As stated earlier, one of the main phytochemical constituents of the *Rubus* genus are the flavonoids. Various *Rubus* species have been shown to possess flavonoids such as quercetin, kaempferol, and ellagic acid (Li, Du et al. 2015). These compounds have been shown to possess potent antioxidant activities in a DPPH assay, which monitors chemical reactions involving free radical molecules. Additionally, one study conducted by Wei et al, isolated two specific flavonoids from the roots of *Rubus crataegifolius,* kaempferol 3-O-b-d-galactopyranoside and polydatin. These two compounds, utilized at a concentration of 50 mg/L demonstrated DPPH free radical scavenging rates of 96.50% and 75.23%, demonstrating the clear antioxidant capacity of certain flavonoids found within the *Rubus* genus (Z. Wei 2012).

Anti-inflammatory and Antibacterial Effects

Rubus has also been shown to exhibit anti-inflammatory and antibacterial properties. In one study, an ethanol extract of *Rubus coreanus* leaves was shown to suppress production of both nitric oxide and prostaglandin E2 factors induced by lipopolysaccharide in a model of murine macrophage-induced inflammation (Park, Oh et al. 2006). Additionally, Sun et al reported that the fruits of *Rubus chingii* exhibited anti-inflammatory activity in a nitrite assay using lipopolysaccharide-induced RAW 264.7 cells, which are murine macrophage cells (Sun, Wang et al. 2013). Furthermore, one study conducted by Sangiovanni et al, investigated the anti-inflammatory activity of

Rubus idaeus and *Rubus fruticosus* at a gastric level, testing extracts prepared from these two species on gastric cell lines. These extracts were shown to have a high protective effect against ethanol injury, significantly reducing gastric lesions by 88% and 75% respectively (Sangiovanni, Vrhovsek et al. 2013). Furthermore, these extracts inhibited the secretion of IL-8, a cytokine widely involved in gastric inflammation (Sangiovanni, Vrhovsek et al. 2013).

Anticancer Activity

Besides possessing strong antibacterial properties, *Rubus* has also been shown to possess some anticancer activity. For example, extracts made from the leaves of *Rubus idaeus* exhibited cytotoxic effects on both human laryngeal carcinoma and colon adenocarcinoma cell lines (Durgo, Belščak-Cvitanović et al. 2012). An ethanolic extract prepared from the unripe *Rubus coreanus* fruit has been shown to suppress the growth of prostate cancer cells (Yuri, Jina et al. 2012). This was indicated by significant reductions in the number of proliferating cells and decreases in the protein levels of these cells. Furthermore, this extract induced mitochondrial-mediated apoptosis in prostate cancer cells (Yuri, Jina et al. 2012). Another study conducted by Zhao et al evaluated the therapeutic efficacy of *Rubus alceaefolius* against hepatocellular carcinoma growth both *in vivo* and *in vitro*. (Zhao, Chen et al. 2013) This study showed that extracts prepared from this *Rubus* species decreased tumor volume by 28% while also decreasing tumor weight by 39% compared to the control group (Zhao, Chen et al. 2013). Additionally, Zhao et al conducted an *in vitro* study of this extract on the viability of human hepatocellular carcinoma cells. They found that treatment with this *Rubus* extract reduced cell viability by 39-65% compared to untreated control cells when incubated for 72 hours

(Zhao, Chen et al. 2013). Other species shown to possess certain phytochemicals that exhibit anticancer activity include *Rubus suavissimus* and *Rubus pileatus* (Li, Du et al. 2015).

Anti-Hyperglycemic and Anti-Hyperlipidemic Effects

The main class of phytochemicals from *Rubus* responsible for anti-hyperglycemic and anti-hyperlipidemic effects is the tripterpenoids (Zhao, Chen et al. 2013). Triterpenoids such as Niga-ichigoside F1 isolated from both *Rubus coreanus* and *Rubus crataegifolius* has been shown to significantly inhibit the increase of blood glucose concentration by 44.8% and 28.7%, respectively, in a diabetic rat model (Choi, Yoo et al. 2008). In addition, treatment with these two extracts inhibited the increase in serum concentrations of triglyceride, total cholesterol, or LDL-cholesterol (Choi, Yoo et al. 2008). In a separate study conducted by Bhandary et al, water extracts prepared from immature *Rubus coreanus* lowered both intracellular and extracellular cholesterol levels in human hepatic cells (Bhandary, Lee et al. 2012).

Hepatoprotective Effects

An alkaloid-rich extract prepared from the roots of *Rubus alceaefolius* decreased the pathologically elevated liver enzymes and cell damage in a rat model of acute liver injury stimulated by carbon tetrachloride (Lin, Zhao et al. 2011). Furthermore, the ethyl acetate fraction of a *Rubus alceaefolius* roots demonstrated significant hepatoprotective activity by reducing the leakage of intracellular enzymes, limiting the oxidation of proteins, and by decreasing the incidence of apoptosis in rat liver cells (Hu, Zhao et al. 2013). Lastly, unripe *Rubus coreanus* (Lee, Yang et al. 2009) and *Rubus chingii* (Giao, Pestana et al. 2010), have also been shown to possess significant hepatoprotective effects.

Cardiovascular Effects

A polyphenol-rich fraction extracted from *Rubus coreanum* has been shown to cause vasorelaxation in thoracic aortic strips isolated from spontaneously hypertensive rats (Lim, Min et al. 2011). More specifically, administration of this extract improved endothelial function by increasing levels of nitric oxide through the activation of nitric oxide synthase in vascular endothelial cells (Lim, Min et al. 2011). This result demonstrates the ability of *Rubus coreanum* to potentially treat cardiovascular diseases.

Anti-Obesity Action

Pancreatic lipase is an enzyme that has been proven to be a key factor affecting the absorption of dietary triglycerides, and therefore it can severely affect obesity. One study conducted by McDougall et al found that cloudberries (*Rubus chamaemorus)* and Arctic bramble (*Rubus stellatus x Rubus arcticus)* were effective inhibitors of pancreatic lipase activity *in vitro* (McDougall, Kulkarni et al. 2009). In addition, one particular component of the raspberry *Rubus idaeus* has been shown to contain anti-obesity activity. This component is the raspberry ketone (4-(4-hydroxyphenyl) butan-2-one) and it is one of the key therapeutic aromatic compounds of the raspberry (Gallois 1982). It is widely used as a fragrance in cosmetics and as a flavoring agent in food (Guichard 1982). In a study conducted by Morimoto et al, mice that were fed a high fat diet plus the raspberry ketone had significantly reduced body weight elevation, and the final weights of the visceral adipose tissues were significantly lower than those simply fed a high fat diet without the raspberry ketone (Morimoto, Satoh et al. 2005).

Other Activities

The diverse genus *Rubus* has also been shown to have analgesic activity (Kanegusuku, Sbors et al. 2007), neuroprotective effects (Im, Nam et al. 2013), smooth muscle relaxant activity (Li, Du et al. 2015), antifatigue effects (Jung, Han et al. 2007), skin protective effects (Bae, Lim et al. 2007), and acetylcholinesterase inhibitory activity (Kim, Choi et al. 2013).

An Investigation of 8 Distinct *Rubus* species

Chemistry Data Table for 7 species

	Tannins	Phenylpropanoid	Terpenes	Benzenoid	Lipid	Quinone	Flavonoid	Other
R. ulm. (Tzouwara-Karayanni and Philianos 1981, Díaz and Olave 1982, Tzouwara-Karayanni and Philianos 1982, Rotundo, Bounous et al. 1998, Flamini, Catalano et al. 2002, Panizzi, Caponi et al. 2002)		Chlorogenic Acid, Ferulic Acid, Quinic Acid, 1-4-Dicaffeoyl, Quinic Acid, 4-Caffeoyl, Caffeic Acid, Caffeoyl-Glucose	Corosine, Corsolic Acid, Euscaphic Acid, Eusaphic Acid 28-Beta-D-Glucside, Oleanolic Acid, Tormentic Acid, 23-Hydroxy, Tormentic Acid-28-Beta-D-Glucoside, Ursolic Acid, Ursolic Acid 28-Beta-D-Glucoside, Nicaichigoside, Hypersoside		Linoleic Acid, Myristic Acid, Oleric Acid, Palmitic Acid	Rubanthrone A, Rubanthrone B, Rubanthrone C	Astragalin, Kaempferol-3-O-Alpha-L-Arabinopyranoside, Kaempferol-3-O-Beta-D-Galactoside, Kaempferol-3-O-Beta-D-Glucuronide, Quercetin, Tiliroside, Luteolin-7-O-Beta-D-Glucuronide, Miquelianin, Cyanidin-7-O-Beta-D-Glucoside, Cyanidin, Chrysanthemin, Kaempferol-3-O-Alpha-L-Galactoside, Kaempferol, Kaempferol-3-O-Caffeoylester, Quercetin, Quercetin-N-3-O-Alpha-L-	

							Galactoside, Hyperoside, Gallic Acid, Ellagic Acid	
R. ursinus, Fruit **(Torre and Barritt 1977, Xue, Aziz et al. 2001, Wada and Ou 2002, Li, Du et al. 2015)**					B-Sitosterol, Daucosterol, Stigmasterol,		Chryanthemin, Yanidin-3-O-Beta-D-Rutinoside, Cyanidin-3-Rutinoside, Cyanidin-3-Glucoside, Pelargonidin-3-glycoside, Ellagic Acid	
R. leucodermis *(Torre and Barritt 1977)*							Cyanidin-3-Rutinoside, Cyanidin-3-Glucoside Cyanidin-3-sambubioside, Cyanidin-3-xylosylrutinoside, Ellagic Acid, Rutin, Isoquercetin , Gallic acid	
R. parvifolius **(Torre and Barritt 1977, Do, Son et al. 1988, Choi, Son et al. 1991, Okuda, Yoshida et al. 1992, Tanaka, Tachibana et al. 1993)**	Lambertianin D, Pedunculagin, Sanguiin H-11, Sanguiin H-6, Tellimagrandin II	Chlorogenic acid	Corsolic Acid, Euscaphic Acid. Suavissimoside R-1, URS-12-ENE-23-28-Dioic Acid, 2-Alpha-3-Beta-19-Alpha-Trihydroxy, Ursolic Acid , parvifolactone A, Rubuside P, fupenzic acid, maslinic acid, 1-Beta-hydroxyeusaphic acid, glucosyl pinfaensate, rubuside J, 2α,3β,19α,23-tetrahydroxyurs-12-en-28-oic acid, 2α,3α,19α,23-tetrahydroxyol	Benzoic Acid, 4-Hydroxy, Protocatechuic Aicd Vanillic Acid,Phloroglucinol	Campesterol , Sitosterol Beta, Stigmasterol, Daucosterol,		Catechin, Chrysanthemin, Cyanidin-3-O-Beta-D-Rutinoside, Ellagic Acid	2-Acetylfuran, Butyl formate, 5-Methyl furfural, Hexanoic acid, trans-3-Hexenoic acid, Benzyl alcohol, Phenyl acetaldehyde, cis-α,α,5-Trimethyl-5-vinyltetrahydrofuran-2-methanol, α-Methyl-

			ean-12-en-28-oic acid, 2α,3α,19α,23-tetrahydroxyurs-12-en-24,28-dioic acid, 2α,3β, 19α-trihydroxyurs-12-en-23,28-dioic acid, Camelliagenin A, Camelliagenin C, Niga-ichigoside F1					α-[4-methyl-3-pentenyl]oxirane methanol, Linalool, Naphthalene, 2,3-Dihydro benzofuran, α,4-Dimethyl-3-cyclohexene-1-acetaldehyde, Nerol, Phenylephrine, 2-Methylnaphthalene, 4-Hydroxy-3-methoxystyrene, 1,1,6-Trimethyl-1,2-dihydronaphthalene, Methyl 4-formylbenzoate, 4-tert-Butylbenzoic acid, Dimethylnaphthalene, α,2,6-Trimethylbenzeneethanamine , 2-Methoxy-4-(prop-1-enyl)phenol , Irisone, Dihydroactinidiolide , 2-(1,3-Butadienyl)mesitylene , Hexahydrofarnes

								ylaceton e, Kaur-16-ene, 3,7,11,15-Tetramethyl-2-hexadec en-1-ol
R. laciniatius Fruit **(Torre and Barritt 1977, Stintzing, Stintzing et al. 2002)**		Cinnamic acid, Caffeic acid, p-Coumaric acid,		3,4-Dihydroxybenzoic acid			Cyanidin-3-rutinoside, Cyandidn-3-glucoside, Pelargonidin-3-glycoside, Cyanidin-3-Dioxalyl-Glucoside, Chrysanthemin, Ellagic Acid, Rutin, Isoquercetin , Gallic acid	
R. allegheniensis **(Torre and Barritt 1977, Ono, Tateishi et al. 2003)**			Niga-Ichigoside-F-1, Rubusside A, Pomolic acid, Tormentic acid, Eusaphic acid, 1-Beta-hydroxyeuscaphic acid, myrianthic acid, ziyu glycoside II, Sericic acid, 19-hydrocy-2,3-secours-12-ene-2,3,28-trioic acid 3-methyl ester				Cyanidin Cyanidin-3-rutinoside, Cyandidn-3-glucoside, Pelargonidin-3-glycoside, Chrysanthemin, Cyanidin-3-O-Beta-Rutinoside	
R. praecox **(Torre and Barritt 1977)**							Cyandidn-3-glucoside	

Table 2.1. Overall chemical data table of *Rubus* species.
Current data on the chemical constituents of the seven known *Rubus* species used in this experiment.

Bioactivity of each species

Rubus ulmifolius:

Antibacterial and Antifungal Activity

Rubus ulmifolius has been shown to possess a wide range of bioactivity, being effective against both yeast strains and gram-positive and gram-negative bacteria such as *Staphylococcus aureus, Bacillus cereus, Pseudomonas aeruginosa, Escherichia coli, Saccharoymces cerevisiae,* and *Candida albicans*. (Panizzi, Caponi et al. 2002). The main compounds responsible for this antibacterial activity are gallic acid (Richards, Durham et al. 1994, Saxena, McCutcheon et al. 1994) and ferulic acid (Baranowski, Davidson et al. 1981, Ravn, Andary et al. 1989, Lattanzio, DE CICC01 et al. 1994, Binutu, Adesogan et al. 1996, Rosenthal, Rosen et al. 1997, Panizzi, Caponi et al. 2002), with a potential influence of caffeoylquinic acids (Daglia, Cuzzoni et al. 1994, Scholz, Heinrich et al. 1994), which has been reported to have antimicrobial activity in the past. *Rubus ulmifolius* has also been shown to inhibit *S. aureus* biofilm formation as well as methicillin-resistant *S. aureus* biofilm formation (Quave, Plano et al. 2008). An extract derived from the roots of *Rubus ulmifolius* has been shown to inhibit the formation of pneuomococcal biofilms in a dose dependent manner (Talekar, Chochua et al. 2014). Furthermore, this extract was shown to completely eradicate overnight cultures of planktonic pneuomococci, including antibiotic resistant strains (Talekar, Chochua et al. 2014). Lastly, this extract was also shown to significantly reduce the population of pneuomococcal biofilms formed on human pharyngeal cells (Talekar, Chochua et al. 2014).

Anti-hyperglycemic activity

Rubus ulmifolius has been shown to reduce serum glucose levels significantly in both alloxan and streptozotocin diabetic rats (2.8% and 29%) (Lemus, Garcia et al. 1999)

Rubus ursinus:

Antioxidant Activity

Rubus ursinus, more commonly known as the marionberry, has been shown to have high antioxidant activity and contain high levels of anthocyanins and phenolic compounds (Wada and Ou 2002). This is thought to be the reason behind their high oxygen radical scavenging capacity.

Transformation Inhibition

Rubus ursinus has been shown to produce a dose-dependent decrease in cell morphological transformation of the Syrian hamster embryo (Xue, Aziz et al. 2001). These results suggests that extracts made from *Rubus ursinus* may display cancer preventive activity (Xue, Aziz et al. 2001).

Rubus laciniatus

Antioxidant Activity

Rubus laciniatus, more commonly known as the evergreen blackberry, has been shown to have high antioxidant activity and contain high levels of anthocyanins and phenolic compounds (Wada and Ou 2002).

Rubus parvifolius

Anti-bacterial Activity

Rubus parvifolius is widely distributed in East and South Asia and is used in herbal medicines to treat various infectious diseases (Yuan, Jiuming et al. 2006, Charles

2012). The leaves of this species have been shown to inhibit the growth of both *E. coli* and *Pastuerella* (Hamil, Apio et al. 2000). Furthermore, a volatile oil, extracted from *Rubus parvifolius* was shown to inhibit the growth of several bacterial strains, including *Pseudomonas aeruginosa, Acinetobacter baumanii, Staphylococcus aureus, Bacillus cloacae, Escherichia coli, Klebsiella pneumoniae, Staphylococcus epidermis, Micrococcus luteus, White candidiasis,* and *Enterococcus faecalis* (Tan, Wang et al. 2002, Thiem and Goślińska 2004, Cai 2012).

Anti-inflammatory Activity

The enzyme cyclooxygenase-2, COX-2, is responsible for mediating inflammation and pain in humans. COX-2 is typically unexpressed under normal conditions; however, its levels are severely elevated during inflammation. In addition, COX-2 is upregulated in many different forms of cancer. The overexpression of this enzyme has been associated with gallbladder carcinomas, and the product of COX-2 can in turn stimulate cancer progression (Legan 2010). Methanol extracts prepared from the roots of *Rubus parvifolius* has been shown to inhibit COX-2 activity (Min, Kim et al. 1996). The extract prepared from *Rubus parvifolius* inhibited COX-2 activity by around 4% at a concentration of 100 μg/mL (Min, Kim et al. 1996). In addition, previous studies have demonstrated that the extracts made from *Rubus parvifolius* were effective in reducing coagulation time while also increasing tolerance to hypoxia (Zhu, Zhang et al. 1990, Wang, Li et al. 2006, Wang, Qiu et al. 2006).

Rubus allegheniensis:

Anti-atherosclerosis activities

Triterpenoids have been shown to have anti-atherosclerosis activities in previous studies (Lee, Im et al. 2006, Zheng, Tang et al. 2009, Fujiwara, Hayashida et al. 2011) leading many researchers to explore these natural products. *Rubus allegheniensis* has been shown to contain many different kinds of triterpenoids and therefore was proposed to possess some anti-atherosclerosis activity. Certain triterpenoids found in *Rubus allegheniensis,* such as tormentic acid, exert inhibitory effects of up to 90% (relative to control) on a model of foam cell formation on human monocyte-derived macrophages induced by acetylated low-density lipoproteins (Ono, Yasuda et al. 2014). This formation of foam cells plays an essential role in the progression of arteriosclerotic lesions (Fujiwara, Hayashida et al. 2011). The ability of the chemical constituents of *Rubus allegheniensis* to inhibit foam cell formation demonstrates its anti-atherosclerotic potential and activity.

Species	Voucher	Plant Part	Site
Rubus ulmifolius	CQ-300	Roots	Basilicata, Italy
Rubus ulmifolius	CQ-300	Stems	Basilicata, Italy
Rubus praecox	TAR-1001	Roots	Oregon, USA
***Rubus* L.**	TAR-1002	Roots	Oregon, USA
Rubus ursinus	TAR-1003	Roots	Oregon, USA
Rubus laciniatus	TAR-1004	Roots	Oregon, USA
Rubus leucodermis	TAR-1005	Roots	Oregon, USA
Rubus parvifolius	TAR-1006	Roots	Oregon, USA
Rubus ulmifolius	CQ-164	Leaves	Basilicata, Italy
Rubus ulmifolius	CQ-164	Woody Stems	Basilicata, Italy
Rubus alleghenensis	CQ-455	Stems	Georgia, USA

Table 2.2. Botanical name of each species and plant part tested with collection site. These species are archived at the Emory University Herbarium.

A


EMORY UNIVERSITY
020202
HERBARIUM
INCORPORATED INTO THE
MEDICAL BOTANY SPECIAL COLLECTION
EMORY UNIVERSITY HERBARIUM
FLORA OF ITALY
Rubus ulmifolius Schott ROSACEAE
Italy, Basilicata, Potenza, Ginestra. 40° 55 60400'N, 15° 43.91200'E. 496 meters
Moist area near stream.
Bush with white flowers, black fruits.
Edible fruits. Medicinal - leaves used for abscesses & furuncles; roots to treat baldness.
Quave, Cassandra L. 137
28 May 2014

B


EMORY UNIVERSITY
020658
HERBARIUM
Thousand Arbor Refuge
28281 Hamm Road, Eugene, OR 97405
ROSACEAE
Rubus ursinus Cham. & Schltdl.
Vines growing in edge of steep slope thicket along old logging road. Roots collected and positively identified with the vouchers from their stems.
TAR 1003
Collected by W.C.McClatchey & K.C.Hall
12 November 2015

C


EMORY UNIVERSITY
020659
HERBARIUM
Thousand Arbor Refuge
28281 Hamm Road, Eugene, OR 97405
ROSACEAE
Rubus laciniatus (Weston) Wild.
Vines growing in full sun at edge of periodic sedge wetland. Roots collected and positively identified with the vouchers from their stems.
TAR 1004
collected by W.C.McClatchey & K.C.Hall
12 November 2015

D


THE MORTON ARBORETUM
153877
HERBARIUM
FAMILY ROSACEAE
PLANTS OF ILLINOIS
THE MORTON ARBORETUM, LISLE, ILLINOIS 60532
Col. No. 173
DuPage Co.
Rubus occidentalis L.
1 mile N of Lisle off I-88. Arbor Lake, west side by Arboretum. Latitude N 41° 48.714, longitude W 88° 04.730. Associates with Prunus, Asclepias, Rhus. Plants are common, perennial, shrub, spontaneous, 1.5 m tall, 7 dm wide, pink flwrs.
Collected by S. Matousek, K. Chenwi, K. Denicker, J. Burke
6/29/2000
SPECIAL COLLECTIONS VOUCHERING THE SPONTANEOUS PLANT OF THE MORTON ARBORETUM--2000
STUDIES OF NORTH AMERICAN RUBUS
Rubus parviflorus L.
Det. M. P. Widrlechner
Iowa State University (ISC)
1991

Figure 2.2. Retention vouchers of all the *Rubus* species tested.
These retention vouchers are archived at the Emory University Herbarium (A). *Rubus ulmifolius.* (B) *Rubus ursinus.* (C) *Rubus laciniatus.* (D) *Rubus parvifolius* (E) *Rubus* L. (F) *Rubus praecox.* (G) *Rubus leucodermis.* (H) *Rubus allegheniensis*

Antibiotic resistance and *Staphylococcus aureus*

Antibiotic Resistance

Antibiotic resistance is one of most urgent global health concerns today due to the increased prevalence of resistant bacteria. Many bacterial strains have become resistant to multiple types of antibiotics, leaving very few treat options available (Control and Prevention 2015). This has led to the gradual inefficacy of standard treatments, increased risk of spread of infection, increased duration of hospital stays, and most importantly, the increased risk of death by bacterial infection (Organization 2003). The total economic cost of antibiotic resistance in the U.S. has been estimated to be as high as $20 billion in excess direct healthcare costs, with additional costs of society for lost productivity as high as $35 billion a year (Control and Prevention 2015). Patients suffering from drug-resistant infections can have additional health care costs from $6,000 to $30,000 compared to those with susceptible infections (Cosgrove 2006).

Common resistant pathogens include *Clostridium difficile*, which causes life-threatening diarrhea and 250,000 infection each year (Control and Prevention 2015). Carbapenem-resistant Enterobacteriaceae (CRE) have become resistant to all or nearly all available antibiotics (Control and Prevention 2015). Patients suffering from bloodstream infections with CRE barely have a fifty percent survival rate (Control and Prevention 2015). *Neisseria gonorrhoeae,* the bacteria responsible for the sexually transmitted disease gonorrhea, is the second most commonly reported notifiable infection in the United States and is easily transmitted (Control and Prevention 2015). There are now over 245,000 drug resistant gonorrhea infections contracted yearly (Control and

Prevention 2015). Other resistant bacteria include *Acinetobacter baumannii, Campylobacter spp., Enterobacter spp., Enterococcu faeciums, Pseudomonas aeruginosa, non-typhodial Salmonella, Shigella, Streptococcus pnumaoniae, Mycobacterium tuberculosis,* and of course, *Staphylococcus aureus*. The severity of this problem is so great that it could lead to a post-antibiotic era, in which common infections and minor injuries become deadly once again (Organization 2003).

