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Amelia Muhs April 6, 2015

Old cures for new wounds: Virulence inhibitors from Brazilian pepper

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An abstract of
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Abstract

Old cures for new wounds: Virulence inhibitors from Brazilian pepper

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Traditional medical remedies are not always well understood despite their persistent use over centuries to safely and effectively treat a variety of ailments. One such example of this is the anti-infective application of *Schinus terebinthifolius,* more commonly known as peppertree, for the treatment of wounds. With the decreased efficacy of many antibiotics in the face of growing drug resistance, it is necessary to identify and develop alternative or complementary therapies. As a result of increased knowledge of chemical synergy and bioactivity of natural products, plants are increasingly being investigated as leads for new drugs. *Schinus terebinthifolius* provides a valuable opportunity for the isolation of bioactive compounds as its use in Brazilian traditional medicine to treat a variety of ailments, including wounds and skin infections, has been well documented. The anti-infective activity of *S. terebinthifolius* suggests that it may be a source of bioactive compounds that could be used to develop alternative or adjuvant therapies in the treatment of invasive Methicillin Resistant *Staphylococcus aureus* (MRSA) infections, listed among the top "serious threats" to human health in the USA. Specifically, plant compounds may be able to inhibit the accessory gene regulator (*agr)* pathway of *S. aureus,* which is critical in quorum sensing and toxin production.

Bioactivity-guided fractionation techniques were employed to prepare a refined extract of *S. terebinthifolius* fruits (430D-F5) with potent quorum quenching activity in the absence of growth inhibition at concentrations as low as 2 μg/mL. Inhibitory effects of 430D-F5 were confirmed at the transcriptional level with the use of *agr* fluorescent reporter strains of *S. aureus* as well as at the translational level through quantification of exotoxin production using HPLC. 430D-F5 was characterized to reveal 47 compounds, currently under further individual evaluation for quorum quenching activity and structure elucidation studies by NMR. Low toxicity to human skin cells as well as considerable quorum quenching activity at low doses both corroborate the traditional medical uses of *Schinus terebinthifolius* to treat skin and soft tissue infections and provide support for further investigation of the plant as an alternative treatment or adjuvant therapy for invasive methicillin resistant *S. aureus* infections.

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CHAPTER 1: INTRODUCTION

Natural products as alternative treatments: an ethnobotanical approach to drug discovery

Ethnobotany is a field of study that combines aspects of anthropology and botany to study how humans interact with plants in their environment (Quave & Pieroni, 2015; Surridge, 2015). Traditional ecological knowledge, which forms the basis of ethnobotanical research, can be defined as "a cumulative body of knowledge, practice and belief evolving by adaptive processes and handed down through generations by cultural transmission, about the relationship of living beings (including humans) with one another and with their environment" (Berkes, 2012). Over the past century, traditional ecological knowledge and ethnobotany has played a crucial role in the development of new drugs and therapies used in allopathic medicine, sometimes referred to as "Western" or "modern" medicine (Gurib-Fakim, 2006). In fact, over 25% of drugs prescribed by physicians have been derived from flowering plants, and nearly 40% originate from other natural sources such as fungi and animals (Houghton, 2001).

A focus on the identification of bioactive compounds from natural products can be a beneficial route to drug discovery. Plants often have very complex and specific secondary metabolites that contribute to protection from predators and microorganisms as well as allow the plant to chemically interact with its environment (Gurib-Fakim, 2006). The complexity of bioactive compounds in plants allows for the isolation and identification of a broad range of chemically diverse compounds that could not be achieved through synthetic chemistry alone (Houghton, 2001). These chemicals are most often the focus of drug discovery. In many cases, traditional medicinal uses of plants are used to guide the process of drug discovery and identify possible botanical sources of bioactive compounds (Gurib-Fakim, 2006).

With the increasing prevalence of multidrug resistant infections, it is necessary to develop alternative solutions to standard treatments, as many have become ineffective. The investigation of natural products is a cost and time-effective solution to this problem. Over the past few years, many pharmaceutical companies have shifted their focus away from the development of new antibiotics to the development of drugs to treat chronic disease. In addition, the development of new antibiotics is expensive and takes a great deal of time, resulting in the development and release of only two new antibiotics in the United States in the last five years (Edwards-Jones, 2013).

Schinus terebinthifolius, more commonly known as Brazilian peppertree, provides a valuable opportunity for the isolation of bioactive compounds as its use to treat a variety of ailments and infections has been well documented. In addition, the plant is widely available and can grow in diverse habitats. Previous research has shown that the plant is responsible for a range of biological activity including, but not limited to, antibacterial, antifungal, anti-inflammatory, anti-infective, and wound healing activity. The anti-infective activity of *S. terebinthifolius* suggests that it may be a source of bioactive compounds that could be used to develop alternative or adjuvant therapies in the treatment of MRSA infections. Specifically, plant compounds may be able to inhibit the accessory gene regulator (*agr)* pathway of *S. aureus,* which is critical in quorum sensing and toxin production. With the increasing threat of antimicrobial resistance, it is important to investigate the activity of natural products, such as those from *Schinus terebinthifolius,* for the development of new, more effective therapies.

Project aims and research questions

The aim of this study is to investigate the anti-infective properties of *Schinus terebinthifolius,* more commonly known as Brazilian peppertree, a traditional medicine used in the treatment of skin and soft tissue infections and wounds. This involves an investigation of traditional uses and preparations as well as bioactivity and any possible cytotoxicity. Further, the study aims to identify chemical compounds present in *S. terebinthifolius* that are able to inhibit virulence and quorum sensing in Methicillin Resistant *Staphylococcus aureus* (MRSA), a pathogen considered by the CDC to be a "serious threat" (Centers for Disease Control and Prevention, 2013a). These quorum-quenching compounds isolated from *S. terebinthifolius* could ultimately be used as alternative or adjuvant therapies to improve patient response to existing drugs that have become increasingly ineffective with the widespread emergence of antimicrobial resistance.

A thorough review of literature related to *Schinus terebinthifolius* and its traditional uses was performed in an attempt to guide laboratory research. Special attention was paid to primary sources and books published by 19th century European naturalists following their travels to Brazil and other regions of South America where the plant was widely used to treat a variety of ailments. References to plant parts used, medicinal preparation, and application were used to guide laboratory tests and plans for future research. In addition to traditional uses, this literature review addresses current knowledge of *S. terebinthifolius* bioactivity and chemical composition.

CHAPTER 2: LITERATURE REVIEW

The status of traditional medicine

A need for research

For an estimated one third of the global population, access to allopathic medicine is limited. Traditional medicine can provide a more accessible and affordable alternative that is often more consistent with the cultural beliefs of a population (World Health Organization, 2001). Many countries have chosen to incorporate aspects of traditional medicine into their healthcare systems and have started to establish policies that encourage the safety and quality of such practices (World Health Organization, 2013). According to the World Health Organization, challenges in the implementation of these policies include evaluating efficacy, safety, and quality of traditional medicine, monitoring advertising and claims, and investing in research and development (World Health Organization, 2013).

Today, traditional medical practices and remedies are becoming increasingly globalized. Regions are exporting traditional medical knowledge and resources and incorporating those from other regions as "complimentary medicine". The WHO defines complimentary medicine as health care practices that are adopted from an outside source but are not fully integrated into the country's healthcare system (World Health Organization, 2013). With this increase in the accessibility of traditional medical practices, it is necessary to encourage the establishment of standards and guidelines supported by scientific research (World Health Organization, 2013). Over the past fifteen years, the number of countries with policies relating to traditional medicine and regulations of herbal medicines has steadily increased (World Health Organization, 2013). In

addition, the number of countries with national research institutes dedicated to the study of traditional medicine and herbal medicine has increased from 19 in 1999 to 73 in 2012 (World Health Organization, 2013). Although there has been an increased focus on research, lack of research data continues to rank as the most common difficulty related to the regulation of traditional medical practices reported by 105 out of 194 WHO member states (World Health Organization, 2013). Moreover, increased guidance for research of quality and safety of herbal remedies is reported as being of great need by the largest number of member states (World Health Organization, 2013).

Traditional medicine in Brazil

Traditional botanical medicine is especially popular in interior regions of Brazil and has gained popularity among all socio-economic groups (de Lima et al., 2006). Current traditional medical knowledge and practices in the country come from a combination of the knowledge of slaves, indigenous peoples, and European settlers (Moura-Costa et al., 2012). Although the knowledge base of traditional medicine is large and phytomedicine represents an important sector in the Brazilian economy, there has been relatively little formal scientific study of the plants being used (de Lima et al., 2006). In addition, destruction of native ecosystems has led to a loss of knowledge about the use of some native medicinal plants (Brandao et al., 2008).