Antibiotics typically work to kill bacteria through four different mechanisms of action: interference with cell wall synthesis, inhibition of protein synthesis, interference with nucleic acid synthesis, and inhibition of a metabolic pathway (Tenover 2006). Resistant bacteria are simply bacteria that are able to change in some manner that reduces or eliminates the therapeutic abilities of antibiotics (Control and Prevention 2015). Bacteria acquire this resistant trait through either random mutation, which is highly exacerbated by their power to replicate extremely rapidly, or by horizontal gene transfer (Tenover 2006). One mechanism of resistance is when the bacteria acquires genes encoding enzymes that destroy the drug before it can bind to its target (Tenover 2006). Additionally, the bacteria may develop efflux pumps that thrust the antibacterial agent out of the cytosol (Tenover 2006). Bacteria can also alter their membrane permeability to the antibiotic, modify the drug itself, or simply utilize an alternative metabolic pathway that circumvents the antibiotic's mechanism (Dever and Dermody 1991). Although an antibiotic treatment may kill the bacteria that have not mutated or altered their genetic makeup, the mutants that survive will still be able to divide and ultimately then transfer their acquired resistance to other bacterial cells. Once this resistance is acquired in a bacterial strain, that antibiotic will no longer be functional for infection caused by that

strain and a new treatment regimen will be needed. Resistance in bacteria is unpredictable and inevitable in the current paradigm, and is truly a global health threat that requires immediate action.

Staphylococcus aureus

Staphylococcus aureus is a gram-positive coccus that is a member of the Micrococcaceae family (Lowy 1998). It is a facultative aerobe, catalase-positive, and coagulase-positive bacterium (Spaulding, Salgado-Pabón et al. 2013). In 2007, the CDC and their collaborators reported that *S. aureus* "is the most significant cause of serious infectious diseases and infectious disease deaths in the United States" (Klevens, Morrison et al. 2007). While *S. aureus* is clearly pathogenic, "30 to 40% of the human population are asymptomatically colonized at any given time on one or more of their mucosal surfaces" (Spaulding, Salgado-Pabón et al. 2013). The ability of this pathogen to be so virulent while also appearing as a normal part of the human flora is due to the bacteria's large number of cell surface and secreted virulence factors (Spaulding, Salgado-Pabón et al. 2013).

S. aurues is responsible for many diseases including bacteremia, endocarditis, metastatic infections, sepsis, and toxic shock syndrome. Additionally, *S. aureus* infection is a major cause of skin, soft-tissue, respiratory, bone, joint, and endovascular disorders (Lowy 1998). Surgical sites are prone to *S. aureus* invasion causing bone and joint infection and prosthetic valve endocarditis (Livermore 2000). Furthermore, *S. aureus* can cause a serious form of pneumonia and can also cause severe diarrhea due to certain enterotoxins that some strains produce.

Resistance and *Staphylococcus aureus*

The discovery of the first antimicrobial drugs in the mid 20th century transformed both medicine and life itself. Before the advent of antimicrobials, infections such as syphilis, standard *Staphylococcal* infections, endocarditis, and many more were considered incurable. Mortality of patients with *S. aureus* bacteremia exceeded 80%, and over 70% developed metastatic infections (Skinner and Keefer 1941). Infectious diseases were the leading cause of death worldwide and the average life expectancy was 47 (Cohen 2000). Medical procedures such as open-heart surgery, organ transplantation, and burn management were not possible without acquiring a severe infection and therefore death (Min (2012).

In 1928, Alexander Fleming fortuitously stumbled upon a fungal agent that inhibited *staphylococci* growth. By the 1940s, the first antibiotic, penicillin, was officially developed for medical use (Hare 1982). Penicillin was highly used in World War II, curing soldiers' battlefield and bomb wounds and saving thousands of lives. By the late 1940s, the drug was largely accessible to the public and was celebrated as a miracle drug. In 1969, the Surgeon General William H. Stewart was documented telling the United States Congress that it is time to "close the book on infectious disease" (Garrett 1992). Despite the early success of antibiotics against this pathogen, resistance began to emerge rapidly. By 1947, only two years after the widespread introduction of penicillin to the public, about 6 % of *S. aureus* strains were resistant to penicillin (Barber and Rozwadowska-Dowzenko 1948). The continued overuse and inappropriate use of penicillin for infected wounds led to a rise in resistance. By 1948, over 50% of hospital *S. aureus* were penicillin-resistant (Barber and Rozwadowska-Dowzenko 1948). This

proportion of penicillin-resistant isolates has since grown to about 80-90% (Henwood, Livermore et al. 2000). Staphylococcal resistance to penicillin is mediated by *blaZ,* a gene that encodes a β-lactamase, which is an enzyme that hydrolyzes the β-lactam ring of penicillin, rendering it inactive (Lowy 2003). Fortunately, this rising resistance to penicillin was curtailed by the new surge of antibiotic development in the 1960s leading to the invention of a new class of antibiotics that penicillin-resistant *S. aureus* were susceptible to: Methicillin.

While the problem of penicillin-resistant *S. aureus* became less severe, the first methicillin-resistant *S. aureus* (MRSA) isolates were discovered in the year 1961 when methicillin had reached the market (Rolinson 1961). Similar to the spread of penicillin resistance, methicillin-resistant isolates were first detected in hospitals and then increasingly recognized within the community (Lowy 2003). Today, within a nosocomial setting, over 50% of *S. aureus* strains causing disease are MRSA (Boucher, Miller et al. 2010). As stated earlier, MRSA kills more Americans each year than HIV/AIDS, Parkinson's disease, emphysema, and homicide combined (Gross 2013, Golkar, Bagasra et al. 2014). In addition to its multi-drug resistance capabilities, MRSA has shown vast versatility in its ability to emerge and spread in different settings over time (hospitals, community, and animals) (Rossolini, Arena et al. 2014). MRSA strains are of a particular concern because resistance to methicillin confers resistance to all β-lactam agents (Lowy 2003). MRSA strains are also resistant to other classes of antibiotics such as macrolides and floroquinolones (Drago, De Vecchi et al. 2007). MRSA strains often cause skin and soft tissue infection, urinary tract infections, pneumonia, and bacteremia (McDonald 2006).

When MRSA is identified within the patient, vancomycin is the standard treatment for that infection. While in the past, MRSA strains have been fully susceptible to vancomycin, there has been an increasing concern about MRSA strains that are less susceptible to vancomycin or completely resistant (Weinstein and Fridkin 2001). This poses an extreme threat because when *S. aureus* becomes resistant to vancomycin, there are very few treatment options available after that. In 1997, the first report of vancomycin intermediate-resistant *S. aureus* (VISA) came from Japan, and additional cases were reported later in other countries (Hiramatsu 1998, Smith, Pearson et al. 1999). Although the CDC reports that from 2002-2013 there have only been 4 identified cases of vancomycin-resistant *S. aureus* within the US and 13 cases total (Control and Prevention 2015), these numbers are only bound to rise based upon previous resistance trends. While the development of new antibiotics has proven to be effective against certain bacteria, this approach is not sustainable.

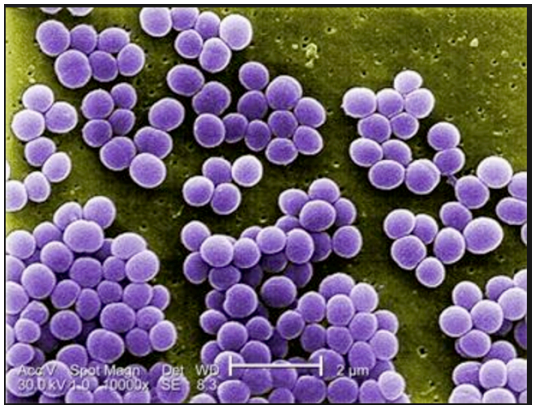


Figure 2.3. *Staphylococcus aureus* (CDC).
Group of *S. aureus* cells visualized by scanning electron microscopy

Biofilms and *Staphylococcus aureus*

What is a biofilm?

A biofilm can be defined as, "a microbially-derived sessile community, typified by cells that are attached to a substratum, interface, or to each other, [that] are embedded in a matrix of extracellular polymeric substance, and exhibit an altered phenotype with regard to growth, gene expression and protein production" (Donlan and Costerton 2002). Biofilms provide a "protected mode of growth that allows survival in hostile environment" (Costerton, Stewart et al. 1999). Biofilms confer the bacteria certain advantages that include an increased ability to sequester and concentrate environmental nutrients, the ability to evade multiple clearance mechanisms, and an increased potential for cellular detachment (Archer, Mazaitis et al. 2011). Biofilm-associated infections tend to lead to longer hospital stays, persistent infection, as well as increased fatalities, creating a larger economic burden than non-biofilm-associated infections (Quave, Estévez-Carmona et al. 2012). Various nosocomial infections such as those related to the use of central venous catheters (Passerini, Lam et al. 1992), urinary catheters (Morris, Stickler et al. 1999), prosthetic heart valves (Hyde, Darouiche et al. 1998), and orthopedic devices (Gristina, Shibata et al. 1994) are associated with biofilms. Today, there has been up to 17 million new biofilm infections each year resulting in up to 550,000 fatalities annually (Wolcott, Rhoads et al. 2010, Wolcott and Dowd 2011). The National Institutes of Health estimates that 80% of all bacterial infection are biofilm-related (Harro, Peters et al. 2010).

Biofilm formation is a major contributing factor to antibiotic resistance. The protection afforded by a biofilm can provide a breeding ground for spontaneous mutants.

Furthermore, the close spatial proximity of bacterial cells within a biofilm may accelerate plasmid transfer (Hausner and Wuertz 1999). One mechanism of biofilm resistance is due to the failure of the antibiotic agent to penetrate the full depth of the biofilm (Costerton, Stewart et al. 1999). The viscous polymeric substance of the biofilm retards the attempted diffusion of antibiotics. An additional possible mechanism of resistance is that certain cells within a biofilm exist in a slow-growing or starved state (Brown, ALLISON et al. 1988). Slow growing or non-growing cells are less susceptible to antibiotic agents (Costerton, Stewart et al. 1999). One suggested mechanism for resistance relies on a specific type of bacterial cell: persister cells. Persister cells are "dormant variants of regular cells that form stochastically in a microbial population and are highly tolerant to antibiotics" (Lewis 2010). When a biofilm-related infection occurs, antibiotics kill the majority of cells, and the immune system eliminates both regular cells and persisters from the bloodstream. The only live cells remaining are the persisters within the biofilm. Once the level of antibiotic drops, persisters are able to repopulate the biofilm, explaining the recalcitrance of biofilm related infections (Lewis 2010).

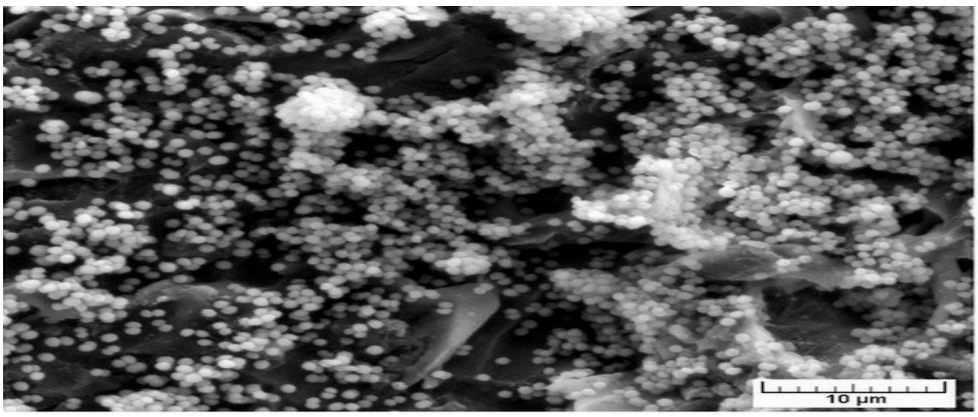
***Staphylococcus aureus* biofilms**

S. aureus possesses the ability to attach itself to the host extracellular matrix proteins and form intricate micro-colonies via biofilms (Figure 2.4.) (Archer, Mazaitis et al. 2011). Many infections caused by *S. aureus* are closely associated with biofilm formation. For example, endocarditis, osteomyelitis, infections from implanted medical devices or foreign bodies, and some skin infections are all highly associated with biofilm formation. These infections deserve more attention than standard non-biofilm infections

because the biofilm matrix and phenotypic characteristics of the bacteria confer resistance to host immune response and the therapeutic action of antibiotics (Yarwood, Paquette et al. 2007, McCarthy, Rudkin et al. 2015) This ability to form biofilms is a key virulence factor, especially within healthcare settings where there is a high use of antibiotics and the formation of a biofilm represents an effective survival mechanism for the bacteria (Høiby, Bjarnsholt et al. 2010).

The compounded effects of biofilm formation/resistance and acquired antibiotic resistance present a major hurdle for the treatment of *S. aureus* infection. Evidently, biofilms are crucial for bacterial proliferation, virulence, and resistance and therefore represent a potential therapeutic target for *S. aureus* infections. Such therapeutics could inhibit colonization, reduce virulence, or be used as adjunct therapies in order to improve the therapeutic effect of conventional antibiotics (Kalan and Wright 2011, Wolcott and Dowd 2011).

A



B

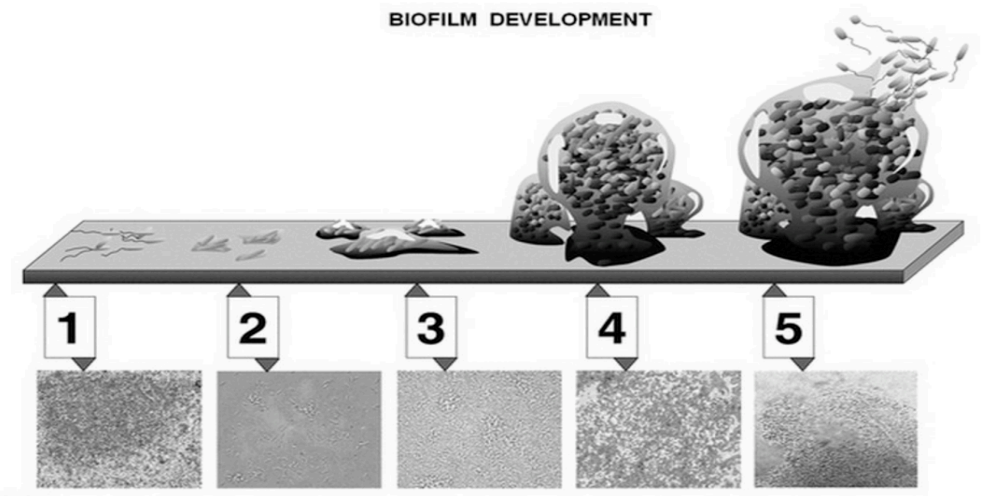


Figure 2.4. *S. aureus* biofilm and biofilm development
(A) *Staphylococcus aureus* biofilms (Otto 2008) (B) Biofilm development: Stage 1, initial attachment; stage 2, irreversible attachment; stage 3, maturation I; stage 4, maturation II; stage 5, dispersion. Each stage of development in the diagram is paired with a photomicrograph of a developing P. aeruginosa biofilm. All photomicrographs are shown to same scale (Monroe 2007). (Image credit: D. Davis)

CHPATER 3: MATERIALS AND METHODS

Literature review:

A thorough literature review was conducted in order to identify what information already exists regarding the diverse genus *Rubus.* The literature review examined the history of *Rubus* pharmacology, the use of *Rubus* in both traditional and folk medicine, the current research on both the chemistry of the genus and biological activity of extracts, and the current research on the chemistry and biological activity of my specific species. Most of the sources that were utilized for the history of *Rubus* pharmacology and the use of *Rubus* in traditional and folk medicine were gathered through the American Society for Horicultural Science database and through review articles that had been published in reputable, peer-reviewed journals. Search terms used to find these articles on the history and traditional uses of *Rubus* included "History of *Rubus,*" "*Rubus* in traditional medicine," and included a wide range of search terms similar to those stated above.

Investigation of the chemistry and biological activity of *Rubus* relied heavily on the use of PubMed, SERNEC, and Napralert databases. Most of the sources gathered had been published in reputable, peer-reviewed journals. Search terms utilized to find this information include simply the name of each of the *Rubus* species tested in this experiment (*Rubus ulmifolius Rubus praecox, Rubus leucodermis* etc.). Furthermore, other search terms included "Chemistry of the genus *Rubus*" and "Biological activity of the genus *Rubus.*" Lastly, the chemistry and biological activity of each individual species were also search terms utilized (ex. Chemical activity of *Rubus praecox* etc.).

Botanical Materials:

The Quave Research Group and collaborators have collected samples of various different *Rubus* species from both Europe and the United States (Table 2.1.). All of our specimens have been botanically validated according to the 2003 WHO Guidelines on Good Agriculture and Collection Practices for Medicinal Plants (Organization 2003) for the collection and identification of any bulk and voucher specimens. In addition to the samples stored within the lab, we have voucher specimens deposited within the Emory University Herbarium, where they are electronically recorded for inclusion in a web-accessible platform for image and collection data. Plant leaves, stems, and roots were separated and manually cleaned of soil and contaminants. Plant material was then dried in a desiccating cabinet over a period of days at low heat. Once dried, plant material was placed into paper bags, sealed, and stored at room temperature.

Generation of *Rubus* Extracts:

Crudes:

Each plant species shown in Table 2.2. was air-dried prior to extraction. To ensure that each *Rubus* species was extracted both consistently and effectually, each species was grounded into a fine powder using a Wiley Mill (2 mm mesh) and then extracted in 80% MeOH (1 gram of plant material:10 mL of 80% MeOH). Once the methanol was added, the solution was sonicated for 20 minutes, filtered through both coarse (Fischer P8) and fine (Fischer P2) filter paper via vacuum filtration, and the filtrate was collected. The plant material was then extracted, sonicated, and filtered two more times with 80% MeOH, and the three filtrates were collected and combined. The filtered extract was

concentrated using rotary evaporation at reduced pressure to remove solvent. The concentrated extracts were then re-suspended in deionized H_2O, dried through lyophilization, and stored at -20°C. Dry extracts were collected and percent yields for crude extracts were calculated (Table 4.1.).

Partitions:

A modified Kupchan partitioning scheme in succession with hexane, ethyl acetate, and butanol was used in the separation of all crude extracts. All ACS (American Chemical Society) grade solvents were obtained from Fisher Chemical. Each crude extract was suspended in water (1 g of extract: 30 mL of water) and placed in a 1 L separatory funnel. Next, the solution was mixed with hexane three times, each time adding 1/3 of the volume of water used to suspend the crude extract originally as the hexane volume. The organic layer was removed and treated with anhydrous sodium sulfate before coarse filtration. This process was then repeated with ethyl acetate and n-Butanol. The remaining aqueous solution was collected and filtered without sodium sulfate treatment. The partitions were dried down via rotary evaporation, dissolved in DI H_2O, frozen, lyophilized, and stored at -20°C. The hexane partition was labeled "B", the ethyl acetate partition was labeled "C", the n-Butanol partition was labeled "D", and the remaining aqueous partition was labeled "E". Percent yields were calculated after collecting the dry partitions.

Minimum Inhibitory Concentration Assay (Growth Inhibition)

Minimum inhibitory concentration (MIC) assays were performed to test for growth inhibition of *S. aureus* by each extract. Samples were prepared within a 1.8 mL

Eppendorf tube at a concentration of 10 mg/mL at a total volume of ~1.5 mL dissolved in DMSO and stored at -20°C. A small volume of *S. aureus* strain UAMS1 taken from frozen stock was plated on Tryptic Soy Agar and allowed to incubate overnight at 37°C. Following incubation, a single colony was obtained from the TSA plate and growth overnight in 6 mL of Tryptic Soy Broth (TSB), purchased from Himedia, at 37°C while shaking at 200 rpm in a Biotek Shaker and Incubator. After a second overnight incubation, when the culture had reached the logarithmic growth phase, it was standardized to x 105 CFU/mL (colony forming units/mL) using OD600 readings, generated from a Biotek Cytation 3 imaging reader and diluted to the necessary concentration in cation-adjusted Mueller-Hinton broth (CAMHB). MICs were determined by the microtiter broth method in sterile flat-bottom 96-well polystyrene plates. Each extract was added to one well at an initial concentration of 256 μg/mL. Serial dilution techniques were used to yield a final well volume of 200 μL and concentrations ranging from 8 – 256 μg/mL. DMSO (negative control) and vancomycin (positive control) were tested at concentrations ranging from 0.5 – 256 μg/mL. Vancomycin was dissolved at 10 mg/mL in DI water. All tests were performed in triplicate. Plates were incubated at 37°C for 18 hours. Optical density was assessed immediately after inoculation and again after 18 hours using a Biotek Synergy II microplate reader. Percent growth inhibition was calculated with this equation to account for extract color on the OD_{600nm} reading:

$$\%\ inhibition = \left(1 - \left(\frac{ODt18 - ODt0}{ODDMSOt18 - ODDMSOt0}\right)\right) \times 100$$

OD_{t18}: OD_{600} of extract treated wells after 18 hours of incubation
OD_{t0}: OD_{600} of extract treated wells at 0 hours
$OD_{DMSOt18}$: OD_{600} of DMSO treated wells after 18 hours of incubation
OD_{DMSOt0}: OD_{600} of DMSO treated wells at 0 hours

The MIC was defined as the lowest concentration that inhibited growth to a level ≥50% (MIC_{50}) and ≥90% (MIC_{90}) relative to negative control treatment.

Assessment of biofilm formation

Assays were performed in sterile flat-bottom 96-well polystyrene plates. Samples were prepared within a 1.8 mL Eppendorf tube at a concentration of 10 mg/mL at a total volume of ~1.5 mL, dissolved in DMSO and stored at -20°C. A small volume of *S. aureus* strain UAMS1 and UAMS929 taken from frozen stock was plated on Tryptic Soy Agar and allowed to incubate overnight at 37°C. Following incubation, a single colony was obtained from each of the TSA plate and growth overnight in 6 mL of Tryptic Soy Broth (TSB), purchased from Himedia at 37°C while shaking at 200 rpm in a Biotek Shaker and Incubator. After a second overnight incubation, when the cultures had reached the logarithmic growth phase, they were standardized to x 105 CFU/mL (colony forming units/mL) using OD_{600} readings, generated from a Biotek Cytation 3 imaging reader, and diluted to the necessary concentration in biofilm media containing sodium chloride, tryptic soy broth, dextrose, and human plasma. To test for the effects of extracts on biofilm formation, each extract was added to one well at an initial concentration of 64 μg/mL. Serial dilution techniques were used to yield a final well volume of 200 μL and concentrations ranging from 8 – 64 μg/mL. DMSO (negative control) and 220D-F2 (positive control) were tested at concentrations ranging from 0.5 – 256 μg/mL. Optical density was assessed immediately after inoculation and again after 24 hour incubation without shaking at 37°C using a Biotek Synergy II microplate reader. The contents of the wells were then aspirated and rinsed 3 times with 200 μl of phosphate-buffered saline to

remove nonadherent cells. Adherent biofilms were fixed with 200 µl of 100% ethanol prior to staining for 15 min with 200 µl of 0.41% (wt/vol) crystal violet in 12% ethanol (Protocol Crystal Violet; Biochemical Sciences, Swedesboro, N.J.). The stain was then aspirated, and the wells were washed several times with tap water and left to dry. Once the wells were fully dry, 200 µl of 10 % Tween 80 was added to all the wells. The stain adhering to the biofilm biomass was then pulled off with the Tween 80 and a total of 50 µL of the eluent was then transferred to a new sterile polystyrene microtiter plate from which the absorbance ($OD_{595\ nm}$) was determined using a plate reader. The minimum biofilm-inhibiting concentration (MBIC) was defined as the lowest concentration of extract in which biofilm formation was inhibited to a level ≥90% (for $MBIC_{90}$) or ≥50% (for $MBIC_{50}$) of that present after the negative control treatments.