As of 2001, there were over 12,000 physicians and 1,300 pharmacists in Brazil who were classified as practicing homeopathic medicine, designated as a medical specialty by the Brazilian government in 1988 (World Health Organization, 2001). In addition, regulation of traditional medicine has been introduced in the form of “La Política de Atención Integral a la Salud Indígena de FUNASA” which encourages respect for and preservation of traditional health

systems (World Health Organization, 2001). Although policies have been established to preserve traditional medical knowledge, there is still a need to document the country's enormous extant flora and conduct ethnobotanical research. It is estimated that of the 55,000 plant species in Brazil, only 0.4% have been formally documented (Gurib-Fakim, 2006). Because Brazil is home to approximately 15-20% of the world's biodiversity, it is important to document and gather information about the wide variety of flora before native ecosystems are destroyed (Moura-Costa et al., 2012).

An investigation of *Schinus terebinthifolius*

Introduction

Schinus terebinthifolius, a tree native to South America, has been heavily utilized in traditional medicine and is currently being investigated as a possible lead for drug development. It is known for its wide-ranging medicinal uses including use as an anti-infective and anti-inflammatory treatment. *S. terebinthifolius* is widespread in many subtropical regions and is classified as an invasive species in the southeastern United States. Its prevalence as well as its wide-ranging biochemical activity makes *Schinus terebinthifolius* an important target for research as well as a lead for possible drug development.

Botanical Description

Schinus Terebinthifolius Raddi, more commonly known as Pepper Tree, is a large shrub or small dioecious tree native to Brazil, Paraguay, and Argentina (Panetta & McKee, 1997). Although native to South America, the plant has been introduced to parts of Central America, the

Bahamas, West Indies, Hawaii, Guam, Mediterranean Europe, South Asia, Northern and Southern Africa, and parts of the United States including Florida, California, and Arizona (Morton, 1978). *S. terebinthifolius* is referred to by many names including Christmas-berry in Hawaii and Guam, false pepper in regions of Europe, chichita in Argentina, copal in Cuba, pimienta de Brasil in Puerto Rico, and Florida holly in parts of the United States. In its native country of Brazil it is called corneiba, or more popularly by variations of the name aroeira, including aroeira negra, aroeira da minas, and aroeira de praia (Morton, 1978).

Schinus terebinthifolius belongs to the family Anacardiaceae, which is composed of over 76 genera and 600 species including poison ivy and poison oak (Carvalho, Melo, Aragao, Raffin, & Moura, 2013). Plants belonging to the Anacardiaceae family have been a focus of research related to the identification of bioactive substances. Commonly studied genera include *Mangifera,* which includes the mango, *Rhus,* which includes sumac, *Anacardium,* containing cashews, *Spondias, Lannea, Pistacia, and Schinus* (Carvalho et al., 2013). The *Schinus* genus includes 29 species, most of which, including *Schinus terebinthifolius,* are native to the Brazilian coast (Carvalho et al., 2013).

S. terebinthifolius has red fruits that appear during the winter months, leading to its export to other countries to be used as an ornamental plant known as “Christmas berry” (Williams, Muchugu, Overholt, & Cuda, 2007). It is a fast-growing shrub or broad tree that can grow to 12 meters in height with a trunk about one meter in diameter (Morton, 1978). The plant has evergreen, pinnate leaves and sprays of white flowers that are followed by one-seeded green fruits that ripen and become red (Figure 2.1) (Morton, 1978).

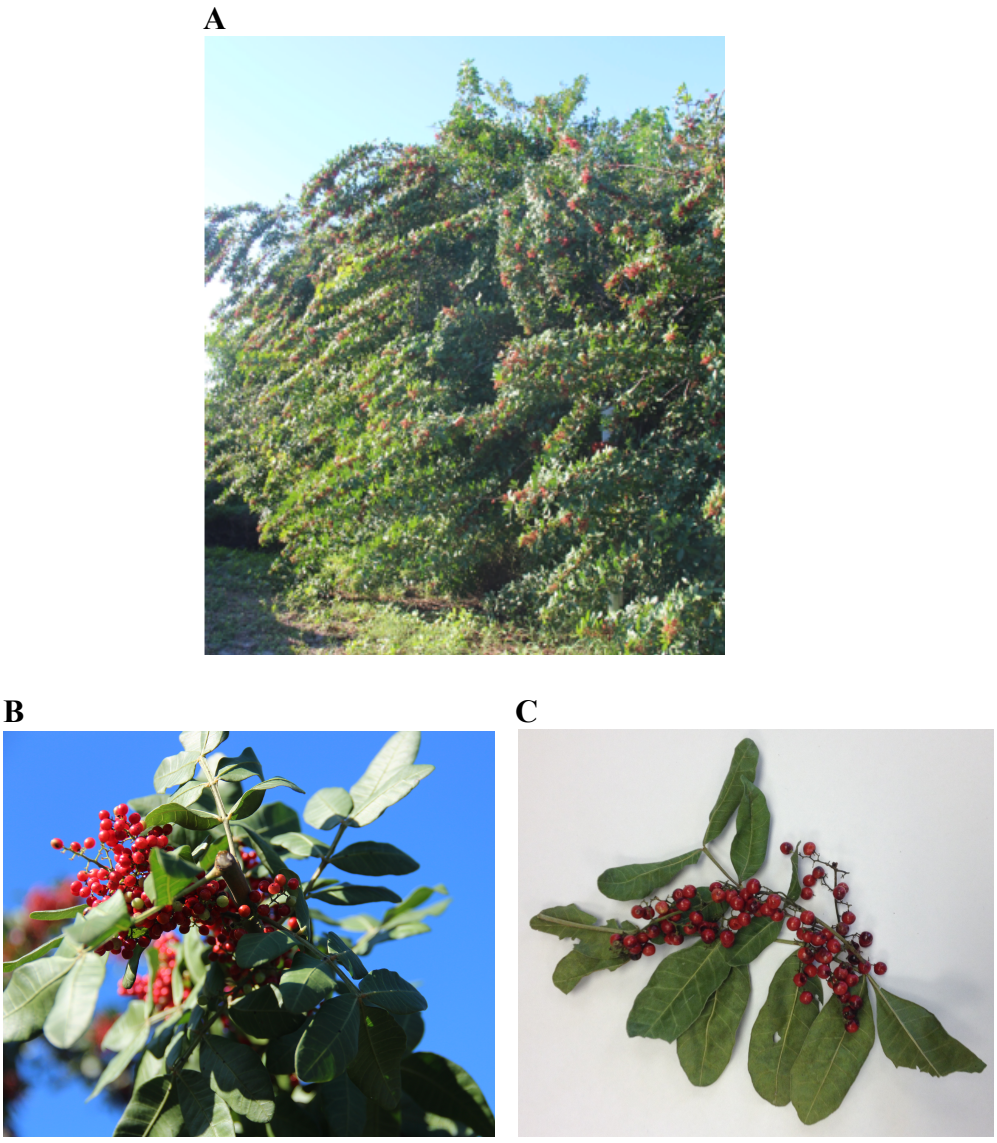


Figure 2.1. *Schinus terebinthifolius* collected in Florida. (A) *S. terebinthifolius* is a fast-growing shrub or broad tree that can grow to 12 meters in height. (B) The plant has evergreen, pinnate leaves and clusters of small red fruits that each contain one seed. (C) Dried specimen.

Although native to South America, *S. terebinthifolius* can be found in over 20 subtropical countries and is considered to be an invasive species in the southeastern United States, particularly in Florida where it has colonized most of the state (Geiger, Pratt, Wheeler, & Williams, 2011). It was first introduced to southern Florida in 1898 where it thrived and eventually became classified as an invasive species (Morton, 1978). The plant has proven to be a

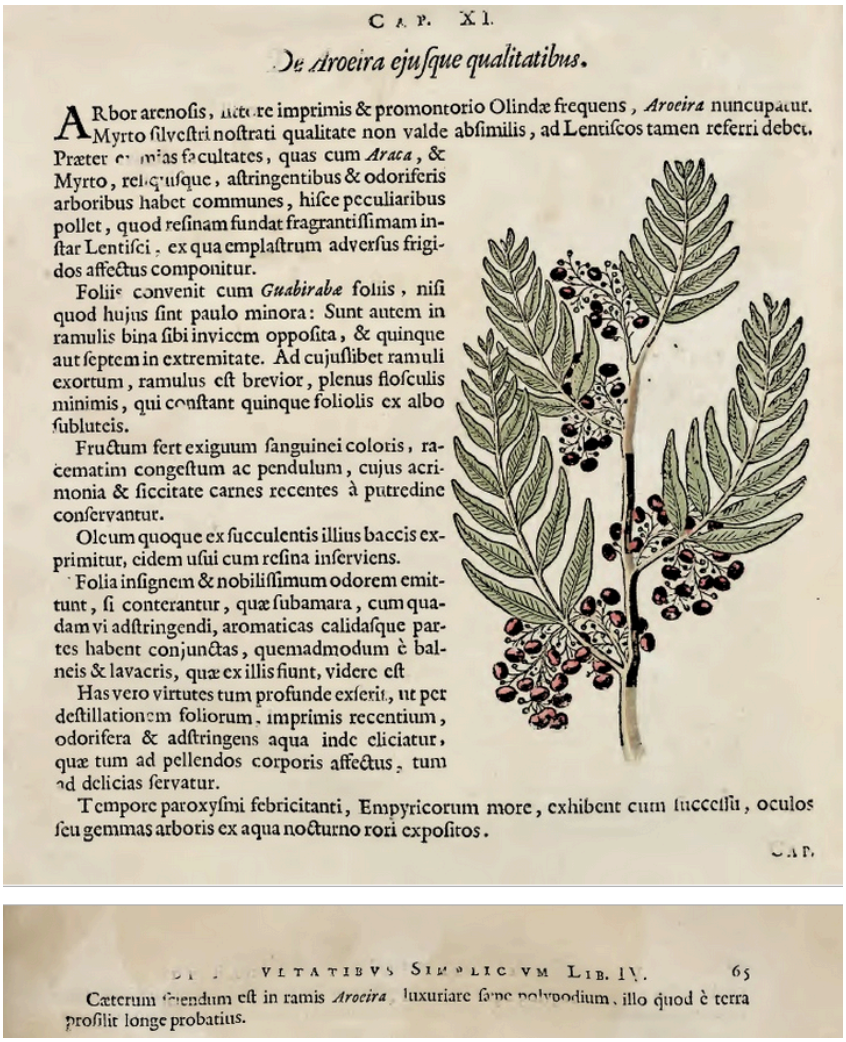
successful colonizer as it can tolerate both shade and periods of drought (Panetta & McKee, 1997). Its successful colonization of a variety of habitats may be due in part to its allelopathic effects, or ability to inhibit the growth of other species in the vicinity (Morton, 1978). Its main pollinators include flies and wasps, while birds, such as mockingbirds and catbirds are thought to be responsible for long-distance dispersal of seeds (Williams et al., 2007).

Traditional Uses

Schinus terebinthifolius, which is included in Brazilian Pharmacopoeia, has served as a staple in Brazilian traditional medicine (Fedel-Miyasato et al., 2014). Almost all parts of the plant, including leaves, bark, fruit, seeds, and resin have been used to treat a variety of ailments (Gundidza, Gweru, Magwa, Mmbengwa, & Samie, 2009). *S. terebinthifolius* has been used traditionally for its anti-septic and anti-inflammatory qualities in the treatment of wounds and ulcers, bruises, hemoptysis, diarrhea, chills, tumors, and arthritis, as well as urinary and respiratory infections (Fedel-Miyasato et al., 2014; Morton, 1978).

Historical References

References to *S. terebinthifolius* in Brazil date back to 1648 when the plant was described by Willem Piso, a Dutch naturalist who lived in northwestern Brazil for 7 years before publishing his book, *Historia Naturalis Brasiliae* (Figure 2.2) (Brandao et al., 2008). In this work, the plant was referred to by its common name, Aroeira (Piso, 1648). Following Piso, the plant was described by multiple 19th century European naturalists including Auguste Saint-Hilaire, Hermann Burmeister, and Richard Burton (Brandao et al., 2008).



CAP. XI.

De Aroeira ejuſque qualitatibus.

ARbor arenoſis, littore imprimis & promontorio Olindæ frequens, *Aroeira* nuncupatur. Myrto ſilveſtri noſtrati qualitate non valde abſimilis, ad Lentiſcos tamen referri debet. Præter communias facultates, quas cum *Araca*, & Myrto, reliquiſque, aſtringentibus & odoriferis arboribus habet communes, hiſce peculiaribus pollet, quod reſinam fundat fragrantiſſimam inſtar Lentiſci, ex qua emplaſtrum adverſus frigidos affectus componitur.

Foliis convenit cum *Guabirabæ* foliis, niſi quod hujus ſint paulo minora: Sunt autem in ramulis bina ſibi invicem oppoſita, & quinque aut ſeptem in extremitate. Ad cujuſlibet ramuli exortum, ramulus eſt brevior, plenus floſculis minimis, qui conſtant quinque foliolis ex albo ſubluteis.

Fructum fert exiguum ſanguinei coloris, racematim congeſtum ac pendulum, cujus acrimonia & ſiccitate carnes recentes à putredine conſervantur.

Oleum quoque ex ſucculentis illius baccis exprimitur, eidem uſui cum reſina inſerviens.

Folia inſignem & nobiliſſimum odorem emittunt, ſi conterantur, quæ ſubamara, cum quadam vi adſtringendi, aromaticas calidaſque partes habent conjunctas, quemadmodum è balneis & lavacris, quæ ex illis fiunt, videre eſt

Has vero virtutes tum profunde exſerit, ut per deſtillationem foliorum, imprimis recentium, odorifera & adſtringens aqua inde eliciatur, quæ tum ad pellendos corporis affectus, tum ad delicias ſervatur.

Tempore paroxyſmi febricitanti, Empyricorum more, exhibent cum ſucceſſu, oculos ſeu gemmas arboris ex aqua nocturno rori expoſitos.

CAP.

DE VIRTUTIBUS SIMPLICIUM LIB. IV. 65

Cæterum ſciendum eſt in ramis *Aroeira* luxuriare ſæpe polypodium, illo quod è terra proſilit longe probatius.

Figure 2.2. A reference to Aroeira in *Historia naturalis Brasiliae*

Source: Piso, W. (1648). *Historia naturalis Brasiliae : auspicio et beneficio illustriss*. Lugdun. Batavorum: Apud Franciscum Hackium, et Amstelodami, apud Lud. Elzevirium.

Information regarding the traditional uses of *S. terebinthifolius* could be found in multiple sources written by 19th century European naturalists following their time spent in Brazil. These naturalists include Richard Francis Burton, Pedro Chernoviz, Carl von Martius, and Nicoláo Moreira. Many refer to the astringent properties of the plant, which have been used to treat a variety of maladies as well as preserve fishing nets. Common uses of the plant include treatment of rheumatic pain and arthritis, tumors, wounds and ulcers, diarrhea, fever, inflammation,

bleeding, infection, dental care, and use as a diuretic (Figure 2.3). The most common uses reported in the four texts listed are treatment of wounds and ulcers as well as infections.

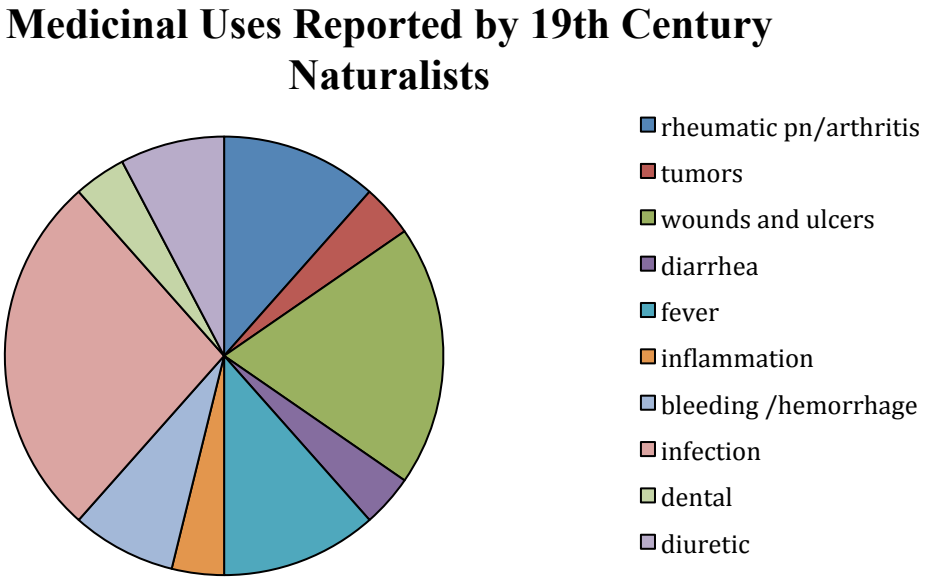


Figure 2.3 Medicinal uses of *Schinus terebinthifolius* reported by 19th century European naturalists after traveling to Brazil. Sources include: Martius (1854), Moreira (1862), Chernoviz (1864), and Burton (1868).