High Performance Liquid Chromatography (HPLC)

Chromatograms of ellagic acid, ellagic acid-rhamnoside, and ellagic acid-xyloside were generated using HPLC and utilized as standards to be compared to the chromatographs of each of the samples being studied. An Agilent Eclipse XDB-C18 4.6 x 250 mm, 5-µm analytical column, with a compatible guard column, was used at 40°C. Each of the extracts were dissolved in DMSO (10 mg/mL), and filtered at 0.2 microns. A 10 µL injection of each extract was run at a flow rate of 1.0 mL/min using a gradient system consisting of (A) 0.1% formic acid in H2O and (B) 0.1% formic acid in acetonitrile (ACN). The mobile phase was 98:2 A:B at time 0 min, 88:12 A:B at 50 min, 75:25 A:B at 70 min, 5:95 A:B at 82 min, 98:2 A:B at 98 min, followed by a hold at 2:98 A:B for 5 min, and ending with a column flush at initial conditions for 5 min.

CHAPTER 4: RESULTS

Literature Review:

After an extensive review of the PubMed, Napralert, Google Scholar, and Web of Science databases, no information on the biological activity of *Rubus leucodermis* or *Rubus praecox* was found. Additionally, there was limited information on the chemical constituents of *R. ursinus, R. leucodermis,* and *R. praecox*. Furthermore, there was limited research and information on the biological activity of *R. laciniatus,* and *R. ursinus* extracts. Lastly, one of my species, extract 729, was collected without knowledge of the specific species.

Extraction:

Table 4.1 shows the percent yield from initial dry plant material to crude extract prior to further liquid/liquid separation. The average percent yield for the eleven different plant parts is 16.7%. Extract 737 or *Rubus allegheniensis* stems produced the greatest yield of dried extract at 25.8% while extract 725 or *Rubus ulmifolius* roots produced the smallest yield of dried extract at 9.2%.

Extract #	Voucher ID	Botanic Name	Plant Part	Mass Extracted (grams)	Volume of 80% MeOH (mL)	Mass of Dry Extract (grams)	Yield (%)
725	CQ-300	*Rubus ulmifolius*	roots	40	400	3.6619	9.15475
726	CQ-300	*Rubus ulmifolius*	stems	90	900	11.4698	12.74422222
728	TAR-1001	*Rubus praecox*	roots	80	800	11.2238	14.02975
729	TAR-1002	*Rubus* L.	roots	40	400	7.48	18.7
730	TAR-1003	*Rubus ursinus*	roots	40	400	7.363	18.4075
731	TAR-1004	*Rubus laciniatus*	roots	40	400	6.6973	16.74325
732	TAR-1005	*Rubus leucodermis*	roots	70	700	12.8012	18.28742857
733	TAR-1006	*Rubus parvifolius*	roots	10	100	1.5288	15.288
734	CQ-164	*Rubus ulmifolius*	leaves	80	800	17.5078	21.88475
735	CQ-164	*Rubus ulmifolius*	woody stem	80	800	10.0939	12.617375
737	CQ-455	*Rubus allegheniensis*	stems	80	800	20.602	25.7525

Table 4.1 List of *Rubus* species extracted and tested
Percent yields of crude extracts from initial dry plant material are listed

Liquid/Liquid Partitioning:

To further separate the crude extracts, a sample of each crude extract was dissolved in water and then partitioned against varying solvents to obtain 4 new extracts. The B partition represents Hexane, the C partition represents Ethyl acetate, the D partition represents butanol, and the E partition represents water. The average yield of all of the partitions was 4.3%. The partitioning methodology resulted in one dry powder sample and one viscous liquid sample due to washing the flask for residual material for

each individual partition; however, the hexane partition always resulted in just one viscous liquid sample. The blank spaces within the table below indicate that after the partitioning methodology was completed, there was no product formed.

Extract #	Partition	Mass Partitioned (g)	Partition Dry Mass (g)	Partition Wet Mass (g)	Yield (g of dry partition per g of plant material extracted)	Yield (%)
725	B	8.25	8.25	0.00519	6.77E-05	6.77E-03
	C	8.25	2.71630	0.079	3.54E-02	3.54E+00
	D	8.25	2.5057	0.0494	3.27E-02	3.27E+00
	E	8.25	2.9246	0.2649	3.82E-02	3.82E+00
726	B	10		0.1544	1.97E-03	1.97E-01
	C	10	0.8292	0.0998	1.06E-02	1.06E+00
	D	10	2.9964	0.0725	3.82E-02	3.82E+00
	E	10	6.5041	0.3026	8.29E-02	8.29E+00
728	B	10		0.02422	3.40E-04	3.40E-02
	C	10	1.5083	No Wet	2.12E-02	2.12E+00
	D	10	2.88063	0.3052	4.04E-02	4.04E+00
	E	10	5.0539	0.6575	7.09E-02	7.09E+00
729	B	6		0.0165	5.14E-04	5.14E-02
	C	6	0.8131	0.0994	2.53E-02	2.53E+00
	D	6	1.491	0.09397	4.65E-02	4.65E+00
	E	6	3.4006	0.4086	1.06E-01	1.06E+01
730	B	6		0.0295	9.05E-04	9.05E-02
	C	6	0.8074	0.0536	2.48E-02	2.48E+00
	D	6	5.3994	2.0786	1.66E-01	1.66E+01
	E	6	3.0557	0.3154	9.37E-02	9.37E+00
731	B	5.0712		0.01685	5.56E-04	5.56E-02
	C	5.0712	0.4521	0.2233	1.49E-02	1.49E+00
	D	5.0712	6.989	2.1279	2.31E-01	2.31E+01
	E	5.0712	2.7268	0.2635	9.00E-02	9.00E+00
732	B	11		0.0174	2.89E-04	2.89E-02
	C	11	2.276	2.0688	3.78E-02	3.78E+00
	D	11	4.3261	0.0468	7.19E-02	7.19E+00
	E	11	5.657	2.8258	9.40E-02	9.40E+00
733	B	1.0059		0.0384	5.84E-03	5.84E-01
	C	1.0059	0.0651	0.0239	9.89E-03	9.89E-01
	D	1.0059	0.0751	0.0761	1.14E-02	1.14E+00
	E	1.0059	0.4867	0.0915	7.40E-02	7.40E+00
734	B	16		0.1294	1.77E-03	1.77E-01
	C	16	1.3283	0.5754	1.82E-02	1.82E+00
	D	16	2.1955	0.077	3.00E-02	3.00E+00
	E	16		0.5589	7.64E-03	7.64E-01
735	B	9		0.15143	2.12E-03	2.12E-01
	C	9	0.4109	0.0784	5.76E-03	5.76E-01
	D	9	2.0482	0.078	2.87E-02	2.87E+00
	E	9	6.5529	0.408	9.19E-02	9.19E+00
737	B	18		0.1293	1.85E-03	1.85E-01
	C	18	1.4281	0.0922	2.04E-02	2.04E+00
	D	18	2.2306	0.1477	3.19E-02	3.19E+00
	E	18	11.7939	0.5345	1.69E-01	1.69E+01

Table 4.2 Partitioning yields

Each crude extract was partitioned into 4 new partitions: Hexane (B), Ethyl Acetate (C), Butanol (D), Water (E). The mass of the crude extracts initially suspended in water is

listed. The yield in grams of both the dry partition and wet partitions are listed along with the percent yield.

S. aureus growth and biofilm inhibition:

Staphylococcus aureus growth inhibition and biofilm inhibition is displayed below for each individual extract tested. Initially, each extract was tested at a screening concentration of 256 μg/mL to further guide MIC analysis. Based upon the results of the preliminary growth inhibition screen, certain extracts were chosen to be serial diluted and tested further in order to discover the lowest concentration that would inhibit *S. aureus* growth by both 90% and 50%. Of the 55 extracts tested, 18 had an MIC_{50} value of 256 μg/mL, 19 had an MIC_{50} value of 128 μg/mL, 1 had an MIC_{50} value of 64 μg/mL, and 1 had an MIC_{50} value of 32 μg/mL. Only 2 extracts had an MIC_{90}: 728D and 729D, both at 256 μg/mL. Based on these MIC values, 7 of the 11 crude extracts, 3 of the 11 hexane partitions, 9 of the 11 ethyl acetate partitions, 10 of the 11 butanol partitions, and 10 of the 11 water partitions inhibited *S. aureus* strain UAMS1 growth by at least 50% at a tested concentration.

Similar to the MIC analysis, each extract was initially screened at a concentration of 256 μg/mL to guide MBIC analysis. At the screening concentration, biofilm inhibition was extremely high for almost all extracts leading to an additional master screen at a new concentration of 64 μg/mL. Based upon the results of this second master screen, certain extracts were chosen to be serially diluted and tested further in order to discover the lowest concentration that would inhibit biofilm formation by both 90% and 50% for each extract. Of the 55 extracts tested, 5 had an $MBIC_{50}$ value of 64 μg/mL, 16 had an $MBIC_{50}$ value of 32 μg/mL, 13 had an $MBIC_{50}$ value of 16 μg/mL, and 15 had an $MBIC_{50}$ value of

8 µg/mL. Only 6 extracts did not inhibit biofilm growth by at least 50% at a tested concentration. Furthermore, 12 had an $MBIC_{90}$ value of 64 µg/mL, 11 had an $MBIC_{90}$ value of 32 µg/mL, 2 had an $MBIC_{90}$ value of 16 µg/mL, and 8 had an $MBIC_{90}$ value of 8 µg/mL. 22 out of the 55 extracts did not inhibit biofilm growth by at least 90%. Based upon the MBIC values, all 11 crude, 11 ethyl acetate, 11 butanol, and 11 water extracts inhibited *S. aureus* strain UAMS1 biofilm formation by at least 50%. 5 out of the 11 hexane partitions inhibited *S. aureus* strain UAMS1 biofilm formation by at least 50%. Furthermore, 10 out of the 11 crude extracts (all but 734), none of the hexane partitions, 8 of the 11 ethyl acetate partitions, all 11 butanol partitions, and 4 of the 11 water partitions inhibited *S. aureus* strain UAMS1 biofilm formation by at least 90%.

All of the specific MIC and MBIC values can be found in Table 4.3, and the corresponding graphs for both growth inhibition and biofilm inhibition for each plant part tested can be found below in Figures 4.1 – 4.11. Figures 4.12 and 4.13 display biofilm inhibition for certain crude, butanol, and water partitions. These extracts were further diluted and tested at lower concentrations due to high levels of biofilm inhibition during initial testing. None of the extracts inhibited biofilm growth by at least 50% at a concentration below 8 µg/mL. Figure 4.14 displays the controls used for both biofilm testing and growth inhibition. The MIC for 220D-F2 shown in the table is taken from a previous study conducted by Quave et al, that utilized the same techniques (Quave, Estévez-Carmona et al. 2012)

MIC & MBIC

	UAMS-1			
	MIC_{50}	MIC_{90}	$MBIC_{50}$	$MBIC_{90}$
220D-F2	64	-	8	-
725	-	-	8	32
725B	-	-	32	-
725C	-	-	32	64
725D	128	-	32	32
725E	128	-	8	8
726	-	-	8	32
726B	256	-	64	-
726C	256	-	16	64
726D	256	-	32	64
726E	128	-	64	64
728	-	-	8	32
728B	-	-	-	-
728C	256	-	32	-
728D	128	256	32	32
728E	128	-	32	32
729	128	-	8	16
729B	-	-	-	-
729C	256	-	16	64
729D	128	256	32	32
729E	128	-	32	32
730	-	-	8	8
730B	-	-	-	-
730C	256		32	-
730D	128	-	32	32
730E	128	-	16	-
731	128	-	8	8
731B	-	-	-	-
731C	256	-	16	64
731D	128	-	8	8
731E	32	-	16	-
732	128	-	8	8
732B	-	-	-	-
732C	256	-	32	64
732D	256	-	8	32
732E	128	-	16	-
733	128	-	8	8
733B	-	-	-	-
733C	256	-	16	64

733D	128	-	8	16
733E	-	-	16	-
734	256	-	64	-
734B	-	-	16	-
734C	256	-	16	64
734D	256	-	64	64
734E	256	-	32	-
735	128	-	64	64
735B	64	-	16	-
735C	-	-	16	64
735D	-	-	8	8
735E	256	-	16	-
737	128	-	32	32
737B	256	-	32	-
737C	256	-	32	-
737D	256	-	8	8
737E	128	-	8	-

Table 4.3. MIC and MBIC value of all extracts against *S. aureus* strain UAMS1.
All MBIC values are compared to control extract 220D-F2 and are all represented in μg/mL.

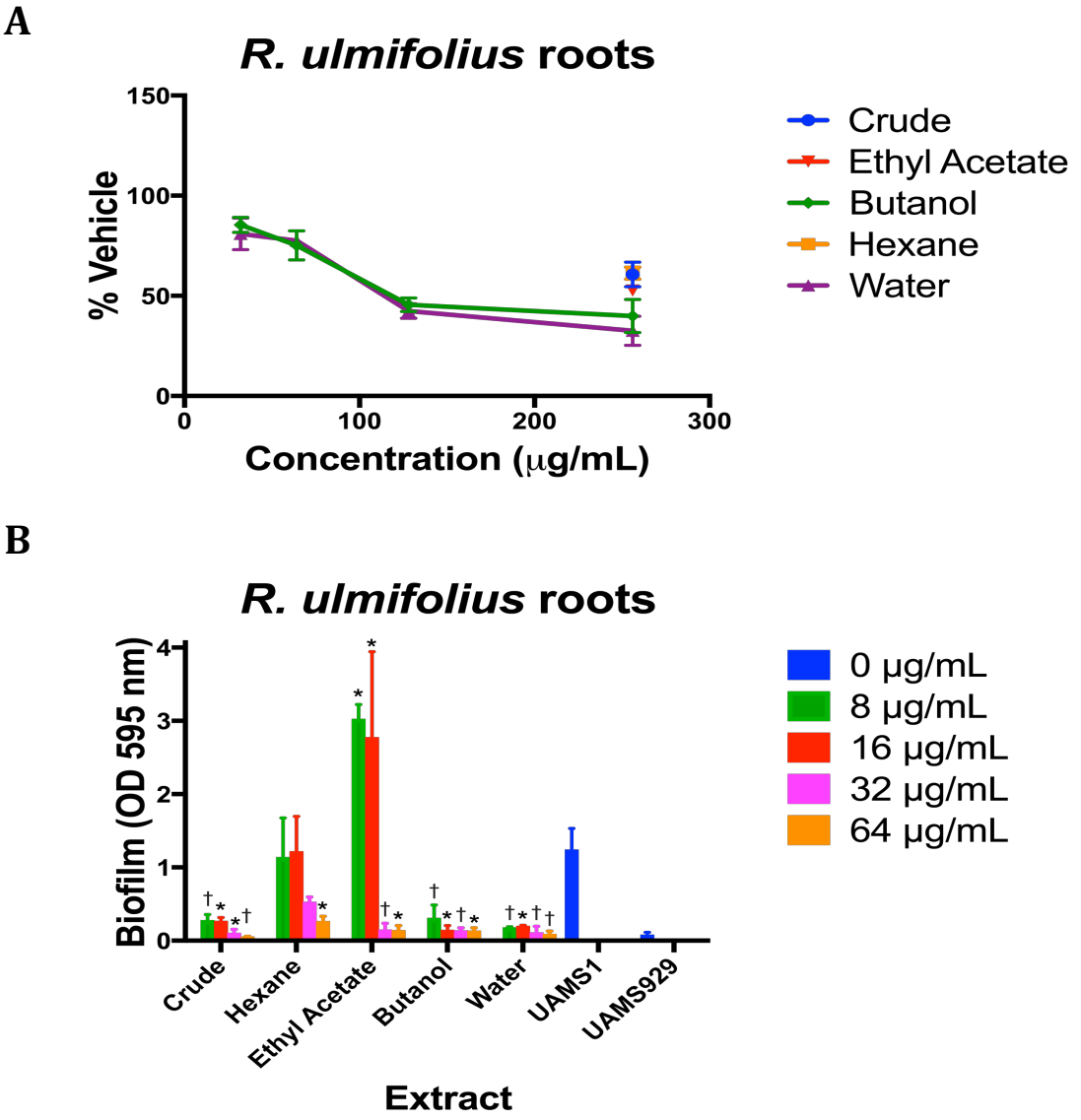
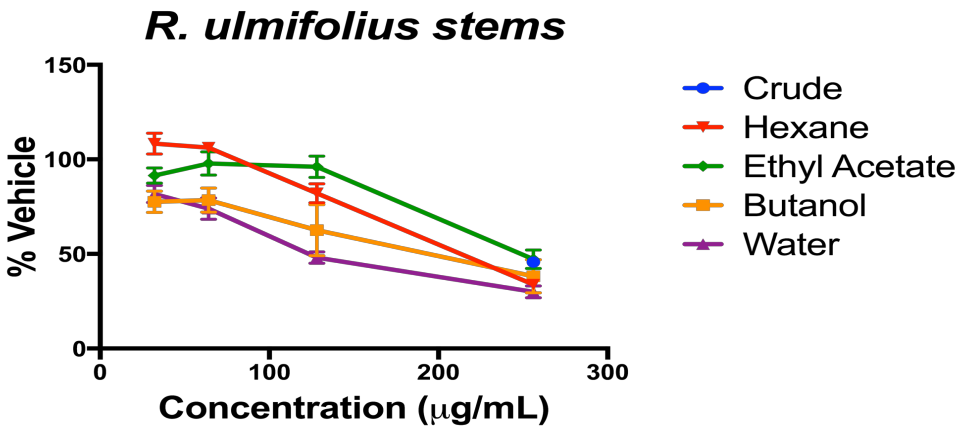


Figure 4.1. *R. ulmifolius* roots growth and biofilm inhibition
(A) Growth inhibition of *S. aureus* by *R. ulmifolius* roots extracts. At 256 μg/mL: the hexane and ethyl acetate partitions showed significance ($0.01 < p\text{-value} < 0.05$); the butanol and water partitions showed significance ($0.001 < p\text{-value} < 0.01$); and the crude extract showed significance ($p\text{-value} < 0.001$). At 128 μg/mL: the butanol and water partitions showed significance ($p\text{-value} < 0.001$). At 64 μg/mL: the water partition showed significance ($p\text{-value} < 0.001$); the butanol partition showed significance ($0.01 < p\text{-value} < 0.05$). At 32 μg/mL the butanol and water partitions showed significance ($0.01 < p\text{-value} < 0.05$). (B) *S. aureus* biofilm inhibition by *R. ulmifolius* roots extracts. ($0.01 \leq p\text{-value} < 0.05$: *, $0.001 \leq p\text{-value} < 0.01$: †, $p\text{-value} < 0.001$: ‡). Significance refers to differences between cultures treated with control (DMSO) and cultures exposed to extracts at varying concentrations.

A



B

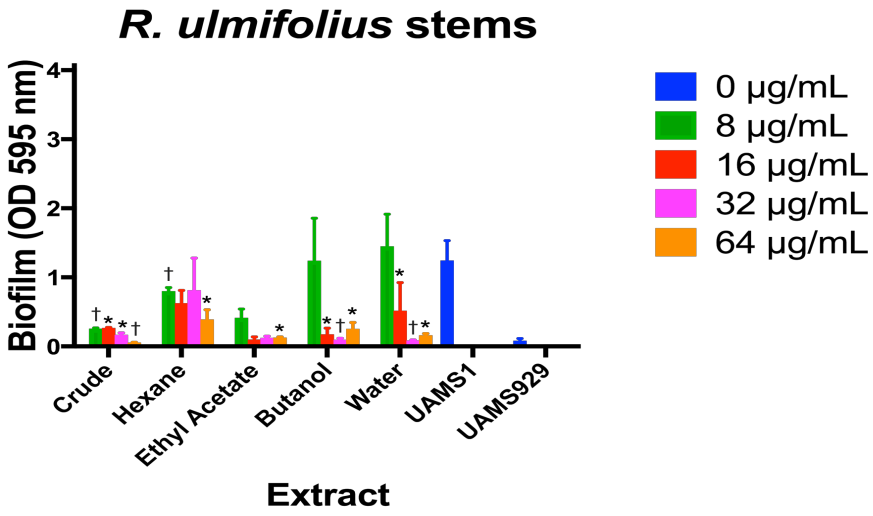
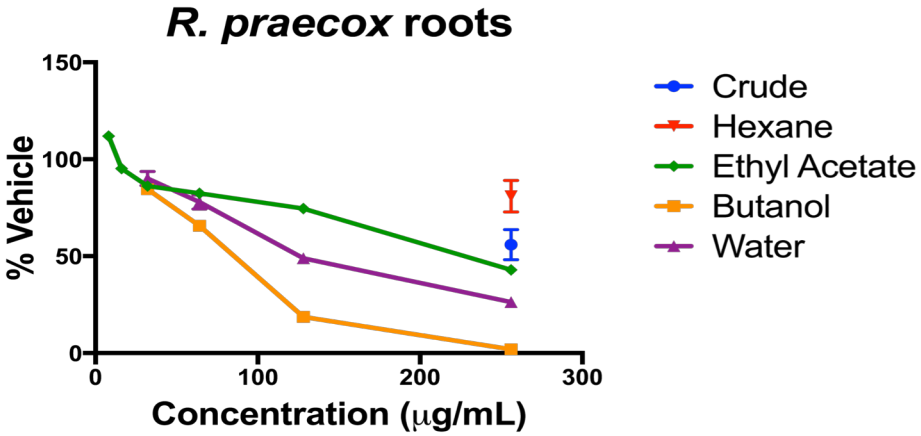


Figure 4.2. *R. ulmifolius* stems growth and biofilm inhibition
(A) Growth inhibition of *S. aureus* by *R. ulmifolius* stems extracts. At 256 µg/mL: the crude and butanol partitions showed significance ($0.001 < \text{p-value} < 0.01$); the hexane, ethyl acetate, and water partitions showed significance ($\text{p-value} < 0.001$). At 128 µg/mL: the hexane and butanol partitions showed significance ($0.01 < \text{p-value} < 0.05$); the water partition showed significance ($\text{p-value} < 0.001$). At 64 µg/mL: the hexane and butanol partitions showed significance ($0.01 < \text{p-value} < 0.05$); the water partition showed significance ($0.001 < \text{p-value} < 0.01$). At 32 µg/mL the butanol and water showed significance ($0.01 < \text{p-value} < 0.05$). (B) *S. aureus* biofilm inhibition by *R. ulmifolius* stems extracts. ($0.01 \leq \text{p-value} < 0.05$: *, $0.001 \leq \text{p-value} < 0.01$: †, $\text{p-value} < 0.001$: ‡). Significance refers to differences between cultures treated with control (DMSO) and cultures exposed to extracts at varying concentrations.

A



B

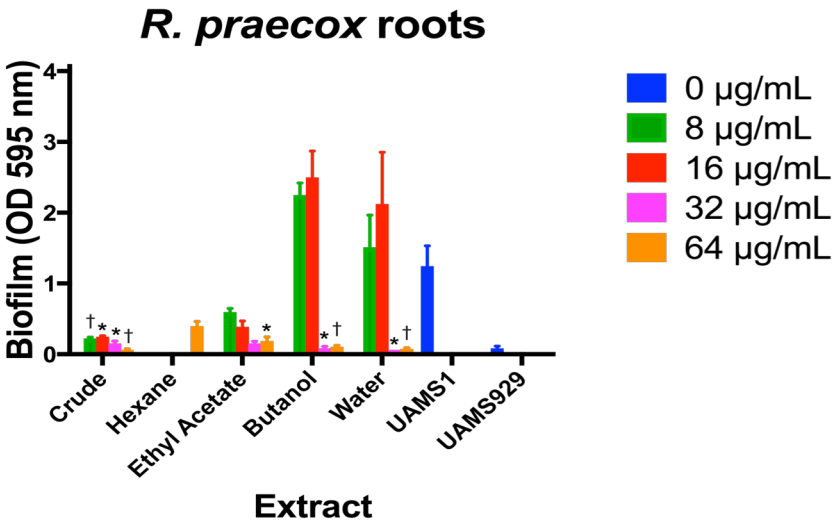
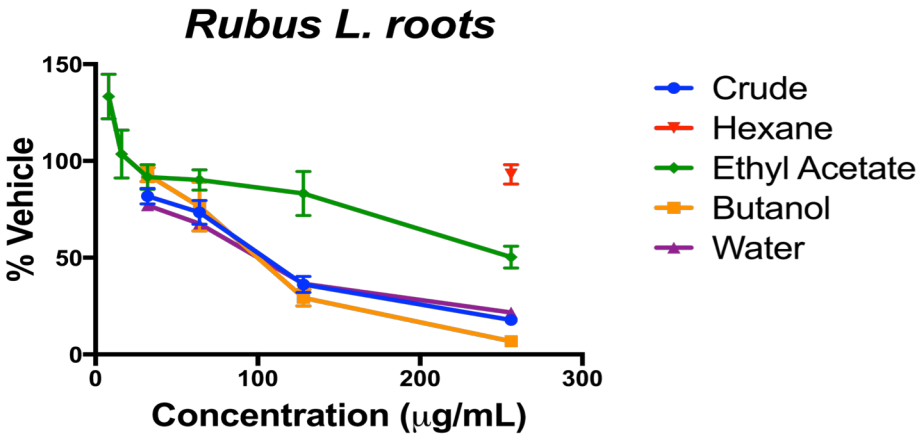


Figure 4.3. *R. praecox* roots growth and biofilm inhibition
(A) Growth inhibition of *S. aureus* by *R. praecox* roots extracts. At 256 μg/mL: the hexane partition showed significance ($0.01 < p\text{-value} < 0.05$); the crude, ethyl acetate, and water partitions showed significance ($0.001 < p\text{-value} < 0.01$); the butanol partition showed significance ($p\text{-value} < 0.001$). At 128 μg/mL: the ethyl acetate, butanol, and water partitions showed significance ($p\text{-value} < 0.001$). At 64 μg/mL: the water partition showed significance ($0.001 < p\text{-value} < 0.01$); the ethyl acetate and butanol partitions showed significance ($p\text{-value} < 0.001$). At 8 μg/mL: the ethyl acetate partition showed significance ($0.001 < p\text{-value} < 0.01$). (B) *S. aureus* biofilm inhibition by *R. praecox* roots extracts. ($0.01 \leq p\text{-value} < 0.05$: *, $0.001 \leq p\text{-value} < 0.01$: †, $p\text{-value} < 0.001$: ‡). Significance refers to differences between cultures treated with control (DMSO) and cultures exposed to extracts at varying concentrations.

A



B

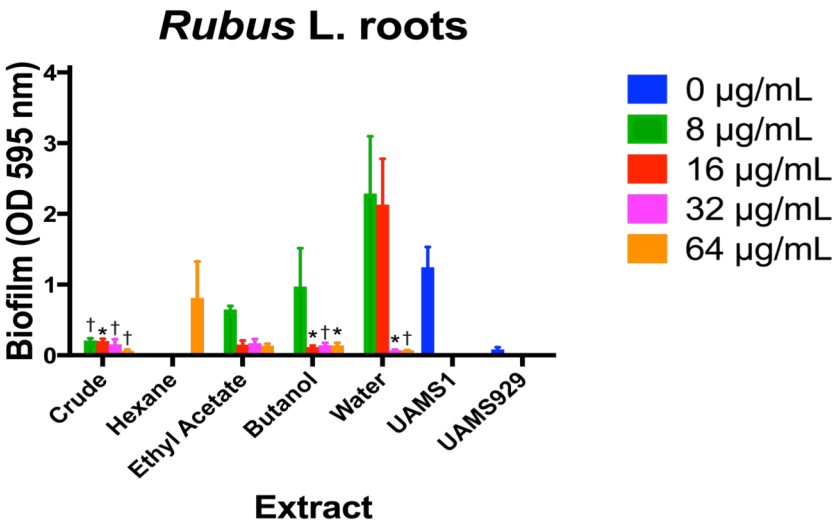
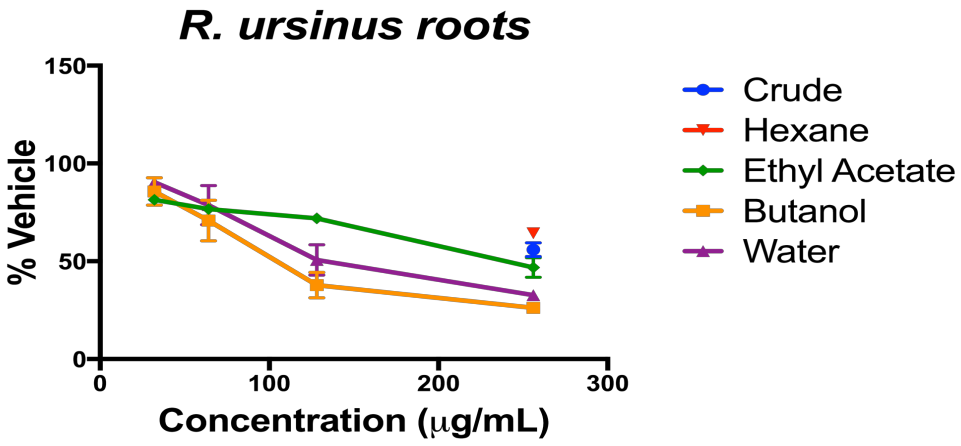


Figure 4.4. *R.* L. roots growth and biofilm inhibition
(A) Growth inhibition of *S. aureus* by *Rubus* L. roots extracts. At 256 μg/mL: the crude and butanol partitions showed significance ($0.001 < \text{p-value} < 0.01$); the ethyl acetate and water partition showed significance ($\text{p-value} < 0.001$). At 128 μg/mL: the crude, butanol and water partition showed significance ($\text{p-value} < 0.001$). At 64 μg/mL: the crude showed significance ($0.01 < \text{p-value} < 0.05$); the water partition showed significance ($0.001 < \text{p-value} < 0.01$). At 32 μg/mL: both crude and water partition showed significance ($0.01 < \text{p-value} < 0.05$). At 8 μg/mL: the ethyl acetate partition showed significance ($0.01 < \text{p-value} < 0.05$). (B) *S. aureus* biofilm inhibition by *Rubus* L. roots extracts. ($0.01 \leq \text{p-value} < 0.05$: *, $0.001 \leq \text{p-value} < 0.01$: †, $\text{p-value} < 0.001$: ‡). Significance refers to differences between cultures treated with control (DMSO) and cultures exposed to extracts at varying concentrations.

A



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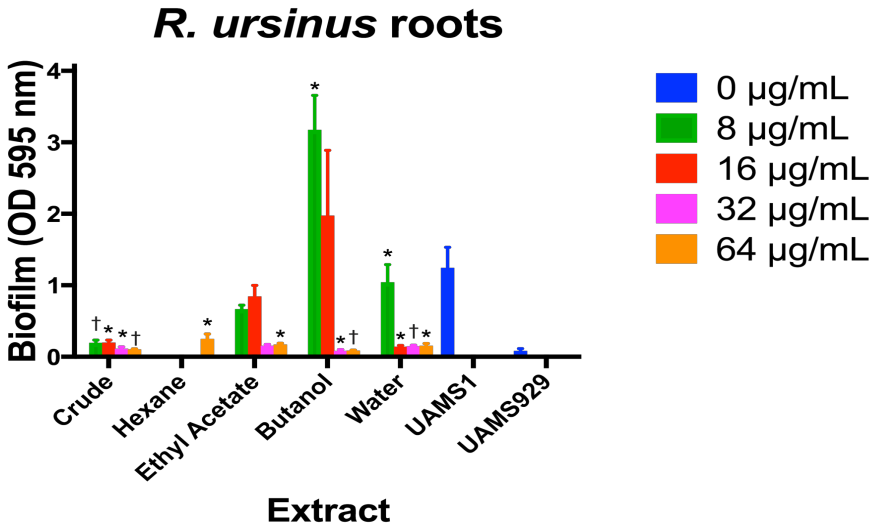
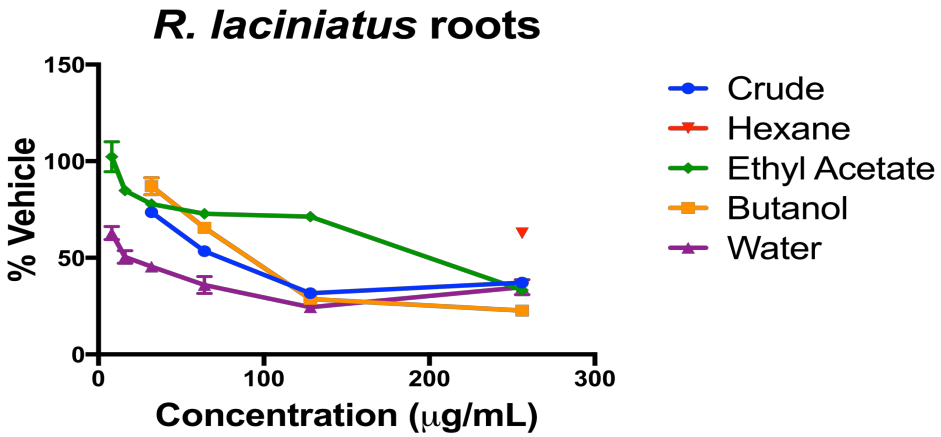


Figure 4.5. *R. ursinus* roots growth and biofilm inhibition
(A) Growth inhibition of *S. aureus* by *Rubus ursinus* roots extracts. At 256 μg/mL: the crude extract showed significance ($0.001 < p\text{-value} < 0.01$); the hexane, ethyl acetate, butanol, and water partitions showed significance ($p\text{-value} < 0.001$). At 128 μg/mL: the butanol and water partition showed significance ($0.001 < p\text{-value} < 0.01$); the ethyl acetate partition showed significance ($p\text{-value} < 0.001$). At 64 μg/mL: the hexane and butanol partitions showed significance ($0.01 < p\text{-value} < 0.05$); the ethyl acetate partition showed significance ($p\text{-value} < 0.001$). At 32 μg/mL: the ethyl acetate partition showed significance ($0.01 < p\text{-value} < 0.05$); the hexane partition showed significance ($0.001 < p\text{-value} < 0.01$). At 16 μg/mL: the ethyl acetate partition showed significance ($0.001 < p\text{-value} < 0.01$). At 8 μg/mL: the ethyl acetate partition showed significance ($p\text{-value} < 0.001$). (B) *S. aureus* biofilm inhibition by *Rubus ursinus* roots extracts. ($0.01 \leq p\text{-value} < 0.05$: *, $0.001 \leq p\text{-value} < 0.01$: †, $p\text{-value} < 0.001$: ‡). Significance refers to differences between cultures treated with control (DMSO) and cultures exposed to extracts at varying concentrations.

A



B

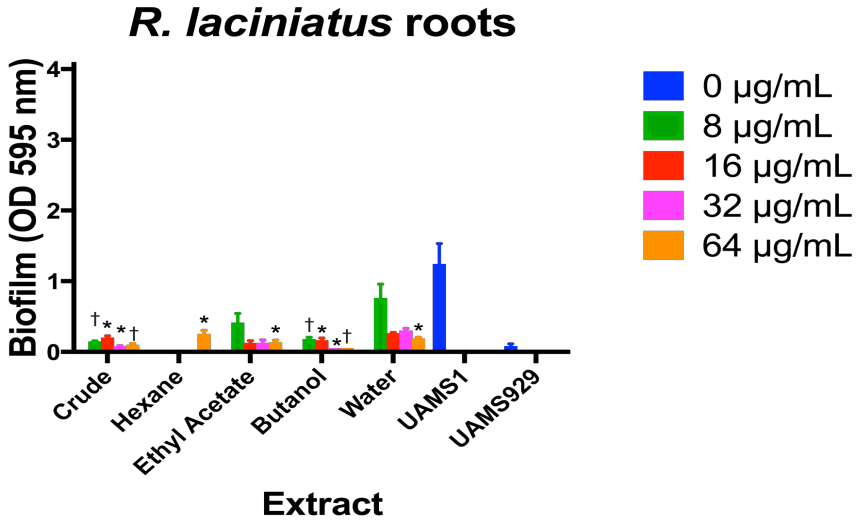
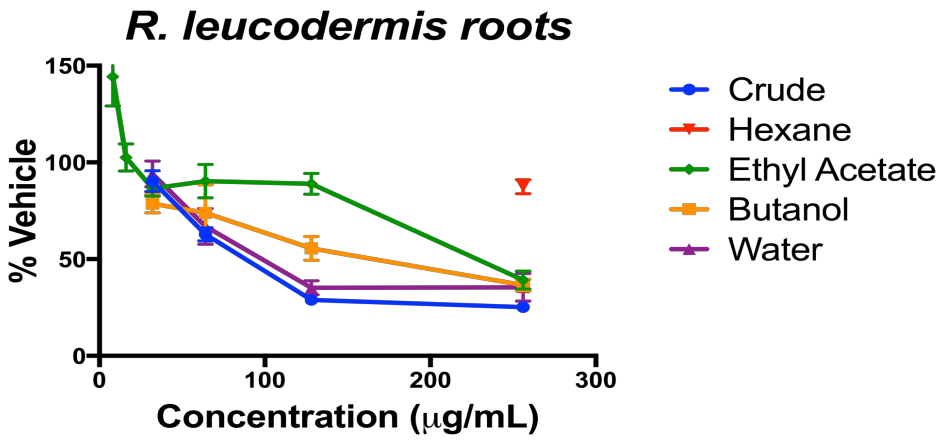


Figure 4.6. *R. laciniatus* roots growth and biofilm inhibition
(A) Growth inhibition of *S. aureus* by *R. laciniatus* roots extracts. At 256 μg/mL: the hexane partition showed significance (0.01 < p-value < 0.05); the crude extract showed significance (0.001 < p-value < 0.01); the ethyl acetate, butanol, and water partitions showed significance (p-value < 0.001). At 128 μg/mL: the crude, ethyl acetate, butanol and water partition showed significance (p-value < 0.001). At 64 μg/mL: the butanol partition showed significance (0.001 < p-value < 0.01); the crude, ethyl acetate, and water partitions showed significance (p-value < 0.001). At 32 μg/mL: the crude, ethyl acetate, and water partitions showed significance (0.01 < p-value < 0.05). (B) *S. aureus* biofilm inhibition by *R. laciniatus* roots extracts. (0.01 ≤ p-value < 0.05: *, 0.001 ≤ p-value < 0.01: †, p-value < 0.001: ‡). Significance refers to differences between cultures treated with control (DMSO) and cultures exposed to extracts at varying concentrations.

A



B

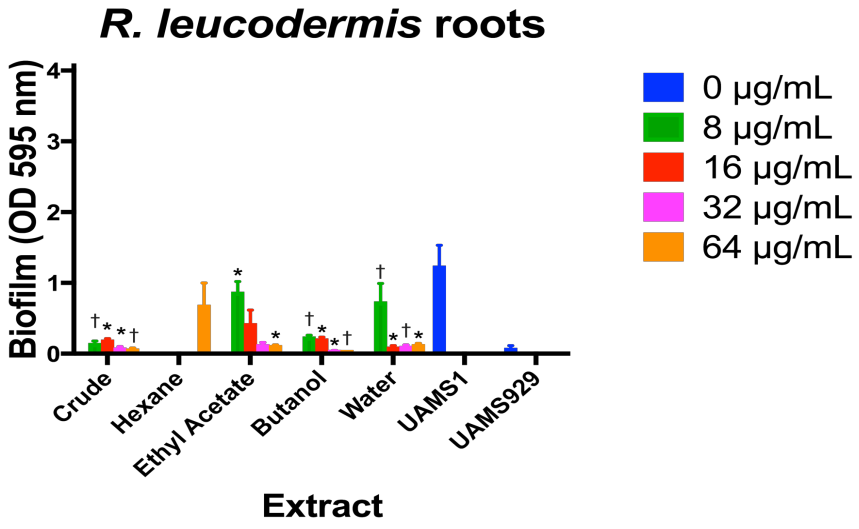
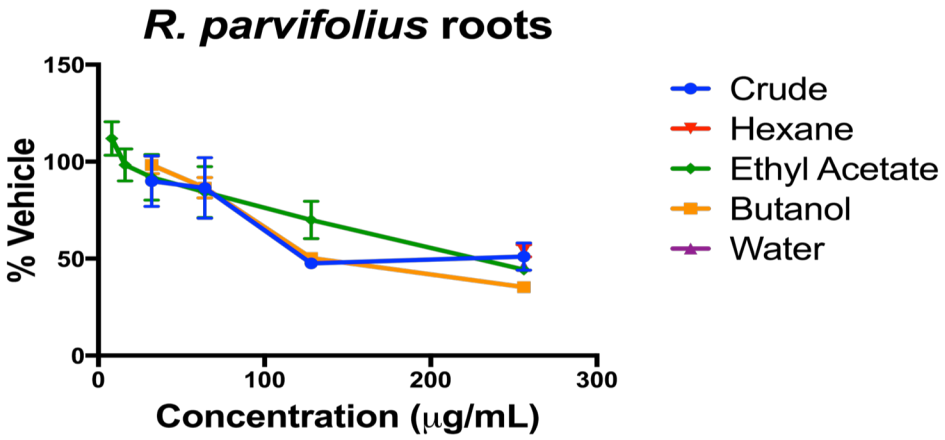


Figure 4.7. *R. leucodermis* roots growth and biofilm inhibition
(A) Growth inhibition of *S. aureus* by *R. leucodermis* roots extracts. At 256 µg/mL: the crude extract showed significance ($0.001 < p\text{-value} < 0.01$); the ethyl acetate, butanol and water partitions showed significance ($p\text{-value} < 0.001$). At 128 µg/mL: the butanol partition showed significance ($0.001 < p\text{-value} < 0.01$); the crude and water partitions showed significance ($p\text{-value} < 0.001$). At 64 µg/mL: the water partition showed significance ($0.01 < p\text{-value} < 0.05$); the crude extract showed significance ($p\text{-value} < 0.001$). At 32 µg/mL the ethyl acetate and butanol partitions showed significance ($0.01 < p\text{-value} < 0.05$). At 8 µg/mL the ethyl acetate partition showed significance ($0.01 < p\text{-value} < 0.05$). (B) *S. aureus* biofilm inhibition by *R. leucodermis* roots extracts. ($0.01 \leq p\text{-value} < 0.05$: *, $0.001 \leq p\text{-value} < 0.01$: †, $p\text{-value} < 0.001$: ‡). Significance refers to differences between cultures treated with control (DMSO) and cultures exposed to extracts at varying concentrations.

A



B

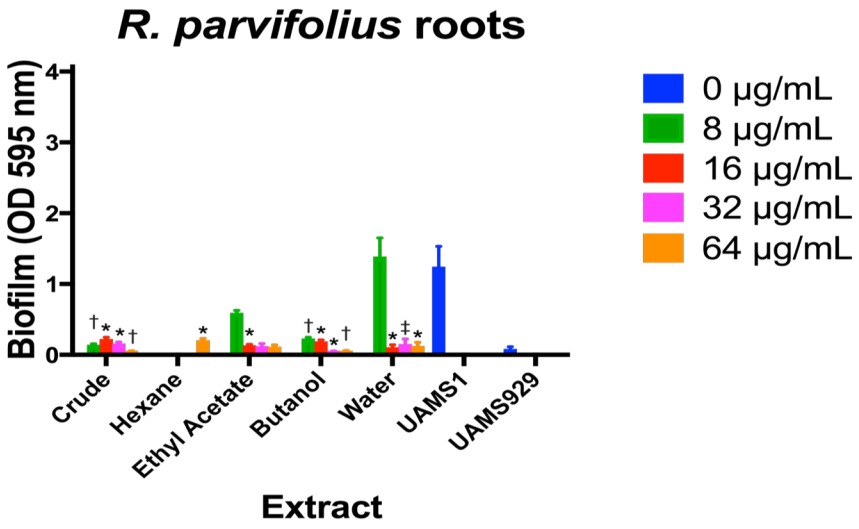
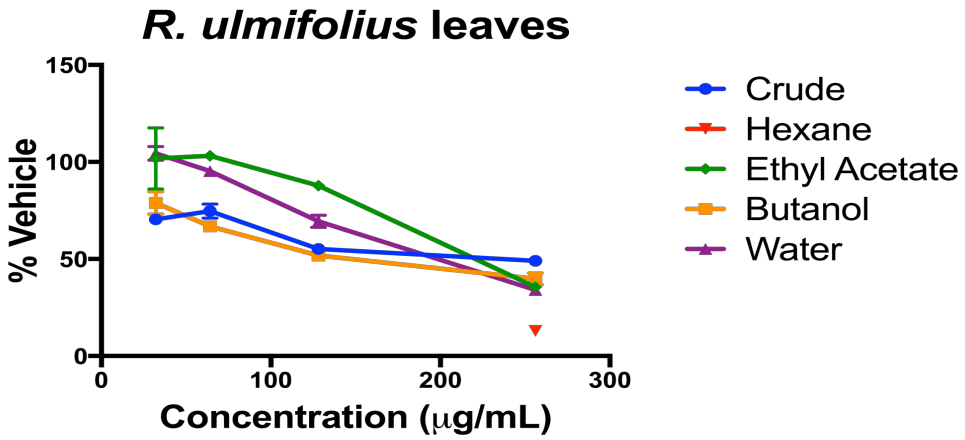


Figure 4.8. *R. parvifolius* roots growth and biofilm inhibition
(A) Growth inhibition of *S. aureus* by *R. parvifolius* roots extracts. At 256 μg/mL: the hexane, ethyl acetate, butanol, and water partitions showed significance ($0.001 < \text{p-value} < 0.01$); the crude extract showed significance (p-value < 0.001). At 128 μg/mL: the ethyl acetate partition showed significance ($0.01 < \text{p-value} < 0.05$); the crude and butanol partitions showed significance (p-value < 0.001). At 64 μg/mL: the butanol partition showed significance ($0.01 < \text{p-value} < 0.05$). (B) *S. aureus* biofilm inhibition by *R. parvifolius* roots extracts. ($0.01 \leq \text{p-value} < 0.05$: *, $0.001 \leq \text{p-value} < 0.01$: †, p-value < 0.001: ‡). Significance refers to differences between cultures treated with control (DMSO) and cultures exposed to extracts at varying concentrations.

A



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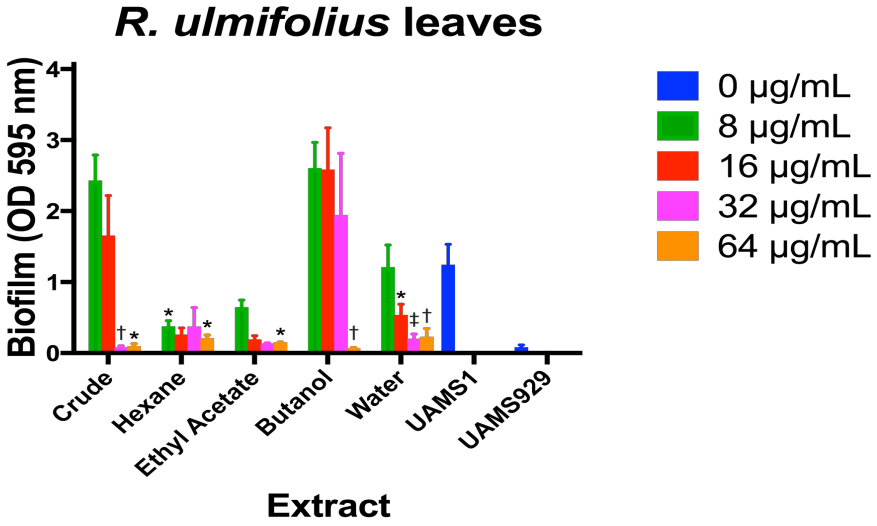
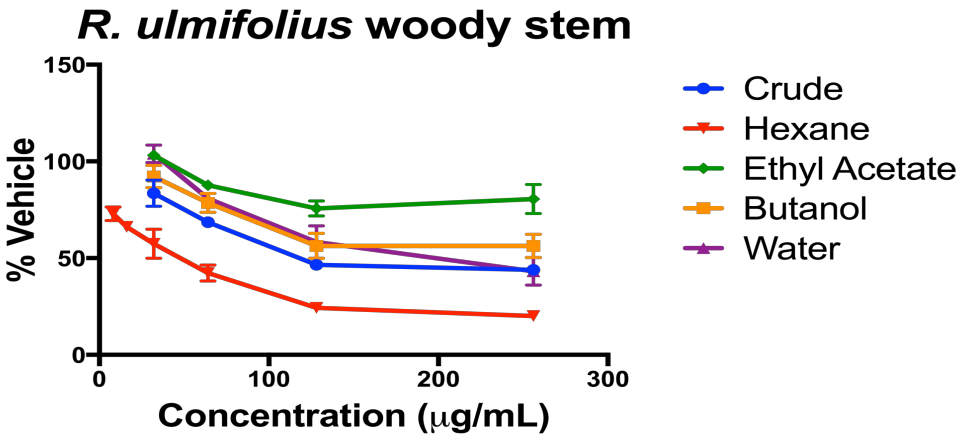


Figure 4.9. *R. ulmifolius* leaves growth and biofilm inhibition
(A) Growth inhibition of *S. aureus* by *R. ulmifolius* leaves extracts. At 256 μg/mL: the crude, ethyl acetate, and water partitions showed significance ($0.001 < \text{p-value} < 0.01$); the hexane and butanol partitions showed significance ($\text{p-value} < 0.001$). At 128 μg/mL: the water partition showed significance ($0.01 < \text{p-value} < 0.001$); the crude, ethyl acetate, and butanol partitions showed significance ($\text{p-value} < 0.001$). At 64 μg/mL: the water partition showed significance ($0.01 < \text{p-value} < 0.05$); the crude extract showed significance ($0.01 < \text{p-value} < 0.001$); the butanol partition showed significance ($\text{p-value} < 0.001$). At 32 μg/mL: the butanol partition showed significance ($0.01 < \text{p-value} < 0.05$); the crude extract showed significance ($0.001 < \text{p-value} < 0.01$). (B) *S. aureus* biofilm inhibition by *R. ulmifolius* leaves extracts. ($0.01 \leq \text{p-value} < 0.05$: *, $0.001 \leq \text{p-value} < 0.01$: †, $\text{p-value} < 0.001$: ‡). Significance refers to differences between cultures treated with control (DMSO) and cultures exposed to extracts at varying concentrations.