In his book, *Viagens aos planaltos do Brasil,* Richard Francis Burton discusses various uses of *S. terebinthifolius* for both medicinal and construction purposes (Burton, 1868). Medicinal uses described by Burton include the heating of leaves to treat rheumatic pain and the use of juice from fresh branches to treat eye disease (Figure 2.4). Furthermore, he states that resin was used as a preservative for ropes and fishing nets. Wood was also of particular importance as it was used as a strong and readily available building material.

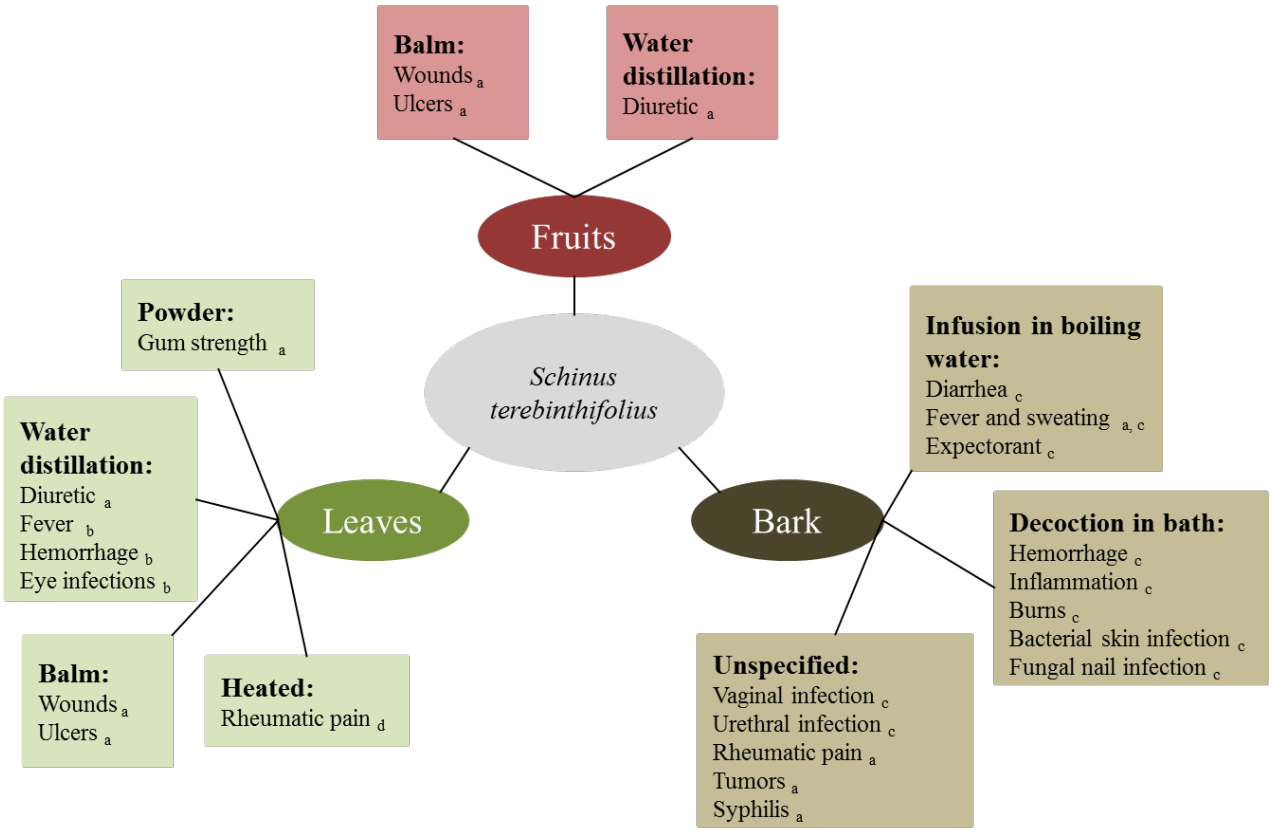
Formulario ou guia medico, by Pedro Luiz Napoleão Chernoviz focuses mainly on medicinal uses of *S. terebinthifolius* bark (Chernoviz, 1864). Preparation of bark for internal use involved making an infusion of bark in boiling water, while preparation for external use involved making a bath from bark decoction in water. The work emphasized the astringent properties of

the bark that were involved in the treatment of intestinal irregularity, diarrhea, sweating, fever, congestion, bleeding and hemorrhage, external inflammation, and burns (Figure 2.4). Bark was also used in the treatment of a variety of skin infections including vaginal and urethral infections and skin infections such as erysipelas and paronychia. Erysipelas is a bacterial skin infection of the dermis usually isolated to the face and legs. It has been traced back to the Middle Ages when it was most commonly referred to as "St. Anthony's Fire" (Da Costa, 1903). The infection manifests as red, tender plaques on the skin. Infections are most commonly caused by *Streptococcus pyogenes* but more recently have also been associated with CA-MRSA and HA-MRSA, *Klebsiella pneumonia,* and *Pneumococcus* species (Schlossberg, 2008). Chernoviz also describes the use of bark for the treatment of paronychia, an infection of the skin around the nails that can be caused by bacteria or fungi (Berman, 2013). In his work, Chernoviz notes that aroeira is commonly used in Brazilian medicine, but is not used in European medicine.

Systema de Materia Medica Vegetal Brasileira by Carl Friedrich Philipp von Martius describes medicinal uses of the bark, leaves, and fruits of *S. terebinthifolius* (Figure 2.4) (Martius, 1854). Martius states that the bark is of "great importance" to the people living in Rio Grande where it is used to treat rheumatic pain and limb weakness as well as "dyscrasia arthritica", an "imbalance" in the blood related to arthritis. In addition, the bark is used to treat those that are believed to have a predisposition for tumors. The author even states that the local people believed that sleeping under the tree would prevent tumors of the joints. In addition to joint pain, parts of the plant were also used in the treatment of wounds and infections. For example, bark was used in the treatment of syphilis, while a balm made of leaves or fruit was used to treat wounds and ulcers. Leaves and fruits were also prepared using water distillation to

be used as a diuretic. Apart from the medicinal uses listed, powdered, dried leaves were used in dental care to strengthen gums.

Diccionario de plantas medicinaes brasileiras, written by Nicoláo Moreira describes uses of both bark and leaves (Moreira, 1862). As in works by other naturalists of the time, the bark is described as having astringent properties, which are employed in the preservation of fishing nets. Moreira also describes medicinal uses of the leaves which were prepared using water distillation to treat fevers, bleeding, and eye infections (Figure 2.4).



a) Martius, 1854
b) Moreira, 1862
c) Chernoviz, 1864
d) Burton, 1868

Figure 2.4. Medicinal uses of *Schinus terebinthifolius* reported by 19th century European naturalists arranged by plant part and preparation. Sources include: Martius (1854), Moreira (1862), Chernoviz (1864), and Burton (1868).

Modern uses and references

Many of the same uses for *Schinus terebinthifolius* described in the publications of the 19th Century persist today. More recent publications describe a variety of uses for almost all plant parts including bark, leaves, fruit, flowers, twigs and branches, wood, and roots. Uses include medicinal uses and as well as food production and construction. Uses of unspecified plant parts include the treatment of gout, muscular atony, arthritis, tendon strain, intestinal ailments, reproductive issues, skin and soft tissue infections, chills, tumors, lymphatic swellings, diarrhea, and hemoptysis (Morton, 1978).

Bark

The bark of the tree is commonly prepared as a decoction or tonic. In Brazil, a decoction of two to three large pieces of bark boiled in water for 20 minutes is used as a soak for individuals suffering from rheumatism, arthritis, and nerve pain, such as sciatica (Brandao et al., 2008; Morton, 1978). In addition, a decoction of bark can be used to treat tumors and leprosy (Schmourlo, Mendonca-Filho, Alviano, & Costa, 2005). Bark tea is also used as a laxative (Gundidza et al., 2009). A common application of the bark's anti-inflammatory and anti-infective qualities can be seen in its use in dental care. For example, *S. terebinthifolius* bark is applied to the gums after tooth extraction and its wood is used to make toothpicks (Morton, 1978; Vieira et al., 2014). In an indigenous reserve in Rio das Cobras located in the Paraná region of south eastern Brazil, use of the bark to treat toothache, oral sores, sore throat, flu, and feminine diseases also has been reported. In this culture, the bark is made into a tea which is then either consumed or used to wash the affected area (Moura-Costa et al., 2012). Individuals from this region also report the use of the bark and seeds prepared as a decoction to treat skin, eye,

nose, and oropharynx diseases, infectious diseases, and musculoskeletal and joint pain (M Bolson, Hefler, Chaves, Gasparotto, & Cardozo, 2014)

Wood, resin, and roots

According to Morton, the juice of macerated roots is used in the treatment of bruises and ganglionic tumors (Morton, 1978). Apart from medicinal uses, wood and tannin-rich resin have been used in construction and for the preservation of fishing equipment. As previously reported by Burton in 1868, *S. terebinthifolius* wood continues to be used in the construction of homes, as well as railroads (Burton, 1868; Morton, 1978).