A



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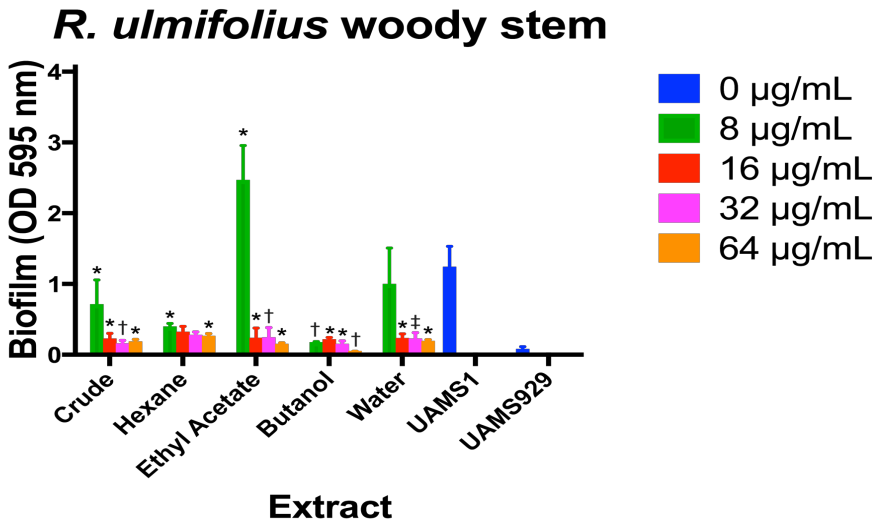
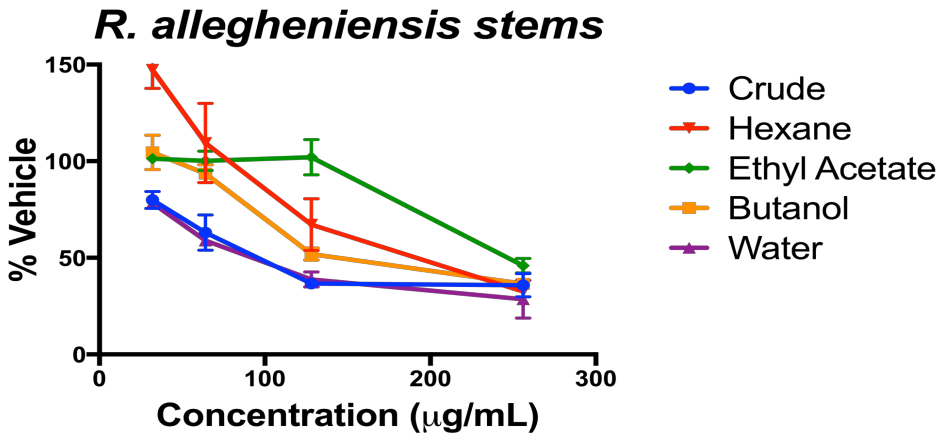


Figure 4.10. *R. ulmifolius* woody stem growth and biofilm inhibition
(A) Growth inhibition of *S. aureus* by *R. ulmifolius* woody stems extracts. At 256 µg/mL: the ethyl acetate partition showed significance ($0.01 < \text{p-value} < 0.05$); the crude and butanol partitions showed significance ($0.001 < \text{p-value} < 0.01$); the water partition showed significance ($\text{p-value} < 0.001$). At 128 µg/mL: the crude, ethyl acetate, butanol, and water partitions showed significance ($0.001 < \text{p-value} < 0.01$); the crude and butanol partitions showed significance ($\text{p-value} < 0.001$). (B) *S. aureus* biofilm inhibition by *R. ulmifolius* woody stems extracts. ($0.01 \leq \text{p-value} < 0.05$: *, $0.001 \leq \text{p-value} < 0.01$: †, $\text{p-value} < 0.001$: ‡). Significance refers to differences between cultures treated with control (DMSO) and cultures exposed to extracts at varying concentrations.

A



B

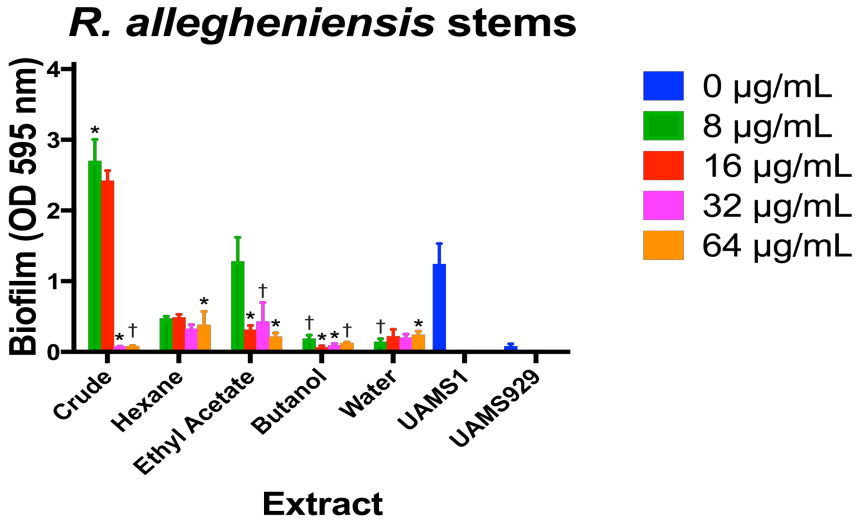


Figure 4.11. *R. allegheniensis* stems growth and biofilm inhibition
(A) Growth inhibition of *S. aureus* by *R. allegheniensis* stems extracts. At 256 μg/mL: the water partition showed significance ($0.001 < p\text{-value} < 0.01$); the crude, hexane, ethyl acetate, and butanol partitions showed significance ($p\text{-value} < 0.001$). At 128 μg/mL: the crude, butanol, and water partition showed significance ($p\text{-value} < 0.001$). At 64 μg/mL: the crude extract showed significance ($0.01 < p\text{-value} < 0.05$); the water partition showed significance ($p\text{-value} < 0.001$). At 32 μg/mL the crude and water partitions showed significance ($0.01 < p\text{-value} < 0.05$); the hexane partition showed significance ($0.001 < p\text{-value} < 0.01$). (B) *S. aureus* biofilm inhibition by *R. allegheniensis* stems extracts. ($0.01 \leq p\text{-value} < 0.05$: *, $0.001 \leq p\text{-value} < 0.01$: †, $p\text{-value} < 0.001$: ‡). Significance refers to differences between cultures treated with control (DMSO) and cultures exposed to extracts at varying concentrations.

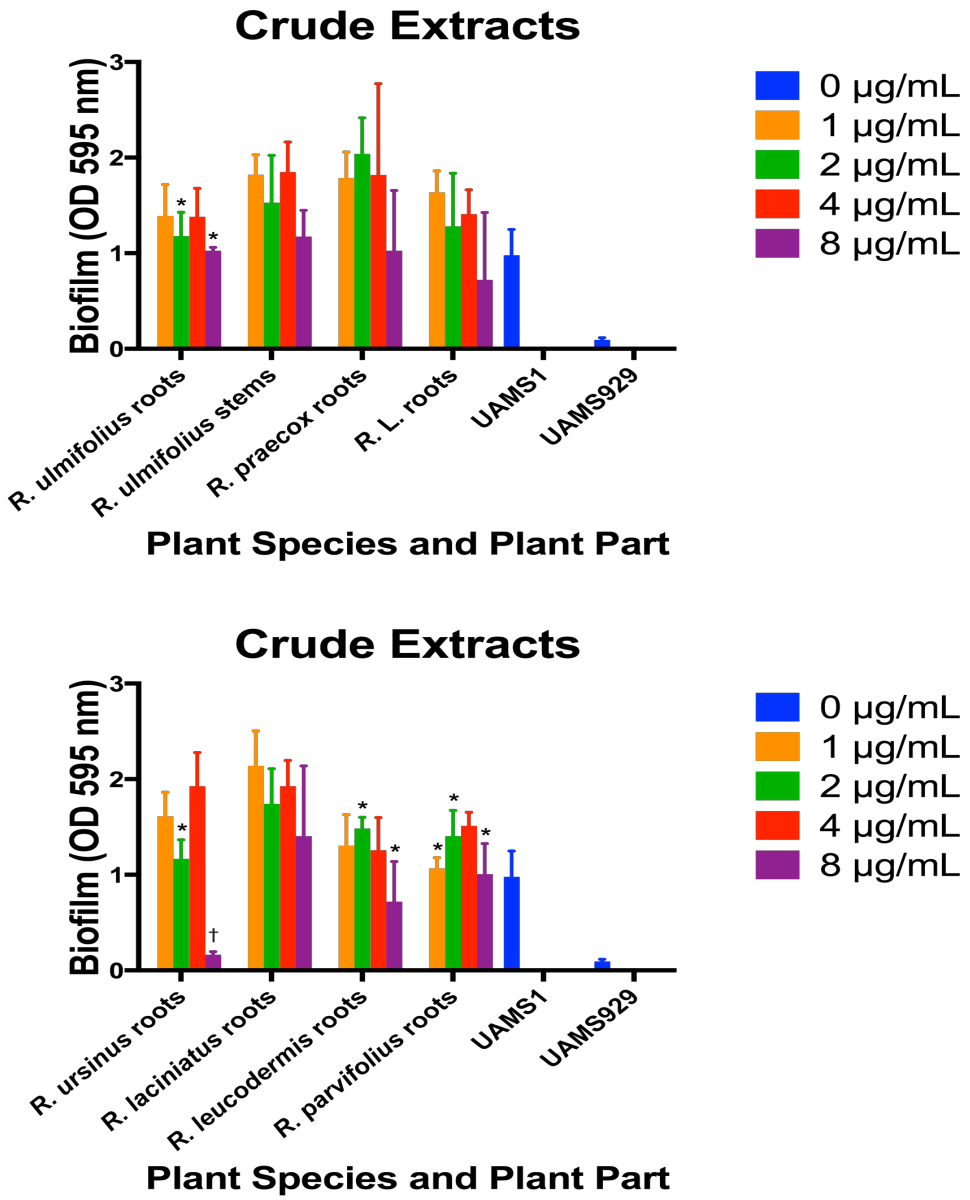


Figure 4.12. Biofilm inhibition of selected crude extracts
Based upon the results of the biofilm testing, the above crude extracts were tested further at lower concentrations for biofilm inhibition against *S. aureus*. ($0.01 \leq$ p-value < 0.05: *, $0.001 \leq$ p-value < 0.01: †, p-value < 0.001: ‡). Significance refers to differences between cultures treated with control (DMSO) and cultures exposed to extracts at varying concentrations

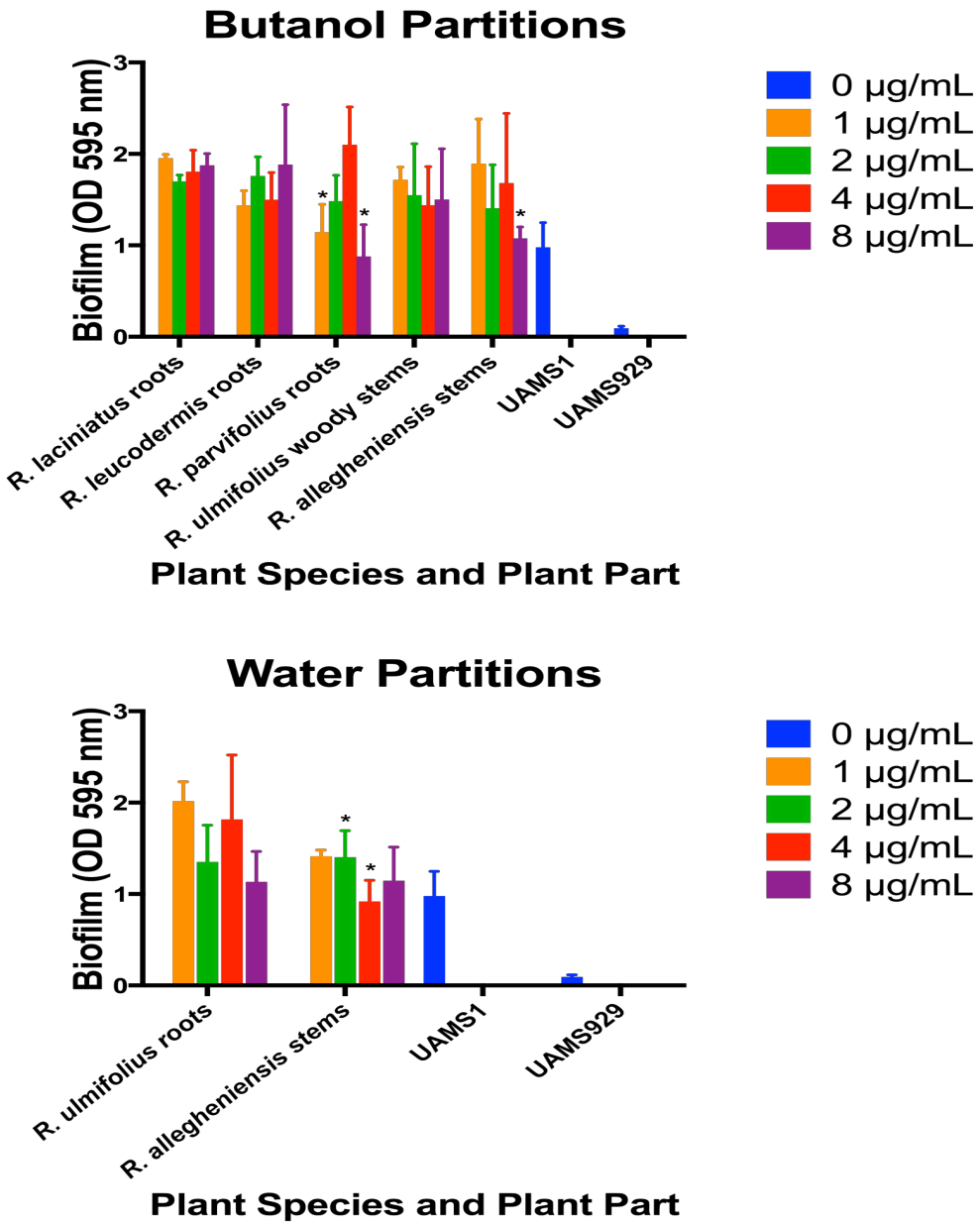
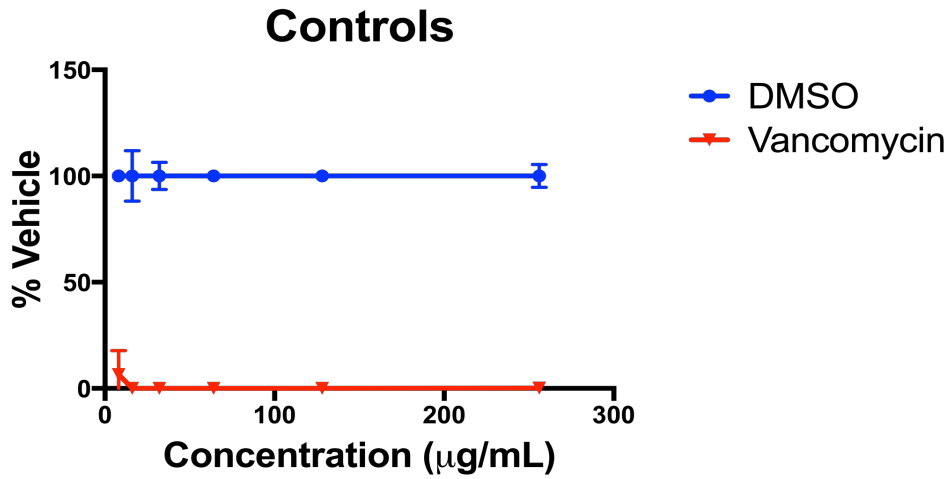


Figure 4.13. Biofilm inhibition of selected Butanol and Water Partitions
Based upon the results of the biofilm testing, the following butanol and water partitions were tested further at lower concentrations for biofilm inhibition against *S. aureus*. ($0.01 \leq$ p-value < 0.05: *, $0.001 \leq$ p-value < 0.01: †, p-value < 0.001: ‡). Significance refers to differences between cultures treated with control (DMSO) and cultures exposed to extracts at varying concentrations

A



B

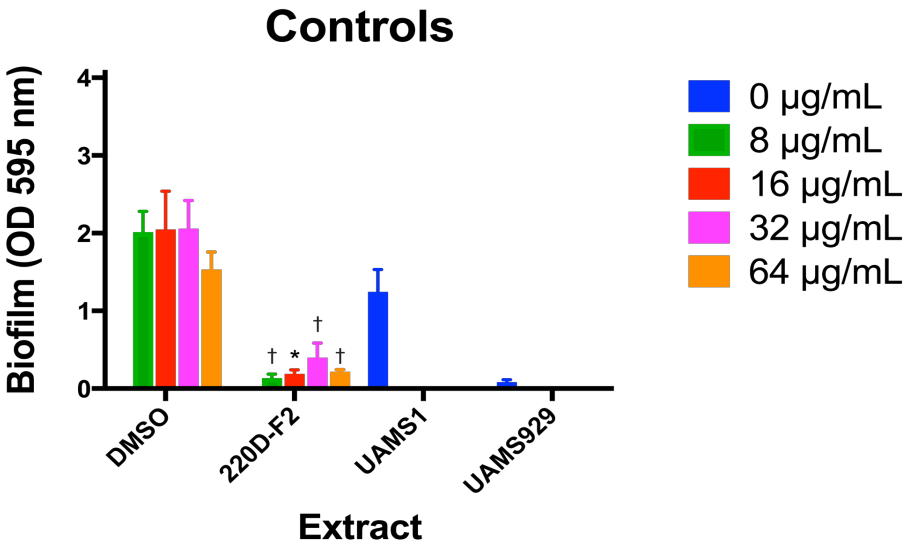


Figure 4.14. Controls used for growth and biofilm inhibition tests
(A) Controls used for the growth inhibition testing. Both Vancomycin and DMSO were used as controls. (B) Controls used for the biofilm inhibition testing. ($0.01 \leq$ p-value < 0.05: *, $0.001 \leq$ p-value < 0.01: †, p-value < 0.001: ‡). Significance refers to differences between cultures treated with control (DMSO) and cultures exposed to extracts at varying concentrations.

HPLC Analysis:

HPLC analysis of all 55 extracts was conducted in order to verify whether each of these extracts contained any of the three active compounds previously found within *Rubus ulmifolius* (Quave, Estévez-Carmona et al. 2012). The three compounds are Ellagic Acid, Ellagic Acid-rhamnoside, and Ellagic Acid-xyloside (Figure 4.15.). In order to compare the active constituents within the varying partitions, the chromatograms were stacked on top of each other, grouped by plant species/part (Figure 4.16.). Of the 55 extracts tested only 11 did not contain ellagic acid. Out of those 11 extracts, 9 of them were hexane partitions and 2 of them were water partitions. None of the extracts contained either of the ellagic acid derivatives. Even though, there were 4 additional signals that were extremely common throughout the extracts. Based upon retention times and UV spectrums, 38 of the 55 extracts shared a common signal at around 56 minutes. Additionally, 42 of the 55 extracts shared a common signal at around 47 minutes; 35 of the 55 extracts shared a common signal at 51 minutes; and 5 extracts shared a common signal at 57 minutes. The UV spectrum for each of the standards used (EA, EA-rhamonoside, EA-xyloside) was identical. Similarly, the common signals found at 56 mins and 57 mins also exhibited the same UV spectrum as the standards tested (EA, EA-rhamonoside, EA-xyloside).