Leaves

Leaves and bark are commonly prepared as a tonic, especially when treating urinary and respiratory infections (Braga et al., 2007; de Lima et al., 2006; Gomes, Procopio, Napoleao, Coelho, & Paiva, 2013; Gundidza et al., 2009). Dried leaves are also prepared as poultices and applied to wounds and ulcers for antiseptic purposes (Brandao et al., 2008; Morton, 1978). Furthermore, leaves are used in the treatment of hemorrhages, inflammation, and menstrual disorders for which they are prepared as a tea (Braga et al., 2007; Gundidza et al., 2009). They are also used as a decoction to treat tumors and have been investigated in laboratory studies for their anti-tumor activity (Bendaoud, Romdhane, Souchard, Cazaux, & Bouajila, 2010; Schmourlo et al., 2005). A decoction of leaves has also been used to treat leprosy (Schmourlo et al., 2005).

Fruits and Flowers

As mentioned previously, a decoction of the fruits and seeds are used in the treatment of skin, eye, ear, nose, and oropharynx diseases, infectious diseases, and musculoskeletal and joint pain (M. Bolson, Hefler, Dall'Oglio Chaves, Junior, & Cardozo Junior, 2014). A decoction of

fruits can also be used as a wound bath (Morton, 1978). In addition to use in traditional medicine, the fruits of the plant are commonly used as a spice and have also been valued as an ornament and decoration (Morton, 1978). Fruits are reported to have an intoxicating quality in birds and have been consumed as part of an alcoholic beverage (Kramer, 1957; Morton, 1978). In Peru, fruits from a very similar species, *Schinus molle,* have been used to make a fermented beverage called Chicha de Molle (Jennings & Bowser, 2008). Archeological investigation of a site in Peru called "Cerro Baúl" showed that this beverage was produced near temples and other ceremonial sites where the fruits also served as offerings for ancestral shrines. The beverage was drunk, mainly by the social elite, during religious feasts. It played an important role in Andean culture and also served as a staple of Incan agriculture (Jennings & Bowser, 2008)

According to Schmourlo, flowers have been used as a decoction for the treatment of tumors and leprosy (Schmourlo et al., 2005). Flowers have also been used in honey production (Feuereisen et al., 2014; Morton, 1978).

Chemistry and Pharmacology

Plants belonging to the family Anacardiaceae commonly contain phenolic lipids, terpenes, and biflavinoids, among other compounds (Carvalho et al., 2013). It is believed that phenolic derivatives present in *S. terebinthifolius* are responsible for the plant's antimicrobial as well as its antioxidant activities. These compounds include, but are not limited to gallic acid, methyl gallate, tannins, and flavonoids, and terpenes (Fedel-Miyasato et al., 2014) (Feuereisen et al., 2014).

Tannins are rich in phenol groups and are known for their ability to bind water-soluble proteins, forming a precipitate. The compounds are common in vascular plants and can be found

in all plant parts, but are present primarily in woods (Cseke et al., 2006). Tannins have been used in the treatment of diarrhea and may also have some anticancer and anti-HIV activity (Gurib-Fakim, 2006). Tannins may play a role in the formation of a protective layer around damaged tissue or mucous membranes due to their antimicrobial activity as well as their ability to interact with and precipitate proteins from cell surfaces (Alves, Freires Ide, de Souza, & de Castro, 2012).

Flavonoids are composed of two benzene rings separated by a propane unit and are classified based on the presence of oxygen containing groups. Classifications include chalcones, flavones, flavanones, flavinols, and anthocyanins (Cseke et al., 2006). These compounds are responsible for pigmentation of flowers and fruits and help to protect plants from UV damage. Flavinoids have demonstrated a wide range of biological activity including anti-inflammatory, analgesic, anti-tumor, anti-HIV, anti-infective, antioxidant, anti-ulcerogenic, and vasodilator activity (Gurib-Fakim, 2006).

Terpenes are commonly found in the essential oils of plants and are known for their fragrant qualities. The compounds often demonstrate allelopathic activity and may also play a role in the plant's chemical defense system by acting as an insecticide, although some are known to attract insect pollinators (Cseke et al., 2006). A high concentration of terpenes can be found in oils extracted from *S. terebinthifolius* leaves with sesquiterpenes and oxygenated monoterpenes making up the largest percentage in fresh leaves, although percentages differ when leaves are dried (Table 2.1) (El-Massry, El-Ghorab, Shaaban, & Shibamoto, 2009). Major compounds found in fruits, most of which are also found in leaves, are summarized in Tables 2.1 and 2.2 (Bendaoud et al., 2010). Monoterpenes are composed of two isoprene units, while sesquiterpenes are composed of three (Cseke et al., 2006). Monoterpenes and sesquiterpenes have been

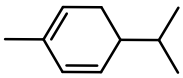
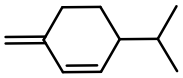
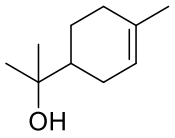
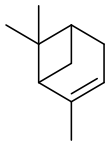
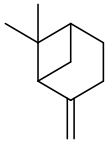
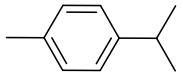
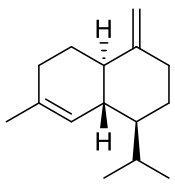
implicated as an important component of the chemical defense system in a variety of flowering plants (Harborne, 1993). It has been suggested that α-Pinene, a monoterpene, plays a role in bacterial degradation of hydrocarbons (Cseke et al., 2006). A group of sesquiterpenes, known as the cardinenes have been associated with antiparasitic activity (Cseke et al., 2006). Other sesquiterpenes have demonstrated antibacterial, antifungal, and antimalarial activity (Gurib-Fakim, 2006).

	Fresh leaves	Dried leaves	Dried fruits
monoterpenes	4.97	15.18	62.77
sesquiterpenes	56.96	39.25	26.81
oxygenated monoterpenes	34.37	8.13	6.29
oxygenated sesquiterpenes	3.32	36.07	2.41

Table 2.1. Terpene composition (percentage) of *S. terebinthifolius* leaves and fruits

Data source for leaves: El-Massry, K. F., El-Ghorab, A. H., Shaaban, H. A., & Shibamoto, T. (2009). Chemical compositions and antioxidant/antimicrobial activities of various samples prepared from *Schinus terebinthifolius* leaves cultivated in Egypt. *J Agric Food Chem, 57*(12), 5265-5270. doi: 10.1021/jf900638c

Data source for fruits: Bendaoud, H., Romdhane, M., Souchard, J. P., Cazaux, S., & Bouajila, J. (2010). Chemical composition and anticancer and antioxidant activities of *Schinus molle* L. and *Schinus terebinthifolius* Raddi berries essential oils. *J Food Sci, 75*(6), C466-472. doi: 10.1111/j.1750-3841.2010.01711.x

Compound	Percent Composition	Structure
α-phellandrene	34.38	
β-phellandrene	10.61	
α-terpineol	5.6	
α-pinene	6.49	
β-pinene	3.09	
p-cymene	7.34	
γ-cadinene	18.04	

*This table only includes compounds present at >3%

Table 2.2 Composition of essential oils from *S. terebinthifolius* fruits

Data source: Bendaoud, H., Romdhane, M., Souchard, J. P., Cazaux, S., & Bouajila, J. (2010). Chemical composition and anticancer and antioxidant activities of *Schinus molle* L. and *Schinus terebinthifolius* Raddi berries essential oils. *J Food Sci, 75*(6), C466-472. doi: 10.1111/j.1750-3841.2010.01711.x

Biological Activity and Current Research

Schinus terebinthifolius has demonstrated a wide range of biological activity in laboratory studies including antibacterial, antiparasitic, antifungal, antioxidant, and antitumor activity. In addition, the plant has shown activity against biofilm formation.