HPLC Summary Table

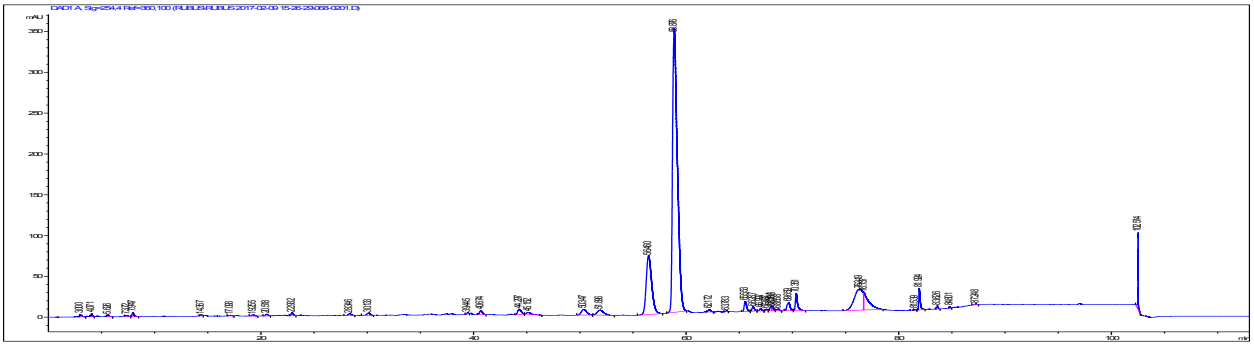
Extract	Ellagic Acid	Ellagic Acid-Rhamnoside	Ellagic Acid- Xyloside
725	+	-	-
725B	-	-	-
725C	+	-	-
725D	+	-	-
725E	-	-	-
726	+	-	-
726B	+	-	-
726C	+	-	-
726D	+	-	-
726E	+	-	-
728	+	-	-
728B	-	-	-
728C	+	-	-
728D	+	-	-
728E	+	-	-
729	+	-	-
729B	-	-	-
729C	+	-	-
729D	+	-	-
729E	+	-	-
730	+	-	-
730B	-	-	-
730C	+	-	-
730D	+	-	-
730E	+	-	-
731	+	-	-
731B	-	-	-
731C	+	-	-
731D	+	-	-
731E	+	-	-
732	+	-	-
732B	-	-	-
732C	+	-	-
732D	+	-	-
732E	+	-	-
733	+	-	-
733B	+	-	-
733C	+	-	-
733D	+	-	-
733E	+	-	-
734	+	-	-

734B	-	-	-
734C	+	-	-
734D	+	-	-
734E	+	-	-
735	+	-	-
735B	-	-	-
735C	+	-	-
735D	+	-	-
735E	-	-	-
737	+	-	-
737B	-	-	-
737C	+	-	-
737D	+	-	-
737E	+	-	-

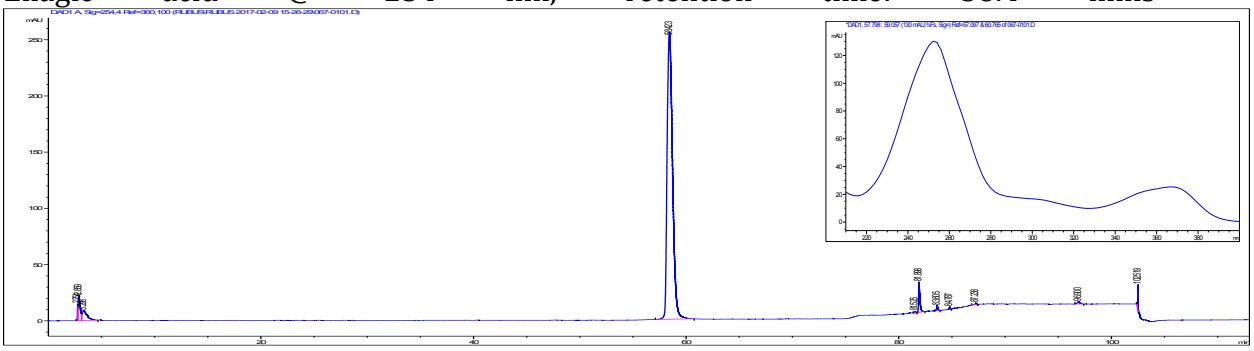
Table 4.4. HPLC Summary table
HPLC was used to determine if three active compounds (Ellagic Acid, Ellagic acid-rhamnoside, Ellagic acid-xyloside) found previously in *Rubus ulmifolius* root extract (220D-F2) are present within these different *Rubus* extracts. Based on retention time and UV spectrum, these compounds were either identified within the extract (+) or absent (-)

HPLC Chromatographs

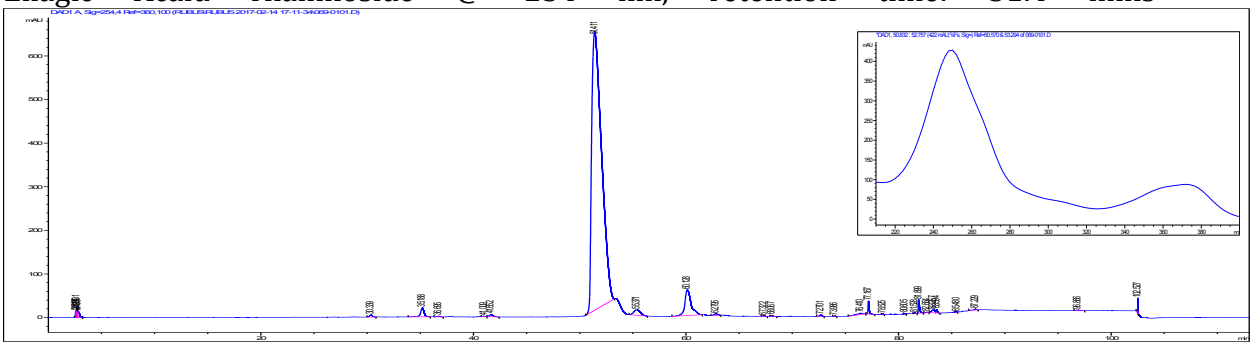
220D-F2 @254 nm



Ellagic acid @ 254 nm, retention time: 58.4 mins



Ellagic Acaid rhamnoside @ 254 nm, retention time: 51.4 mins



Ellagic acid xylsoside @254 nm, retention time: 46.8 minutes

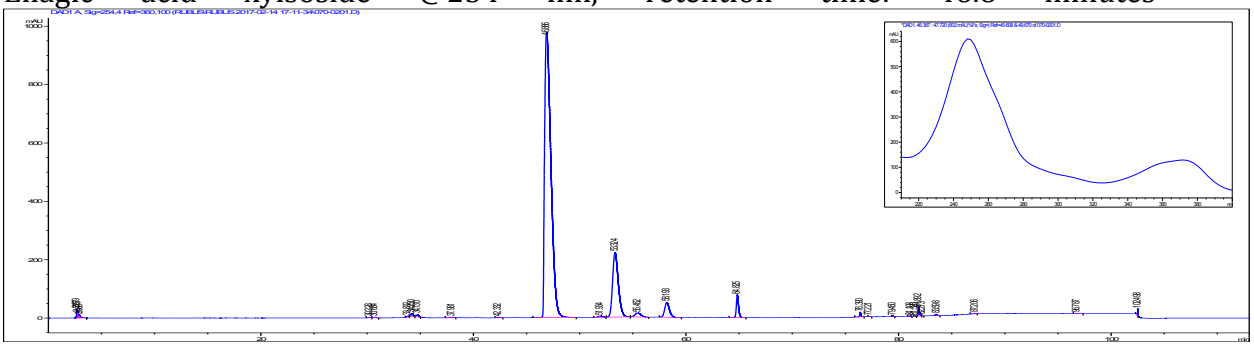
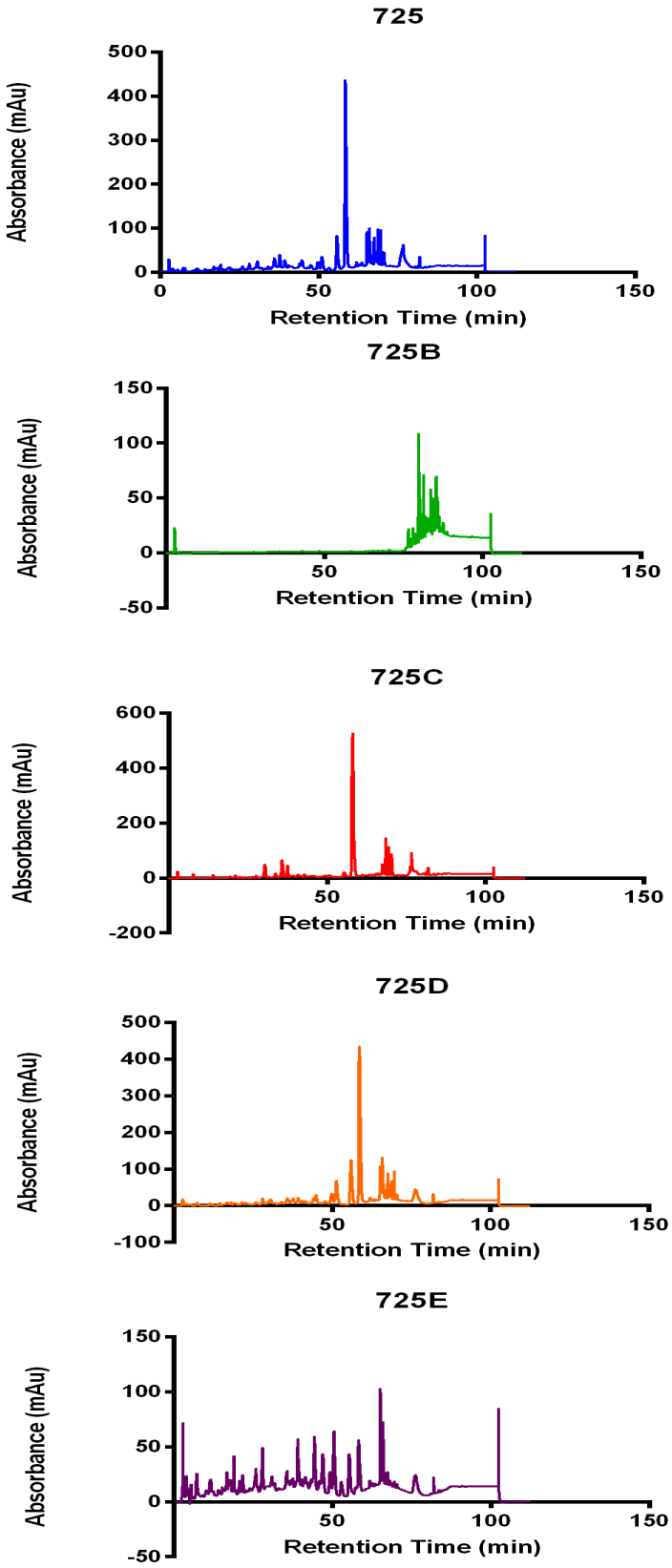
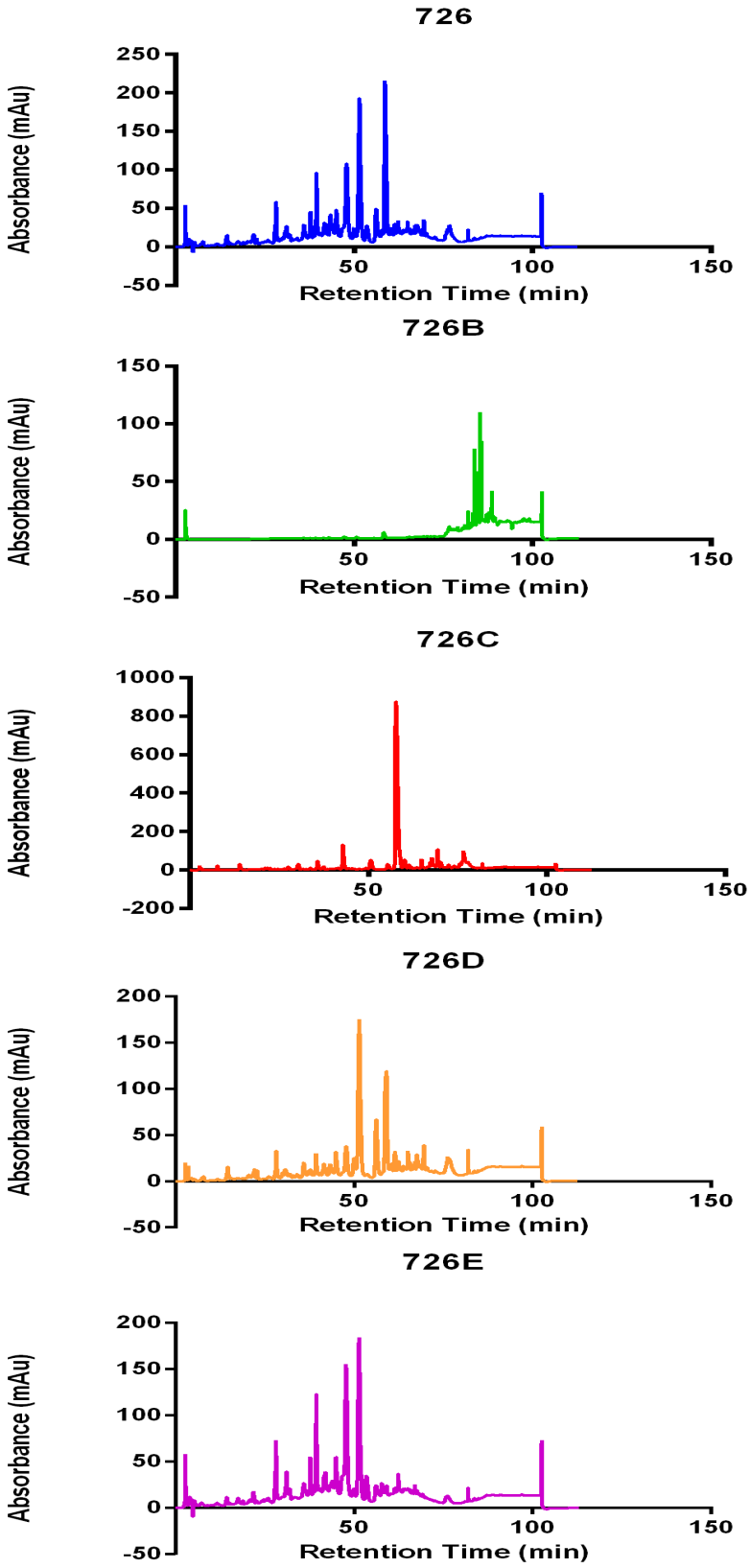


Figure 4.15. Chromatographs and retention time for standards and control (220D-F2)
In the top right of each chromatograph, the UV spectrum is displayed. The large peak shown in all 4 chromatographs is ellagic acid.

A


725
Absorbance (mAu)
500
400
300
200
100
0
0
50
100
150
Retention Time (min)
725B
Absorbance (mAu)
150
100
50
0
-50
50
100
150
Retention Time (min)
725C
Absorbance (mAu)
600
400
200
0
-200
50
100
150
Retention Time (min)
725D
Absorbance (mAu)
500
400
300
200
100
0
-100
50
100
150
Retention Time (min)
725E
Absorbance (mAu)
150
100
50
0
-50
50
100
150
Retention Time (min)

B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100
50
0
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50
100
150
Retention Time (min)
726C
Absorbance (mAu)
1000
800
600
400
200
0
-200
50
100
150
Retention Time (min)
726D
Absorbance (mAu)
200
150
100
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100
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Absorbance (mAu)
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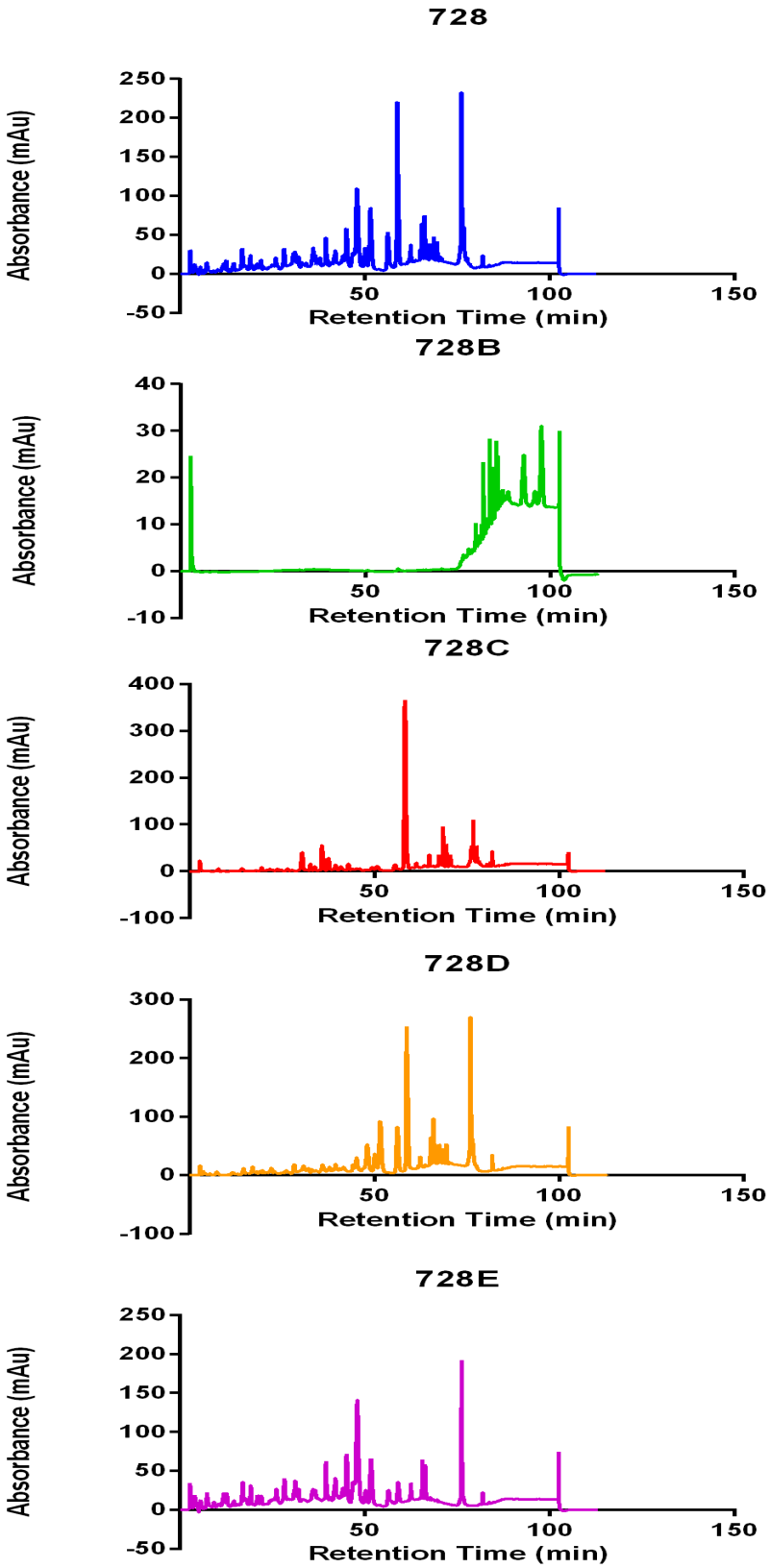
150
Retention Time (min)

C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20
10
0
-10
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100
150
Retention Time (min)
728C
Absorbance (mAu)
400
300
200
100
0
-100
50
100
150
Retention Time (min)
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Absorbance (mAu)
300
200
100
0
-100
50
100
150
Retention Time (min)
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100
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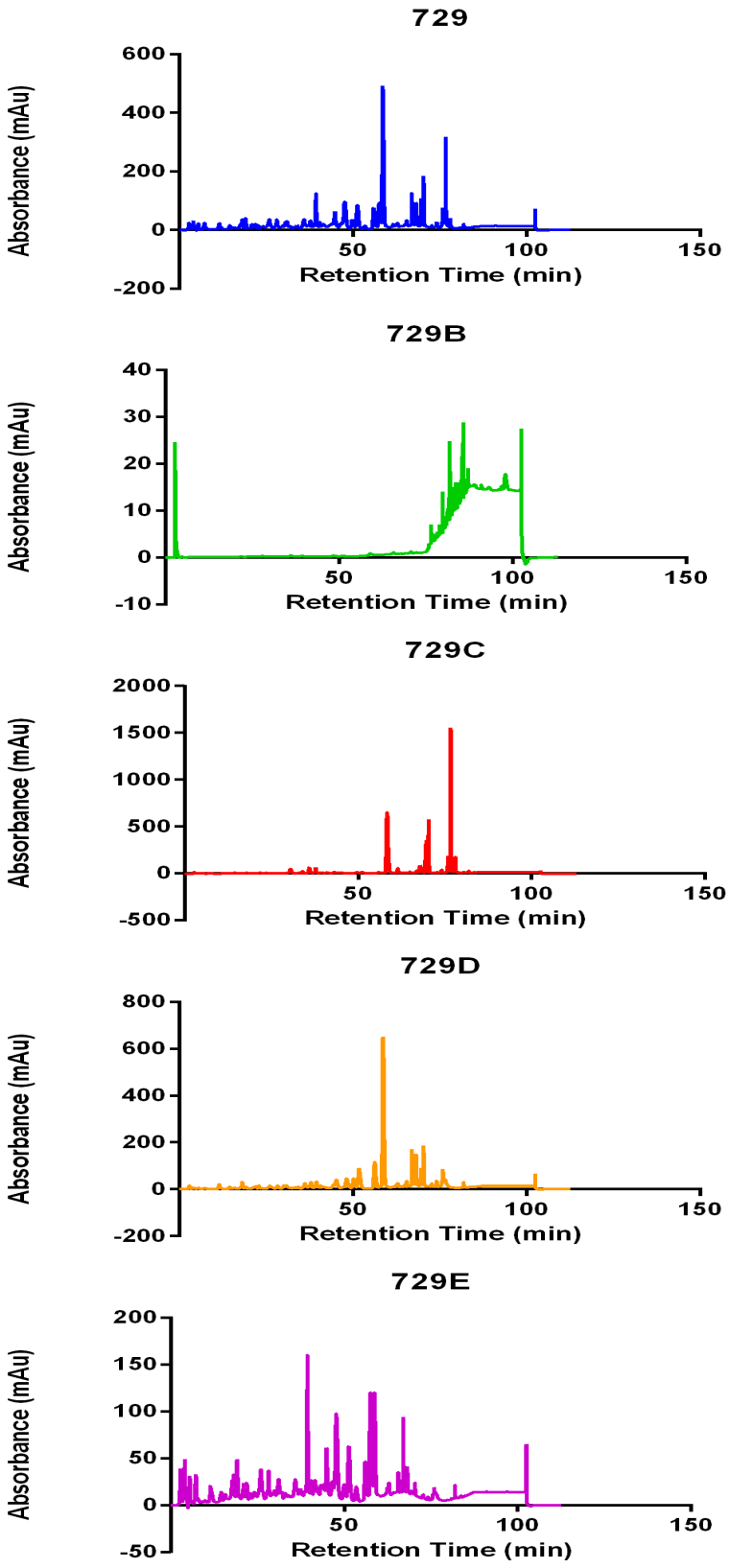
Retention Time (min)

D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20
10
0
-10
50
100
150
Retention Time (min)
729C
Absorbance (mAu)
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1500
1000
500
0
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50
100
150
Retention Time (min)
729D
Absorbance (mAu)
800
600
400
200
0
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100
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Absorbance (mAu)
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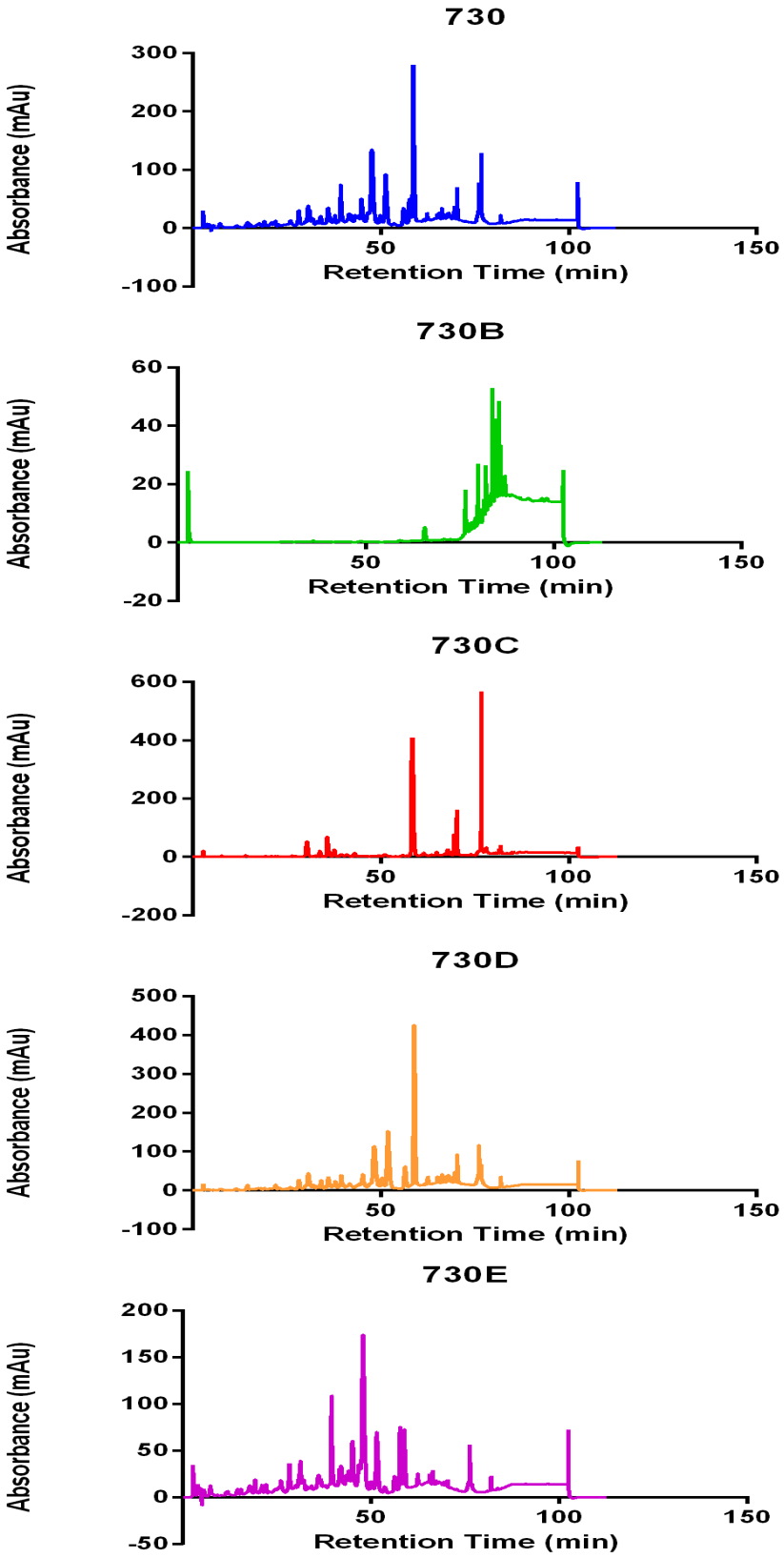
150
Retention Time (min)