Antibacterial activity

Schinus terebinthifolius has shown antibacterial activity against a variety of possible pathogens including *S. aureus, Pseudomonas aeruginosa, and Aspergillus* species (El-Massry et al., 2009). Many studies related to antibacterial activity focused on testing bark extract. In one study, hexane, chloroform, and ethyl acetate partitions of the stem bark showed considerable antibacterial activity against strains of *S. aureus* resistant to fluoroquinolone and macrolide drugs, while ethanolic extract of the leaves showed activity against a non-drug-resistant strain (de Lima et al., 2006). An alcoholic extract of bark also demonstrated activity against *Pseudomonas aeruginosa* (Moura-Costa et al., 2012). In another study, a bark extract injected directly into the abdominal cavity of rats showed promise as a possible treatment for peritonitis (Melo, Gadelha, Oliveira, & Brandt, 2014). Peritonitis is a bacterial infection resulting from perforations or lesions of the digestive organs. It can be life threatening and may progress to sepsis, a severe systemic inflammatory response to bacterial infection. The study by Melo et al. (2014) suggests that *S. terebinthifolius* should be further investigated as an alternative treatment to broad spectrum intravenous antibiotics, the current standard treatment for peritonitis (Melo et al., 2014). Findings of this study may also be related to the possible anti-inflammatory activity of the plant associated with wound-healing properties investigated in other studies (dos Santos et al., 2012; Estevao et al., 2013).

Multiple studies have been performed in an effort to confirm antibacterial properties associated with the plant's use in traditional medicine in Brazil. As mentioned previously, *S. terebinthifolius* has been used in dental care (Vieira et al., 2014). When tested for its activity against *Streptococcus mutans*, a common cause of dental biofilms, *S. terebinthifolius* hydroalcoholic bark extract caused considerable dose dependent growth inhibition at concentrations ranging from 5-100 mg/mL (Vieira et al., 2014). In another study ethanol, hexane, and butanol extractions of leaves and bark showed growth inhibition of *S. mutans* and *S. aureus*, both of which are associated with dental infections (Pereira et al., 2011).

Biofilm inhibition

The investigation of *S. terebinthifolius* as a biofilm inhibitor is important because biofilms can lead to increased antibiotic resistance while serving as a barrier to many drugs. Research involving anti-biofilm activity of *S. terebinthifolius* has focused mainly on dental applications and oral biofilms formed by *Candida* and *Streptococcus* species. In one study, methanolic leaf extract showed a 50% reduction in *C. albicans* biofilm formation and 40% reduction in *S. mutans* biofilm formation (Barbieri et al., 2014).

Anti-parasitic activity

In laboratory tests, *S. terebinthifolius* leaves showed activity against *Leishmania* at relatively low concentrations (Braga et al., 2007). In addition, an aqueous extract of the bark showed anti-leishmanial activity (Moura-Costa et al., 2012). Classes of compounds that may be responsible for this activity include alkaloids, triterpenoids, tannins, flavonoids, and anthraquinones (Braga et al., 2007).

Antifungal activity

S. terebinthifolius extracts have demonstrated activity against fungi, such as *Candida* species, in multiple laboratory studies. This has important medical implications because *Candida* is responsible for oral thrush as well as dangerous opportunistic infections in long-term catheter users and individuals with compromised immune systems (Alves et al., 2012; Braga et al., 2007). In multiple studies, ethanolic extract of the bark and leaves showed growth inhibition of *Candida albicans* (Moura-Costa et al., 2012; Pereira et al., 2011). Both alcoholic and hydroalcoholic extracts of the bark also showed activity against two more *Candida* species: *Candida parapsilosis* and *Candida tropicalis* (Moura-Costa et al., 2012). In another study, a leaf extract containing alkaloids, triterpenoids, tannins, flavonoids, and anthraquinones demonstrated activity against *C. albicans* (Braga et al., 2007).

Antioxidant activity

In laboratory studies, *S. terebinthifolius* essential oils have shown considerable antioxidant activity at higher levels than other *Schinus* species, such as *S. molle*. It is believed that this activity may be the result of the presence of antioxidant compounds, such as γ-terpinene, terpinoline, cedrene, and sabinine (Bendaoud et al., 2010).

Anti-inflammatory activity

Ethyl acetate extractions of *S. terebinthifolius* fruits were found to inhibit phospholipase A, an enzyme associated with inflammatory responses. Specifically, the study found that two types of triterpenoids were responsible for this inhibitory activity (Jain et al., 1995). This finding may partially explain the plant's traditional use in the treatment of rheumatism and other inflammatory disorders (M. Bolson et al., 2014; Fedel-Miyasato et al., 2014).

Antitumor activity

S. terebinthifolius fruits have demonstrated dose dependent cytotoxicity of cells isolated from breast tumors, possibly as a result of high levels of sesquiterpenes (Bendaoud et al., 2010). This activity may also be related to the plant's antioxidant activity.

Wound healing

S. terebinthifolius has been applied in traditional medicine to accelerate wound healing. When studied in rats with surgically created stomach lesions, treatment with an ethanolic extract of *S. terebinthifolius* bark resulted in accelerated healing (dos Santos et al., 2012). The rats treated with injections of the extract at a 100mg/kg dose showed no sign of infection, a lower acute inflammatory response than the control, and increased strength of the healed incision (dos Santos et al., 2012). In another study, rats that received cutaneous lesions were treated with ointment containing 5% *S. terebinthifolius* leaf oil. This study also observed improved wound healing upon treatment with *S. terebinthifolius* extract (Estevao et al., 2013). Similar findings of improved healing of cutaneous wounds in beef cattle have also been observed following treatment with bark extract for 17 days (Lipinski, Wouk, da Silva, Perotto, & Ollhoff, 2012). This study also observed improved clotting and the establishment of a fibrin net in the wounds (Lipinski et al., 2012). Improved would healing may be related to a decreased, although still present, acute inflammatory response (dos Santos et al., 2012).

Cautions and Toxicity

Contraindications of *S. terebinthifolius* have not been investigated in detail in the current literature. Negative effects of *S. terebinthifolius* can include development of a rash or blisters when exposed to leaves or cut bark for an extended period of time and airway inflammation with

inhalation of smoke given off by the burning plant (Carvalho et al., 2013). Irritation of the skin and airways caused by exposure to the tree worsens when the tree is in bloom (Morton, 1978). In Brazil, a common treatment for rash and swelling caused by *S. terebinthifolius* is *Hydrophyllum canadense*, also known as the bluntleaf waterleaf (Morton, 1978). In addition, consumption of the unripe berries has been known to cause vomiting and chills (Morton, 1978).

Multiple studies have been conducted to investigate the toxicity of *S. terebinthifolius*. In one study, both male and female rats were given water containing leaf extract for an extended period of time. Males, who were given the extract for 83 days, showed slight weight loss and a decreased red blood cell count. When mated with untreated females, males did not show a decrease in fertility or mating ability and offspring did not have any notable morphological changes. Females that were fed the extract throughout pregnancy showed a similar decrease in red blood cell count and produced offspring that had slight weakening and deformities of the ribs, but there was no change in the number of stillbirths (Carlini, Duarte-Almeida, & Tabach, 2013). Although some negative effects were observed in individuals treated with large amounts of extract, they were relatively minimal. More trials would need to be performed to confirm the toxic effects of oral consumption of *S. terebinthifolius* leaves, especially during pregnancy.

Staphylococcus aureus **and Antimicrobial Resistance**

Antimicrobial Resistance

The development of antimicrobial resistance poses a threat to the treatment of a wide variety of diseases caused by bacteria, parasites, fungi, and viruses. Development of

antimicrobial resistance is a natural process that is caused by an increase in the prevalence of resistance genes in a population through reproduction and natural selection (World Health Organization, 2014b). Antimicrobial resistance can be accelerated with misuse and overuse of antimicrobials, particularly antibiotics.

Antimicrobial resistance can result in the failure of standard treatments and medications in the treatment of a variety of infections. Loss of efficacy by standard treatments can result in prolonged illness, higher health care costs, a greater risk of serious infection and death, and increased likelihood of spreading the drug resistant microorganisms to others (World Health Organization, 2014b). It is estimated that in the United States alone, antimicrobial resistance has cost the healthcare system between 21 to 34 billion dollars and has caused an extra 8 million days of hospital stays (World Health Organization, 2014a). Individuals with drug resistant infections can have additional healthcare costs from $6,000 to $30,000 compared to those with susceptible infections (Cosgrove, 2006). Antimicrobial resistance also has a negative impact on other areas of healthcare apart from infectious disease, including surgery, organ transplantation, and chemotherapy, as all often involve the use of antibiotics (World Health Organization, 2014b).

Staphylococcus aureus

Staphylococcus aureus is a gram-positive coccus and facultative aerobe. It is coagulase and catalase positive and causes β-hemolysis (Spaulding et al., 2013). While it can be pathogenic, *S. aureus* is part of the normal human microbiome and is present in 30 to 50 percent of healthy adults (Lowy, 1998; Spaulding et al., 2013). Although the bacterium is not always pathogenic, colonized adults are at a higher risk of infection (Lowy, 1998).