E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100
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Retention Time (min)
730C
Absorbance (mAu)
600
400
200
0
-200
50
100
150
Retention Time (min)
730D
Absorbance (mAu)
500
400
300
200
100
0
-100
50
100
150
Retention Time (min)
730E
Absorbance (mAu)
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150
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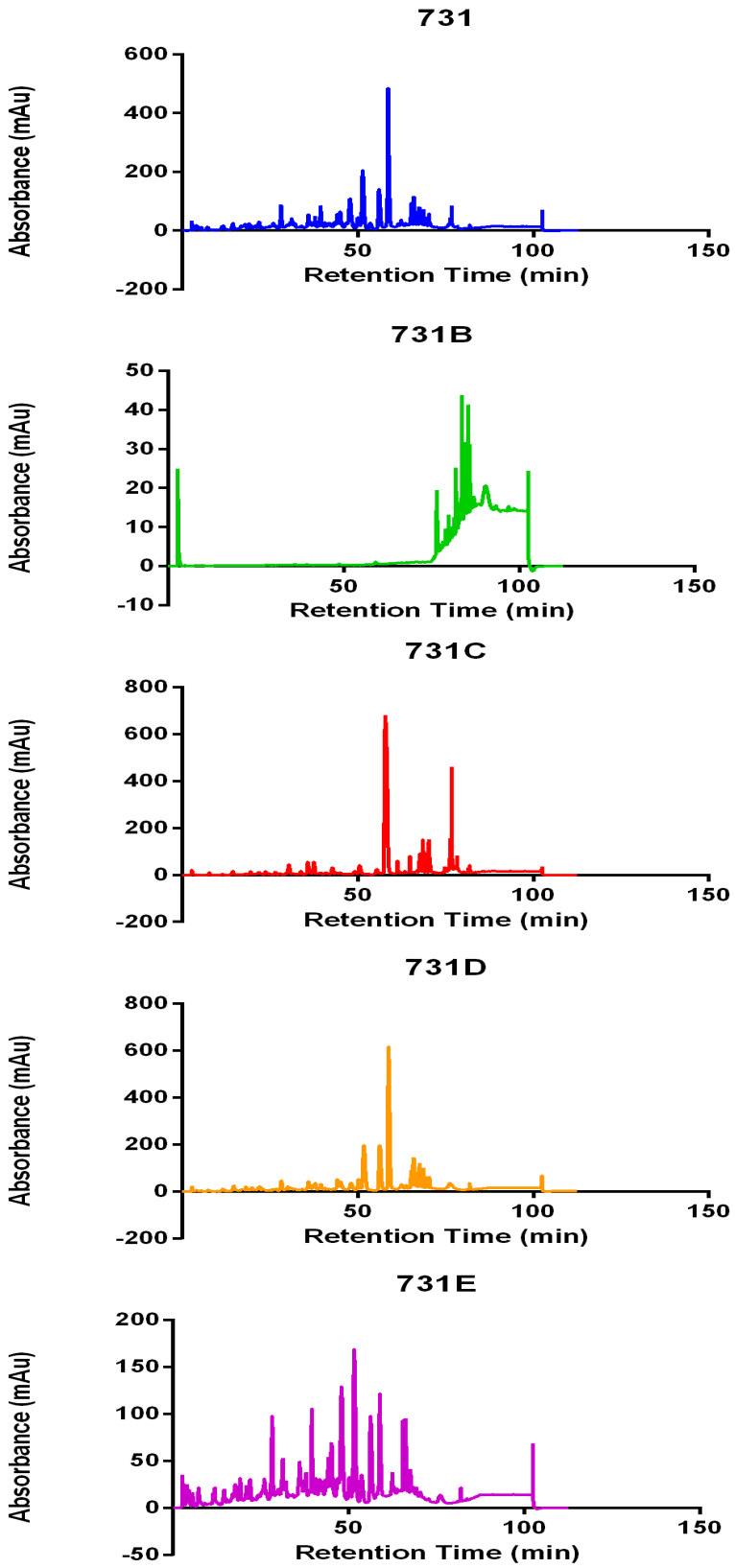
50
0
-50
50
100
150
Retention Time (min)

F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Absorbance (mAu)
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20
10
0
-10
50
100
150
Retention Time (min)
731C
Absorbance (mAu)
800
600
400
200
0
-200
50
100
150
Retention Time (min)
731D
Absorbance (mAu)
800
600
400
200
0
-200
50
100
150
Retention Time (min)
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Absorbance (mAu)
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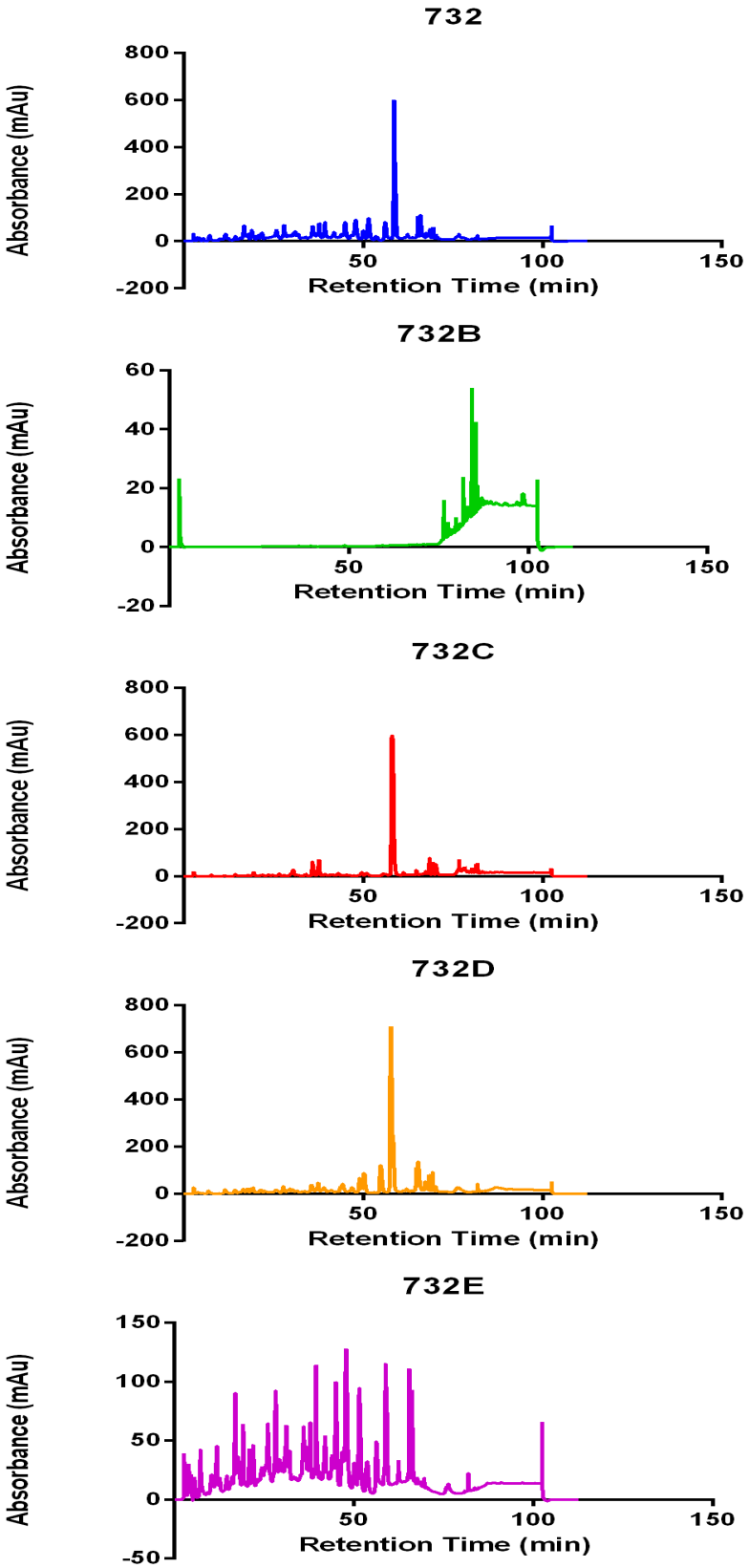
100
150
Retention Time (min)

G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0
-20
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100
150
Retention Time (min)
732C
Absorbance (mAu)
800
600
400
200
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-200
50
100
150
Retention Time (min)
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Absorbance (mAu)
800
600
400
200
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-200
50
100
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Retention Time (min)
732E
Absorbance (mAu)
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100
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0
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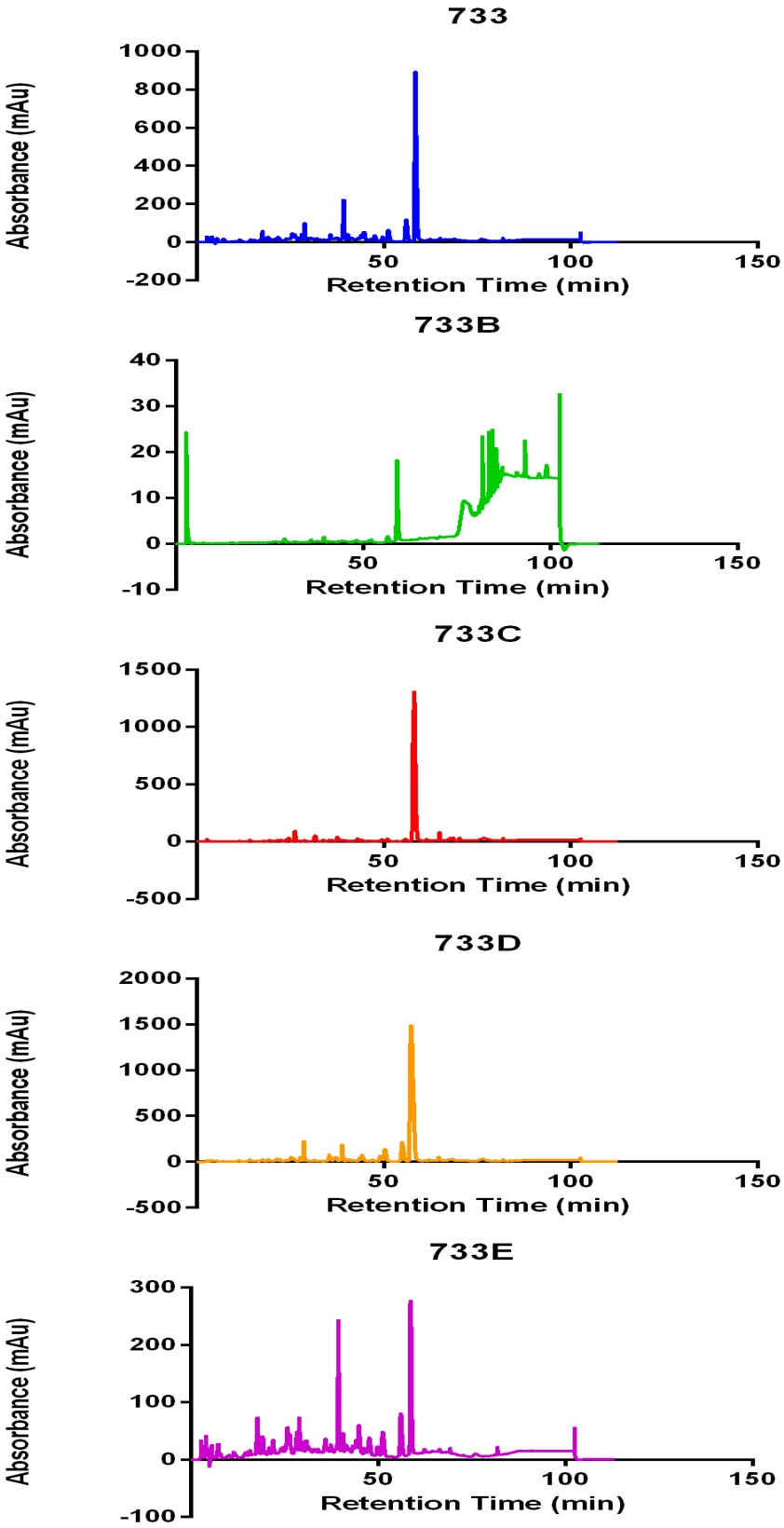
100
150
Retention Time (min)

H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30
20
10
0
-10
50
100
150
Retention Time (min)
733C
Absorbance (mAu)
1500
1000
500
0
-500
50
100
150
Retention Time (min)
733D
Absorbance (mAu)
2000
1500
1000
500
0
-500
50
100
150
Retention Time (min)
733E
Absorbance (mAu)
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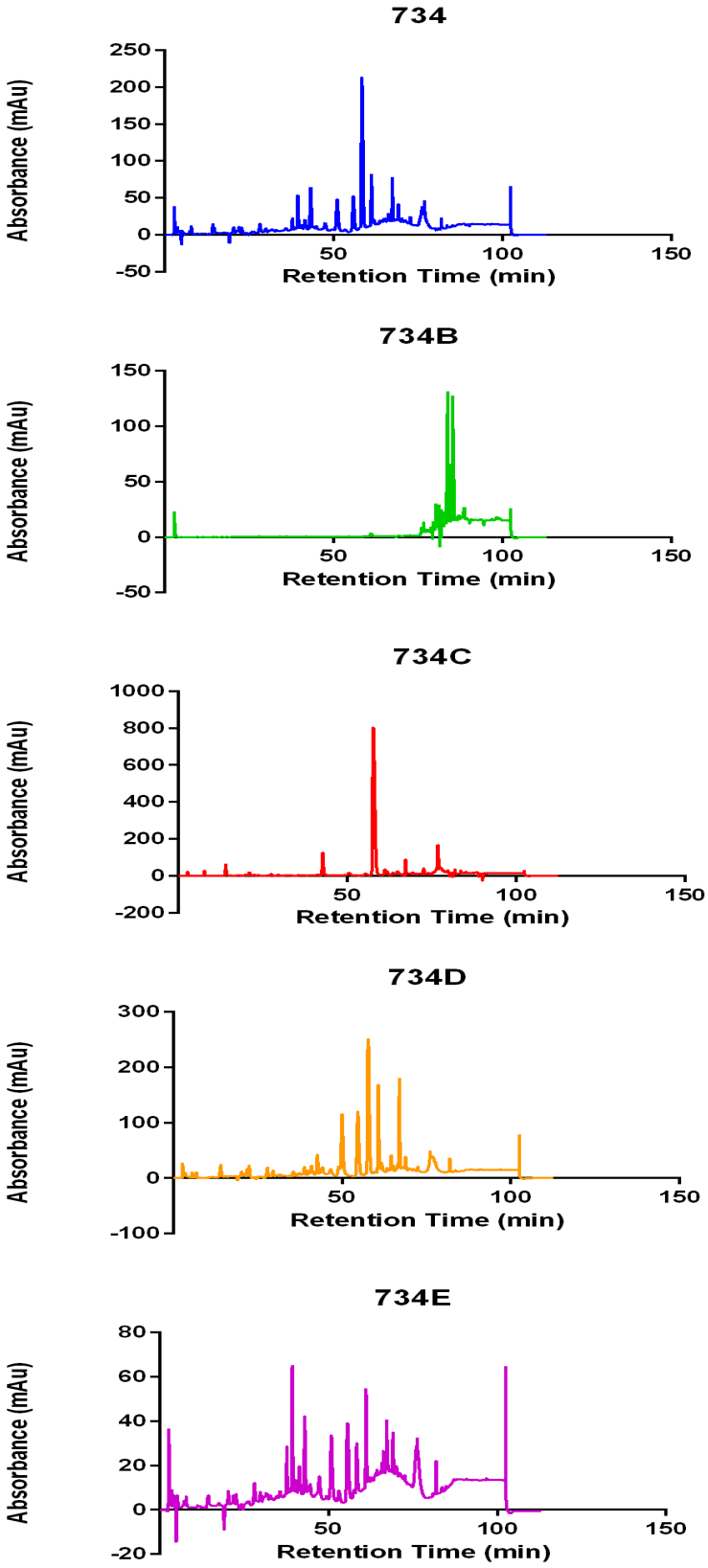
150
Retention Time (min)

I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100
150
Retention Time (min)
734C
Absorbance (mAu)
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800
600
400
200
0
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150
Retention Time (min)
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150
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Absorbance (mAu)
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100
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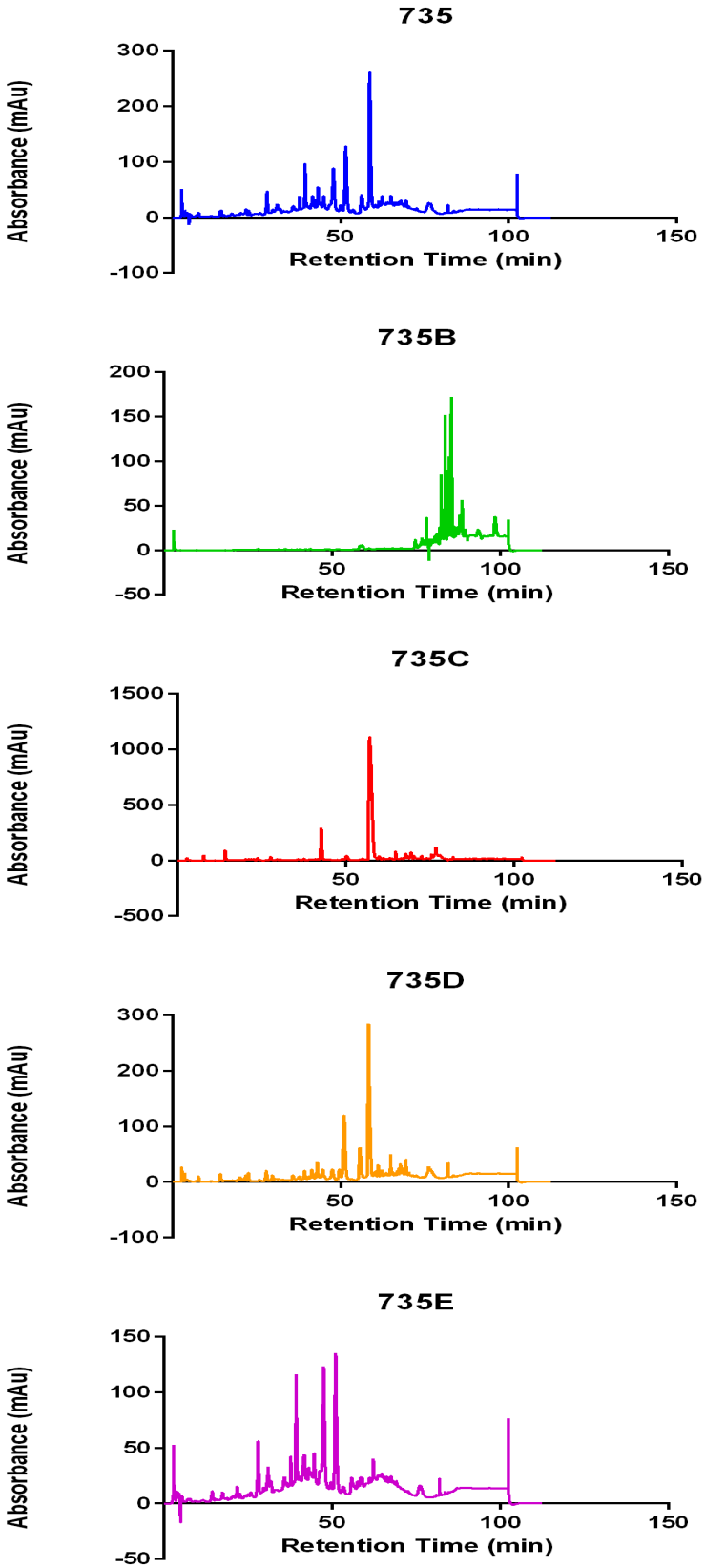
Retention Time (min)

J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50
0
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100
150
Retention Time (min)
735C
Absorbance (mAu)
1500
1000
500
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-500
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150
Retention Time (min)
735D
Absorbance (mAu)
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Retention Time (min)
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Absorbance (mAu)
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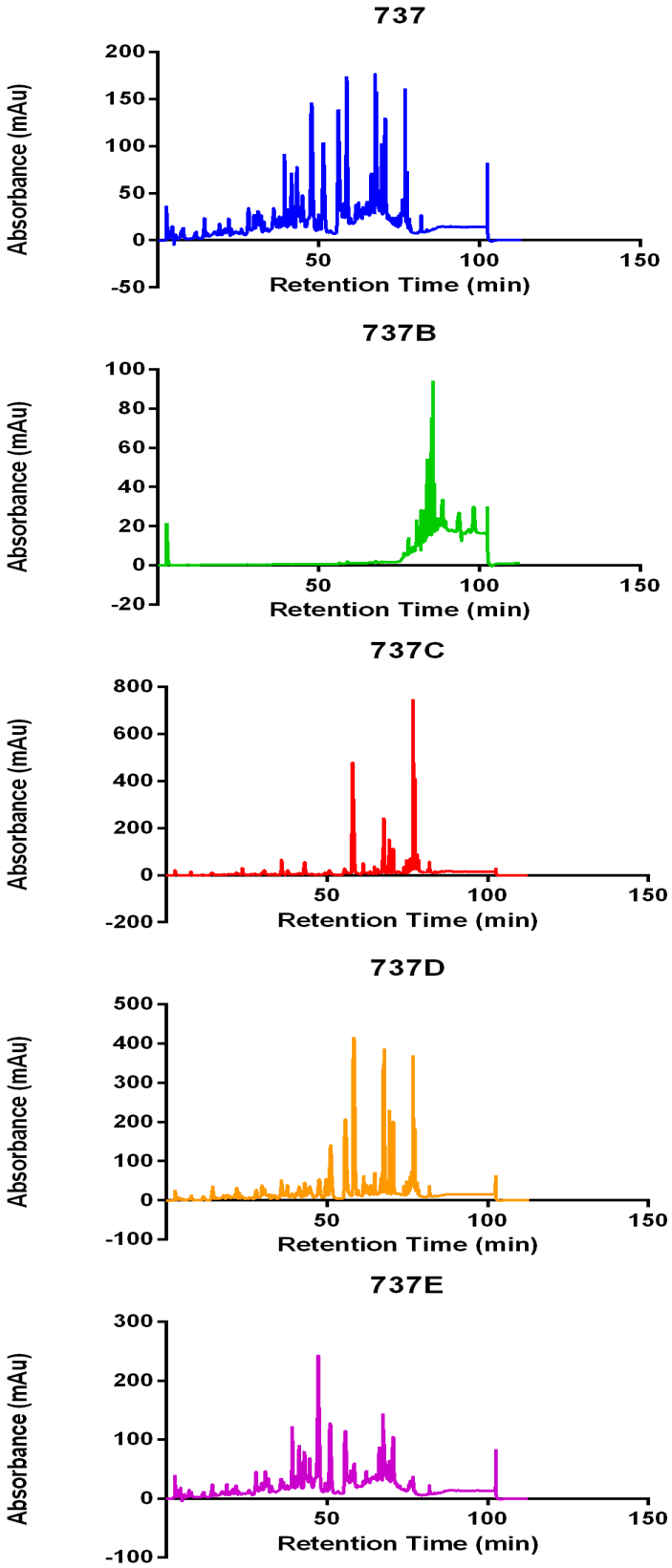
150
Retention Time (min)

K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Absorbance (mAu)
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80
60
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50
100
150
Retention Time (min)
737C
Absorbance (mAu)
800
600
400
200
0
-200
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100
150
Retention Time (min)
737D
Absorbance (mAu)
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400
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100
0
-100
50
100
150
Retention Time (min)
737E
Absorbance (mAu)
300
200
100
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-100
50
100
150
Retention Time (min)

Stacked chromatograms for each plant part tested.
(A). *Rubus ulmifolius* roots. (B) *Rubus ulmifolius* stems (C) *Rubus praecox* roots. (D) *Rubus* L. roots. (E) *Rubus ursinus* roots. (F) *Rubus laciniatus* roots. (G) *Rubus leucodermis* roots (H) *Rubus parvifolius* roots. (I) *Rubus ulmifolius* leaves. (J) *Rubus ulmifolius* woody stems. (K) *Rubus allegheniensis* stems.

CHAPTER 5: DISCUSSION

The inhibitory effects of various *Rubus* species against *Staphylococcus aureus*

This experiment began with 11 different plant parts derived from 8 different *Rubus* species producing 11 separate crude extracts via sonication extraction with 80% methanol. From there, 4 new extracts were produced from the crude extract through liquid-liquid partitioning creating a total of 5 separate extracts per plant part tested (Crude, Hexane, Ethyl Acetate, Butanol, Water). These 55 different extracts were then tested on *S. aureus* for growth inhibition. Of the 55 different extracts tested, only 16 extracts did not inhibit bacterial growth by at least 50% at a tested concentration. Although 16 different extracts did not produce significant results, at least two of the five extracts from each plant part tested inhibited growth by at least 50%. Overall, these results support the initial hypothesis that the antibacterial properties of *Rubus* are conserved across the genus. Each of the species tested inhibited *S. aureus* growth by at least 50% at concentrations ranging from 32 – 256 μg/mL.