Staphylococcus aureus is associated with a wide range of disease pathogeneses including sepsis, toxic shock syndrome, and a variety of skin and soft tissue infections ranging from pneumonia to skin abscesses (Lowy, 1998). Symptoms of *S. aureus* infections are caused largely by toxins and virulence factors produced by the bacteria. Production of many of these toxins and virulence factors are associated with the *agr* (accessory gene regulator) system.

Quorum sensing and the *agr* system

The *agr* system in *S. aureus,* encoded by the *agr* locus, plays an important role in toxin production, quorum sensing, and biofilm formation. Virulence factors produced by the *agr* system are associated with a range of infection processes including invading host cells, evading the host immune system, and breaking down tissus (Novick, 2003). Activity of this system is associated with greater severity of disease (Novick, 2003). For this reason, the *agr* system is an ideal target in the development of novel therapies for invasive *S. aureus* infections.

The *agr* system is activated during the transition from the exponential to stationary growth phase (Vuong, Saenz, Gotz, & Otto, 2000). The *agr* system is cell density dependent and accomplishes signaling through the production of autoinducing peptides (AIPs) (Figure 2.5). When a certain AIP concentration is reached, AIPs bind to the AgrC histidine kinase receptor on the cell surface that initiates a regulatory cascade through the phosphorylation of AgrA. The phosphorylation of AgrA promotes transcription of RNAIII, which causes increased production of virulence factors, including enzymes, toxins, and immunomodulators that allow for Staphylococcal infection (Pang et al., 2010). The reliance on AIP concentration makes the *agr* system a cell density dependent mechanism. The regulatory cascade induces expression of virulence factors and toxins (Boles & Horswill, 2008). Inhibition of the *agr* system has become a

focus of treatment development for *S. aureus* infections, because many symptoms of infection are the result of toxins produced by the pathway (Quave & Horswill, 2014). Studies have shown that inhibition of the *agr* response, and thus quorum sensing, has resulted in decreased toxin production and infection symptoms. One study showed that inhibition of the *agr* system by artificially created pheromone molecules resulted in decreased δ-toxin production (M. Otto, Sussmuth, Vuong, Jung, & Gotz, 1999). In another study, inhibition of the *agr* system by similar pheromone molecules resulted in decreased severity of skin lesions caused by *S. aureus* (Mayville et al., 1999).

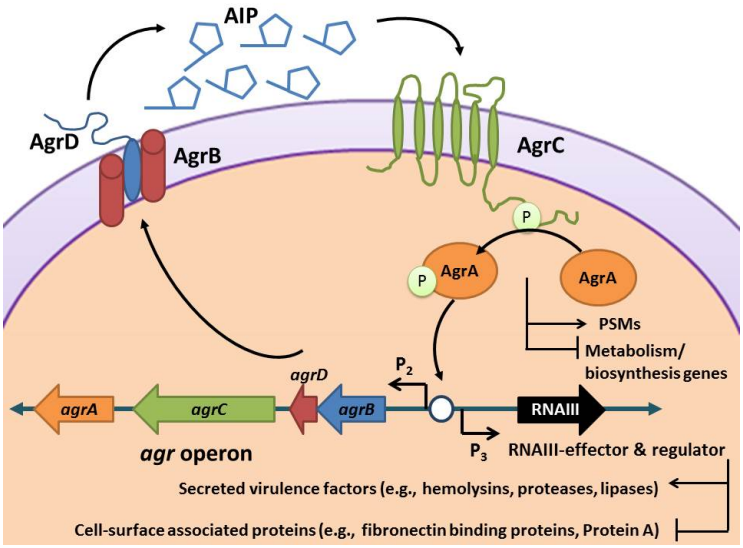


Figure 2.5. The *agr* system in *S. aureus*. Strains of *S. aureus* are organized into four allelic groups depending on the AIP produced by the system.
Source: Quave, C. L., & Horswill, A. R. (2014). Flipping the switch: tools for detecting small molecule inhibitors of staphylococcal virulence. *Frontiers in microbiology, 6*.

S. aureus strains are divided into four main allele groups based on the AIP and corresponding receptor produced by the *agr* system (Jarraud et al., 2002). AIPs produced by a member of an *agr* group will activate the *agr* response in members of the same group and inhibit the *agr* response in members of other *agr* groups (Jarraud et al., 2002). Different *agr* groups

have been associated with different syndromes and symptoms caused by *S. aureus* infection (Figure 2.6). *Agr* groups I and II have been associated with the production of staphylococcal enterotoxins, while *agr* III has been associated with the production of Toxic Shock Syndrome Toxin-1 (Jarraud et al., 2002). Together, these toxins are known as pyrogenic toxin superantigens and cause life-threatening illness through the overstimulation of the innate immune inflammatory response resulting in high fever, capillary leakage, and organ failure (Lowy, 1998; Spaulding et al., 2013). *Agr* IV has been associated with the production of exfoliative toxins, which serve as a protease that cleaves superficial layers of skin. This disease is known as Scalded Skin Syndrome and is common in neonates (Jarraud et al., 2002). Another family of toxins that play a role in the pathogenesis of *S. aureus* infection comprises the hemolytic toxins, α-toxin and δ-toxin. These toxins target many different cell types and form pores in the cell membrane causing lysis (Berube & Bubeck Wardenburg, 2013). They are associated with necrosis, the formation of abscesses, and sepsis (Vivekananda, Salgado, & Millenbaugh, 2014).

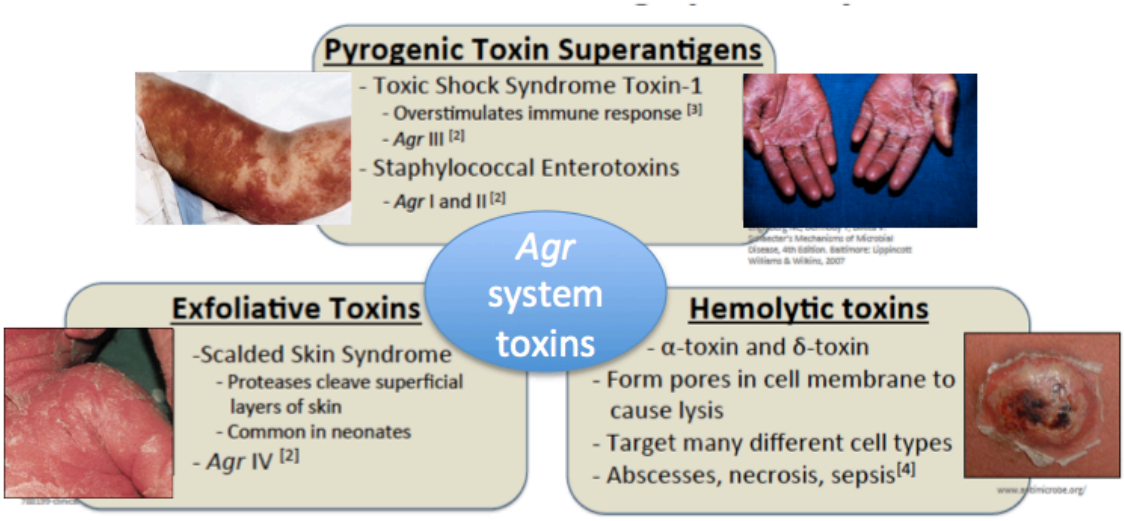


Figure 2.6. Diseases and associated toxins produced by different agr groups of *S. aureus*

Antibiotic resistance in *S. aureus*

Over the course of the past 50 years, many strains of antibiotic resistant *S. aureus* have emerged. One of the first forms of resistance noted in these strains was penicillin resistance. During the 1940s, penicillin was a common treatment for *S. aureus* infections, but resistance resulting from the production of β-lactamase began to emerge (World Health Organization, 2014a). β-lactamase also causes inactivation of, and thus resistance to, related drugs including amoxicillin and ampicillin. Because of this, drugs that would not be affected by beta lactamase were developed. These included methicillin and cloxacillin. However, these new drugs did not remain effective for long as strains of methicillin-resistant *S. aureus* (MRSA) began to emerge in the 1960s (World Health Organization, 2014a). Initially, MRSA infections became prevalent in healthcare settings and were the most common cause of nosocomial infection in the United States in the 1990s (Lowy, 1998). Over time, community associated infections have become more common, affecting even healthy individuals (Lowy, 1998). Although rates of nosocomial MRSA infections have decreased by roughly 50% since 2005, the increase in community acquired cases makes MRSA a continuing concern (Centers for Disease Control and Prevention, 2013b). The highest rates of community-associated MRSA (CA-MRSA) in the United States are in Georgia, where CA-MRSA infections make up 20% of all MRSA infections. Almost 80% of these cases are skin and soft tissue infections. Less common pathologies include urinary tract infections, pneumonia, and bacteremia (McDonald, 2006). Stronger, second-line treatments such as vancomycin can still be used to fight MRSA infections but have more adverse side effects, are more expensive, and are becoming less effective (World Health Organization, 2014a). A study by the World Health Organization found that, compared to individuals with non-drug resistant infections, patients with drug resistant *S. aureus* infections had higher mortality rates, longer

hospital and ICU stays, increased prevalence of septic shock, and a greater likelihood to be discharged to a long term care facility (World Health Organization, 2014a). It is postulated that, through identification of small molecule *agr* inhibitors, adjuvant therapies may be developed that render antibiotic therapy more efficacious and perhaps even reduce time courses for antibiotic therapy, decreasing overall antibiotic use in the treatment of *S. aureus* toxin-mediated infection. Decreased antibiotic use could decrease the risk of resistance and lessen negative side effects associated with stronger second-line antibiotics.