Although each species significantly inhibited *S. aureus* growth, the potency and effectiveness of each extract did differ slightly. For example, of the 11 hexane partitions developed, 8 did not inhibit bacterial growth by at least 50%. Out of these 8 extracts, 7 of them were derived from *Rubus* roots and one was derived from *Rubus* leaves (Table 4.1.). This result is likely due the fact that hexane is an extremely non-polar solvent and is therefore able to dissolve non-polar compounds. Most non-polar compounds found within plants include waxes and fats, which are typically found on the leaves and stems of the plant and therefore would not be present within *Rubus* roots leaving very few

compounds in a hexane partition of root material. Interestingly, the three hexane partitions that did possess inhibitory activity were derived from *Rubus* stems. In fact, the hexane partition from *Rubus ulmifolius* stems had an MIC_{50} of 64 μg/mL, which is one of the lowest MIC_{50} present within the experiment. In contrast, the ethyl acetate and butanol partition of *Rubus ulmifolius* stems did not inhibit *S. aureus* growth to a practically significant level. This demonstrates that it is extremely important to use the appropriate solvent during extraction. Hexane has been shown to be an effective solvent for stems rather than roots, where it was not very effective at dissolving therapeutic compounds. Furthermore, the fact that almost all of the hexane partitions did not inhibit *S. aureus* growth significantly suggests that the active chemical constituents inhibiting growth are most likely not lipophilic.

There were only two extracts that were able to inhibit *S. aureus* growth by at least 90% at the concentrations tested. The two extracts were 728D (*Rubus praecox*. roots, butanol partition) and 729D (*Rubus* L. roots, butanol partition). Notably, both are butanol partitions. In addition, of the 11 butanol partitions tested, 10 inhibited *S. aureus* growth by at least 50%. This suggests that some of the most active chemical compounds may be amphiphilic due to the amphiphilic nature of butanol. Furthermore, these results also support previous research on this genus. For example, the most active partition found within *Rubus ulmifolius* roots was the butanol partition, leading Quave et al, to further fractionate and test this partition as 220D-F2 (Quave, Estévez-Carmona et al. 2012). Besides the butanol partitions, the water partitions were also highly active with only 1 out of the 11 not inhibiting growth by at least 50%. In addition, extract 731E (*Rubus laciniatus* roots, water partition), had the lowest MIC_{50} at a concentration of 32 μg/mL,

further supporting the proposed efficacy of the water partitions. Overall, the butanol and water partitions inhibited *S. aureus* by at least 50% at lower concentrations suggesting that the majority of the active chemical constituents within *Rubus* species are in fact amphiphilic or polar.

When comparing the plant parts, there were no major differences in MIC values between the roots, stems, and leaves; however, this study is limited by the fact that there were 7 different roots tested, 2 different stems, and only 1 extract derived from leaves.

In a previous study, an ethanolic crude extract derived from *Rubus ulmifolius* roots and *Rubus ulmifolius* woody stems both exhibited an MIC_{50} of 512 μg/mL (Quave, Plano et al. 2008). In the present study, the crude extract of *Rubus ulmifolius* roots did not inhibit *S. aureus* growth by at least 50% at concentrations ranging from 32 – 256 μg/mL; however, the extract was never tested at the high concentration of 512 μg/mL. At the highest concentration tested, 256 μg/mL, this crude extract inhibited growth by around 40%. Further testing is needed to verify whether or not the higher concentration could significantly inhibit *S. aureus* growth by at least 50%. On the other hand, the crude extract of *Rubus ulmifolius* woody stem had an MIC_{50} of 128 μg/mL. This contrasting result is most likely due the extraction methodology because the MIC protocol used in their experiment does not differ from the one used in the present investigation. For my extraction, I utilized 80% methanol and used a sonication extraction methodology. In Quave et al, they performed a maceration with 95% ethanol (Quave, Plano et al. 2008). The possible constant agitation of the sonicator bath may have led to a more effective extraction than a simple maceration.

Rubus ursinus, Rubus laciniatus, Rubus allegheniensis, Rubus praecox, and *Rubus leucodermis* have not, to our knowledge, been shown previously to possess antibacterial properties in any studies. Based on the experimental data, it is clear that these species do in fact possess antibacterial properties against *S. aureus* as each one was able to inhibit growth by at least 50%. In addition, *Rubus parvifolius* was found in a previous study to possess antibacterial properties against *S. aureus* (Cai 2012); however, the MIC value reported in that study is very different from our results. In Cai et al, the extract prepared had an MIC_{50} of 5 mg/mL (Cai 2012), a significantly higher concentration. Although this MIC value was significantly higher, their *Rubus parvifolius* extract was a volatile oil extracted from the leaves of the plant whereas our extract was derived from the roots of the plant. Based on these results, utilizing the roots of the plant rather than the leaves seems to produce a more potent effect against *S. aureus*. Overall, the results of the growth inhibition analysis reported herein support the original hypothesis that the antibacterial properties of *Rubus ulmifolius* roots would be conserved across the genus.

Anti-biofilm activity of *Rubus* in *S. aureus*

Only 6 of the 55 extracts did not inhibit biofilm growth by at least 50%. Even though those 6 extracts showed no significance, every plant species and plant part had at least 4 out of their 5 extracts inhibiting biofilm growth by at least 50%. This supports the initial hypothesis that the anti-biofilm activity of *Rubus ulmifolius* against *Staphylococcus aureus* is conserved across the genus. Furthermore, the 6 extracts that did not inhibit biofilm growth significantly were all hexane partitions (Table 4.3.). This finding supports

what was detailed earlier: that the main active constituents within these *Rubus* species are not nonpolar or lipophilic.

The majority of the extracts tested inhibited biofilm growth by at least 90%. Only 22 of the 55 extracts did not exhibit an $MBIC_{90}$. All 11 hexane partitions, 1 out of the 11 crude extracts, 3 out of the 11 ethyl acetate partitions, 0 out of the 11 butanol partitions, and 7 out of the 11 water partitions did not inhibit biofilm formation by at least 90%. Once again, it is clear that the bioactivity is not found within the hexane partition. Furthermore, based upon this experimental data, almost all of the crude extracts and every single butanol partition displayed significant anti-biofilm activity suggesting that these extracts contain very potent biofilm inhibitors.

Based on the initial biofilm assays, multiple crude, butanol, and water extracts needed to be further serially diluted in order to test whether or not these extracts inhibited biofilm growth by at least 50% at a lower concentration than 8 μg/mL (Figure 4.13., 4.14.). 8 crude extracts, 5 butanol partitions, and 2 water partitions were tested further. The only species to have its crude, butanol, and water partition further tested due to its high level of inhibition was *Rubus allegheniensis.* The ability of this species to inhibit biofilm growth through multiple different extracts suggests that it may possess the most diverse range of active constituents. Although these 15 extracts were further diluted, none of them were able to significantly inhibit biofilm formation by at least 50% at a concentration below 8 μg/mL.

Although almost all of the extracts tested inhibited biofilm formation, 2 of the 55 actually promoted biofilm production at a low concentration of 8 μg/mL relative to control. These two extracts were 725C (*Rubus ulmifolius* roots, ethyl acetate partition)

and 730D (*Rubus ursinus* roots, butanol partition). While these two extracts did promote growth, this was only displayed at the lowest concentration tested, and at all other concentrations, this was not the case. The mechanism by which this may occur is unknown.

It is important to take into consideration whether or not the biofilm inhibition is a direct result of the extract specifically working on the biofilm or if it is simply the extract inhibiting growth and killing the bacteria, therefore rendering it unable to produce a biofilm. Additionally, if a certain extract were able to inhibit biofilm formation without having an effect on planktonic cell growth, this would represent a highly promising candidate for anti-biofilm development with a less likelihood of a direct selective pressure typical of antibiotics. When a bacterium is exposed to a drug that they are susceptible to, they often develop various resistance mechanisms in order to combat the foreign entity. Although bacterial growth inhibition is important for eradicating infections, specifically targeting biofilm mechanisms lessens the opportunity for resistance mechanisms to take place, while also rendering the bacteria more susceptible to existing antibiotics.

Two extracts reported herein do in fact exhibit the selective property potently inhibiting biofilm production while having no detectable effect on bacterial growth: 730 and 735D. Both of these extracts inhibited biofilm growth by at least 50% and 90% at the low concentration of 8 μg/mL without inhibiting planktonic growth by at least 50% at the tested concentrations ranging from 32 – 256 μg/mL. These extracts are highly promising candidates for antibiotic adjuvant drug development and can potentially significantly improve the efficacy of current antibiotics.

All of the extracts tested that were able to inhibit biofilm growth, did so at a concentration at which they were unable to inhibit planktonic growth by at least 50%. The concentration range tested for growth inhibition was from 32 μg/mL to 256 μg/mL compared to that of biofilm inhibition, which was from 64 μg/mL to 8 μg/mL. Even extracts that inhibited planktonic growth at a concentration of 64 μg/mL or 32 μg/mL inhibited biofilm still at a lower concentration. Although it is significant that all extracts that inhibited biofilm growth did so at a concentration that was unable to inhibit planktonic growth by at least 50%, most of these extracts did still inhibit growth to some degree, potentially selecting for resistance. Certain extracts however were able to inhibit biofilm growth by at least 90% without inhibiting planktonic growth by at least 50% at any of the concentrations tested and therefore, did not significantly inhibit planktonic growth. Extracts 725, 725C, 726, 728, 730, 735C, and 735D produced this specific anti-biofilm effect detailed above. In addition, 725B, 733E, and 734B were able to inhibit biofilm formation by at least 50% without inhibiting planktonic growth significantly. Although there is no major trend displaying which species, plant part, or partition produced this effect, these extracts should be further explored in order to discover the constituents yielding the observed specific bioactivity.

Previous research has shown that a butanol partition that was further fractionated (220D-F2), derived from the roots of *Rubus ulmifolius*, displayed "dose-dependent inhibition of formation of pneumococcal biofilms" (Talekar, Chochua et al. 2014). This extract was also shown to significantly reduce preformed biofilm biomass as well. Although this study was performed on *Streptococcus pneumonia,* this same extract was able to significantly inhibit biofilm formation in *S.* aureus (Quave, Estévez-Carmona et

al. 2012). In addition, one study showed how a crude extract of *Rubus ulmifolius* roots that was extracted in 95% ethanol inhibited biofilm growth in methicillin-resistant *S. aureus* by at least 50% at a concentration of 8 μg/mL (Quave, Plano et al. 2008). While in the immediate study, the crude extract was developed through a sonication extraction using 80% methanol, the results of the biofilm assay are the same as those of the aforementioned study. The $MBIC_{50}$ of the crude extract of *Rubus ulmifolius* roots was also 8 μg/mL in this study; however, our assay was performed on *S. aureus* strain UAMS1, which is susceptible to methicillin. Besides *Rubus ulmifolius,* no other *Rubus* species has been explored, to our knowledge, with regards to its anti-biofilm activity against *S.aureus* or any other bacteria. This is the first study to explore these 7 other species and their bioactivity. Based upon the experimental results, various *Rubus* species must be further explored and considered when developing biofilm inhibitors and therapeutics and extracts 730 and 735D should be investigated most closely.

Ellagic acid and *Rubus*

In the present study, HPLC analysis was conducted on all 55 extracts in order to detect the presence of 3 specific compounds: Ellagic Acid (EA), Ellagic Acid-rhamnoside, and Ellagic Acid-xyloside. Presence of one of these three compounds was determined based upon retention time and UV spectrums as compared to the three standards listed above, which were first analyzed using the same HPLC method. Of the 55 extracts tested, 11 did not contain ellagic acid (725B, 725E, 728B, 729B, 730B, 731B, 732B, 734B, 735B, 735E, 737B). Once again, out of the 11 extracts that did not contain ellagic acid, 9 of them were hexane partitions. This result is consistent with both the growth and biofilm inhibition assays, suggesting that ellagic acid is one of the main

active constituents within these *Rubus* species. None of the 55 extracts tested contained either of the two EA derivatives tested. This was surprising due to the fact that EA derivatives have previously been displayed in various *Rubus* species (Quave, Estévez-Carmona et al. 2012, Oszmiański 2015). Due to the ubiquity of both rhamnose and xylose in plants, these two derivatives were chosen; however, our results suggest that other derivatives may be present within these extracts.

Although the scope of this analysis was focused only on these three compounds/peaks, 4 other signals were identified that were either common among most of the extracts or displayed a UV spectrum that was identical to that of EA, EA-rhamnoside, or EA-xyloside, suggesting that this peak may be an EA-derivative. EA had a retention time of 58.4 mins, EA-rhamnoside had a time of 51.4 mins, and EA-xyloside had a time of 46.8 mins. All three compounds displayed the exact same UV spectrum, indicating strong structure similarity. The four other common signals had retention times at around 56 mins, 47 mins, 51 mins, and 57 mins. The signal seen at 56 mins possessed the same UV spectrum as EA and 38 of the 55 extracts displayed this same peak and UV spectrum. The signal at 47 mins did not display the same UV spectrum as EA; however, 42 of the 55 extracts displayed this same peak with identical UV spectrums. Although there was a common peak at 51 mins, which would match the retention time of EA-rhamnoside, the UV spectrum of this common peak differed from that of the standard. 35 out of the 55 extracts presented this retention time of 51 mins and shared an identical UV spectrum, though different from that of EA-rhamnoside. Lastly, 5 out of the 55 extracts displayed a significant peak at 57 mins with a UV spectrum identical to that of the three standards tested, which all exhibited the same UV spectrum. Mass spectrometric analysis

needs to be conducted in order to elucidate the identities of these potentially novel therapeutic compounds.

EA and EA-derivatives have been shown to exhibit a wide variety of medicinal properties in many recent studies and have been discovered in various *Rubus* species; this is why they were the focus of the present study. For example, EA-xylopyranoside and EA-mannoppyranoside, two EA-derivatives, have been previously reported within *Rubus ulmifolius* (Quave, Estévez-Carmona et al. 2012). One previous study demonstrated that EA was the main phenolic compound in the berries of *Rubus*, constituting 77-88% of the total phenolics (Häkkinen, Heinonen et al. 1999). Although this study did not specify specific *Rubus* species, this result is consistent with our HPLC analysis, as every species tested contained EA. In addition, EA and EA-rhamnoside were both identified in blackberry extracts based upon mass spectral data and a comparison of retention times to standards and other published data in one previous study (Oszmiański 2015). Furthermore, previous studies have determined EA as a main constituent in fresh, dry and processed fruits of various *Rubus* species (Daniel, Krupnick et al. 1989, Rommel 1993, Häkkinen, Heinonen et al. 1999, Amakura, Okada et al. 2000). In addition, water, 50% methanol, and 100% methanol extracts of *Rubus caesius* have been shown to contain EA as the dominating constituent (Grochowski, Paduch et al. 2016). Similarly, shoot extracts developed from *R. idaeus* have been shown to be a major source of EA (Krauze-Baranowska, Głód et al. 2014). EA has also been reported in the leaves of various *Rubus* species including: *R. saxatilis, R. idaeus, R. occidentalis, R, fruticosus, R. caesius, R. nessensis,* and *R. odoratus* (Gudej and Tomczyk 2004).

Furthermore, EA alone has been shown to possess potent anti-biofilm properties at concentrations ($MBIC_{50}$ < 50mM) well below those that impact bacterial growth (MIC_{90} <2000 mM) (Quave, Estévez-Carmona et al. 2012). Further still, there have been reports regarding the anti-biofilm properties of EA against *Escherichia coli* (Huber, Eberl et al. 2003, Hancock V 2010), *Streptococcus dysgalactiae* (Dürig, Kouskoumvekaki et al. 2010), *Pseudomonas putida* (Huber, Eberl et al. 2003), *Burkholderia cepacia* (Huber, Eberl et al. 2003), and *S. aureus* (Quave, Estévez-Carmona et al. 2012). In addition, EA has been reported previously to exhibit activities such as radical scavenging, cancer preventive and suppressive effects, antiviral and antibacterial activities (Nohynek, Alakomi et al. 2006, Ross, McDougall et al. 2007, Goodwin, Atwood et al. 2009). Lastly, EA and its derivatives have been shown to be DNA damaging agents (Xu, Deng et al. 2003), which suggests that they could potentially be developed for cancer chemotherapy. In previous studies, EA and EA derivatives inhibited many important enzymes such as DNA gyrase (Ohemeng, Schwender et al. 1993) from *E. coli*, glucosyltransferase (Sawamura, Tonosaki et al. 1992) from mutant *Streptococci*, aldose reductase (Terashima, Shimizu et al. 1990) from rat lens, HIV-1 reverse transcriptase and polymerases α and β (Take, Inouye et al. 1989), as well as human DNA topoisomerases I and II (Constantinou, Stoner et al. 1995).

Although much of the previous research demonstrates the large presence of EA and EA-derivatives within *Rubus* species, no previous reports to our knowledge have shown that both *R. praecox* and *R. allegheniensis* contain EA as one of their main constituents as we present in the present study. Based upon both the previous research and this current study, *Rubus* species are potential reservoirs for both EA and EA-

derivatives and should therefore be further explored as potential new therapeutics for a wide variety of ailments. Overall, our data supports the initial hypothesis since all *Rubus* species studied here displayed a somewhat similar chemical profile to that of *Rubus ulmifolius.*

The potential of biofilm inhibitors

This study began with the goal of comparing the chemistry and bioactivity of 8 different *Rubus* species and their varying plant parts to that of *Rubus ulmifolius* due to the highly recognized bioactivity of this species. Although the results do in fact show that the extracts of the various *Rubus* species inhibit bacterial growth significantly, the ability of these extracts to inhibit biofilm formation is far more significant (Table 4.3.). As discussed earlier in this paper, the antibiotic pipeline is running dry and resistance in various different strains of bacteria has been steadily increasing. In order to combat this pressing issue, development of new sustainable and innovative therapeutics is needed to prevent a regression to a time where a small wound or simple operation had a high chance of killing the patient. Rather than simply focusing on therapeutics that target *in vitro* viability and are therefore similar to conventional antibiotics, targeting essential functions for infection, such as biofilm formation or other virulence factors may be a more sustainable and successful approach to drug discovery aimed at improving patient outcomes (Clatworthy, Pierson et al. 2007). Advantages to this approach include expanding the repertoire of bacterial targets, preserving the host endogenous microbiome, and exerting less selective pressure on bacteria for the development of resistance (Clatworthy, Pierson et al. 2007).

As stated earlier, around 80% of infections are thought to be biofilm-related and the recalcitrance of bacteria is often due to their ability to produce a biofilm (Harro, Peters et al. 2010). In fact, when cells exist in a biofilm rather than in a planktonic phase, they can become 10-1000 times more resistant to the effects of antimicrobial agents (Nickel, Ruseska et al. 1985, Evans and Holmes 1987, Gristina, Hobgood et al. 1987, Prosser, Taylor et al. 1987). Although the mechanisms for this acquired resistance are not fully known or understood, there have been multiple studies that provide a rational explanation for the obduracy of biofilm infections. For example, one study suggests that due to the production of the exopolysaccharide matrix, or glycocalyx, many antibiotics are prevented from accessing the bacterial cells embedded within the biofilm (Stewart 1996). In addition, previous studies have shown that when a bacterial cell culture transitions from exponential to slow or no growth, there is an increase in resistance to antibiotics (Tuomanen, Cozens et al. 1986, Tuomanen, Durack et al. 1986). Sensitivity to antibiotics has been shown to increase with increasing growth rates in planktonic cultures and biofilms of *P. aeruginosa*, *Escherichia coli* and *S. epidermidis* (Evans, Allison et al. 1991, Duguid, Evans et al. 1992, Duguid, Evans et al. 1992). This is due to the nature of antibiotics. Antibiotics specifically target rapidly diving cells and are therefore more effective on faster growing cultures. One characteristic of bacteria found within mature biofilms is in fact slow growth (Brown, ALLISON et al. 1988, Wentland, Stewart et al. 1996) and, therefore, this could be an additional mechanism of resistance. Furthermore, one proposed mechanism of resistance is that communities of bacteria hidden within a biofilm develop an altered biofilm-specific phenotype resulting in the expression of various new mechanism to combat the severe effects of antibiotics (Gilbert, Das et al.

1997, Cochran, McFeters et al. 2000, Maira-Litran, Allison et al. 2000, Maira-Litrán, Allison et al. 2000). Although the current research is not fully conclusive regarding how or why bacteria growing in a biofilm develop this increased resistance, the proposed mechanism outlined above may be potential answers. Additional studies need to be performed to broaden our understanding of how these biofilms are so protective against antimicrobial agents. However, it is clear that biofilms significantly prolong and improve the fitness of bacteria. This understanding provides a potential target for new therapeutics to help combat antibiotic resistance.

Based upon the experimental results, every *Rubus* species tested should be further investigated as biofilm inhibitors; however extracts 730 and 735D should be studied most intensely because they are the most promising. Previous studies have shown that the use of multiple drugs with different targets provides more effective therapies by targeting a variety of disease processes, thereby also decreasing risk of drug resistance (Wagner 2011). Utilizing these biofilm inhibitors as adjuncts to antibiotics could be a starting solution to this worldwide problem by decreasing both mortality and resistance.

Limitations and Future Directions:

Although this study provided promising results, there are some limitations present within this experiment. First, out of the 11 *Rubus* samples extracted and tested, 7 of them were roots, 3 of them were stems, and only 1 sample consisted of leaves. Due to the unbalanced and minimal variation in plant parts, it is difficult to conclude that one plant part was the most bioactive over the other two. Although this is a clear limitation, there were very consistent levels of bioactivity across the genus and all plant parts tested. Future research should aim to fill gaps with respect to plant part extracts. In addition, in

the present study, the extracts were only tested for the ability to inhibit biofilm formation/growth; however, none of the extracts were tested against a preformed biofilm, which would have been more robust. Future research should explore the ability of *Rubus* species to not only inhibit the formation of a biofilm, but also eradicate a preformed biofilm.

One additional limitation of this study is that HPLC analysis and identification of constituents were performed based solely on the retention time and UV spectrum without mass spectroscopy. Furthermore, although 4 other major peaks that were common between the samples were identified, only 3 standards were run. This is a small sample of standards; as a result, the lack of matches identified is not surprising. Future research should perform mass spectrometry on the most active extracts in order to confirm the identity of the most highly active constituents. Although many of the extracts tested displayed promising bioactivity, due to the amount of time available, I was unable to perform human keratinocyte cytotoxicity testing. Future research should perform this toxicological test to ensure that none of the extracts are harmful to human skin cells.

Additionally, this study only explored the antibacterial effects of various *Rubus* extracts on one strain of *S. aureus.* Previous studies have shown that the activity of *Rubus ulmifolius* was conserved across 15 genotypically-diverse clinical isolates of *S. aureus* (Quave, Estévez-Carmona et al. 2012); however, in the present study, only the strain UAMS1 was tested against. Future research should test all of the active extracts on various strains of *S. aureus*, including resistant and highly virulent strains. Lastly, efficacy and safety in animal models should be assessed to discover if the activity is

conserved in animal models and if there are any potential negative side effects or toxicity issues.

Conclusion

In this project, we found that the eight distinct *Rubus* species and their various plant parts tested did in fact share similar bioactivity and chemical makeup to that of *Rubus ulmifolius*. Although the full chemical makeup of each species was not elucidated, ellagic acid was identified in every *Rubus* species as one of the main constituents. This result was consistent with the previous paper this research expands upon because ellagic acid was also the main active constituent identified. In addition, this study discovered that the anti-biofilm activity of *Rubus ulmifolius* against *S. aureus* was conserved across all *Rubus* species tested. This is significant because there is an urgent need for the development of new antimicrobials that are sustainable and effective. The innate bioactivity of the *Rubus* genus needs to be further explored, particularly anti-biofilm activity, and considered when developing new therapeutics to combat a rapidly evolving and concerning issue today: antimicrobial resistance. To this end, we present extracts 730 and 735D as promising candidates for the development of novel natural product inhibitors of biofilm formation. Given that they exhibit such selective and potent biofilm inhibitory activity yet do not demonstrate any significant growth inhibitory effects, we recommend further studies and bioassay-guided fractionation be done to develop these compositions into antibiotic adjuvant therapeutics against *S. aureus*.

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