CHAPTER 3: MATERIALS AND METHODS

Literature review

In order to identify possible sources of anti-infective compounds and guide later laboratory work, a thorough literature review was performed while paying particular attention to traditional medicinal uses of *Schinus terebinthifolius*. In addition to traditional and current medicinal uses in Brazil, the literature search also focused on current research into the biological activity of *S. terebinthifolius.* Most sources related to current biological research were gathered through the PubMed and Web of Science databases and had been published in reputable, peer-reviewed journals. Search terms utilized to find articles related to current biological research include "*Schinus terebinthifolius"* and *"Schinus terebinthifolius* biological activity", which yielded roughly 70 results.

Investigation of medicinal uses of *S. terebinthifolius* also used PubMed and Web of Science, but relied more heavily on print sources and scanned copies of books originally published in the 1800s. These scanned books were obtained through the Biodiversity Heritage Library and Internet Archive ("Biodiversity Heritage Library ", 2015; "Internet Archive," 2015). References from the 19th Century were chosen because they were cited by many recently published papers that referenced traditional medicinal uses of the plant. When attempting to locate information regarding uses in traditional medicine, a wider range of search terms (*Schinus terebinthifolius* uses*,* peppertree, Brazil traditional medicine, aroeira) was used to retreive older references which mainly referred to *S. terebinthifolius* by its common names. Many documents referencing traditional uses were written in Portuguese and required translation to English with the assistance of an automatic translator (Google translate) and online Portuguese dictionaries.

Once information about medicinal uses was gathered, they were organized based on plant part, medical ailment, preparation, and geographical region of use.

Experimental overview

A series of biological assays and chemical analyses were performed to investigate the bioactivity of fruit and leaf extracts of *Schinus terebinthifolius* (Figure 3.1). Through a process of bioassay guided fractionation, extracts were purified and tested further to identify compounds capable of *agr* inhibition without biocide activity.

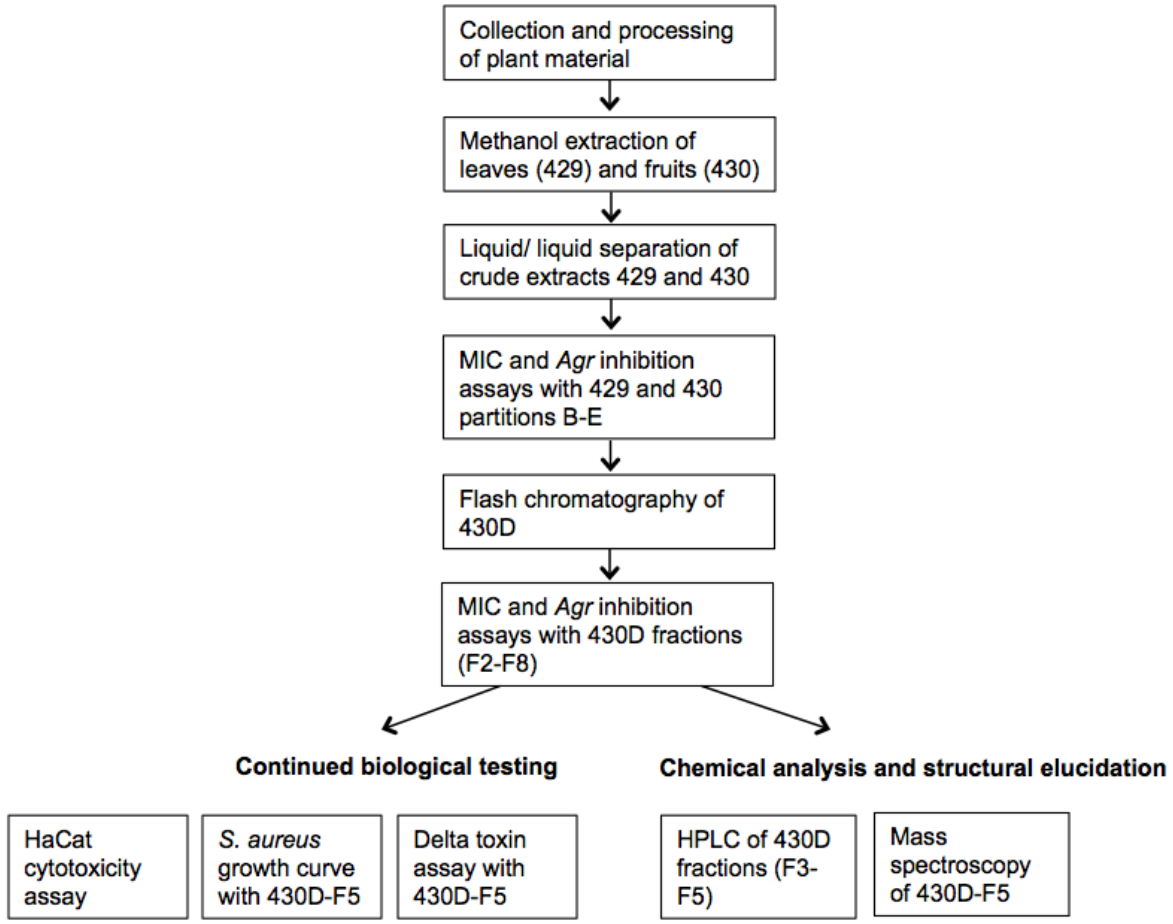


Figure 3.1. Experimental overview

Plant Material

Schinus terebinthifolius leaves, stems, and fruits were collected in bulk from private lands in DeSoto County, Florida in November of 2013 and 2014 after obtaining permission from the land owner. Procedures from the 2003 WHO Guidelines for good agricultural and collection practices (GACP) for medicinal plants were followed for the collection and identification of bulk and voucher specimens (World Health Organization, 2003). Vouchers were deposited at the Emory University Herbarium and were identified using the standard Flora for Florida (Wunderlin & Hansen, 2003). Plant leaves, stems, and fruits 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 sealed in paper bags and stored at room temperature.

Figure 3.2. *Schinus terebinthifolius* voucher specimen deposited in the Emory Herbarium

Extraction

For each extraction, 50 g of dried *Schinus terebinthifolius* fruits or leaves were blended with 500 mL methanol (MeOH) and sonicated for 20 minutes. After sonication, the extract was vacuum filtered and 500 mL of fresh MeOH was added to the remaining plant material. The mixture was again sonicated for 20 minutes in a water bath sonicator and vacuum filtered. For each extraction, this process was repeated a total of three times. Filtered extracts were combined and concentrated using rotary evaporation at reduced pressure. The concentrated extract was then re-suspended in deionized H_2O, dried through lyophilization, and stored at -20˚C. The leaf extract was labeled 429, and the fruit extract was labeled 430.

Separation

A modified Kupchan partitioning scheme in succession with hexane, ethyl acetate, and butnol was used in the separation of extracts 429 and 430. All ACS (American Chemical Society) grade solvents were obtained from Fisher Chemical. 16 g of 429 or 430 extract was dissolved in 500 mL of 20% MeOH/ DIH_2O solution. The solution was placed in a 1 L separatory funnel and mixed with 500 mL of hexanes. The organic layer was removed and filtered through anhydrous sodium sulfate (Na_2SO_4). 500 mL of hexanes was mixed with the aqueous solution two more times. This process was repeated with ethyl acetate and n-Butanol. The remaining MeOH/ H_2O was collected and filtered without sodium sulfate. Each partition was rotary evaporated, dissolved in DI H_2O, frozen, lyophilized and stored at -20˚C. The hexane partition was labeled “B”, the EtOAc partition was labeled “C”, the n-Butanol partition was labeled “D”, and the final remaining aqueous partition was labeled “E” (Table 3.1).

Extract name	Plant part	Description
429	Leaves	Crude extract
429B		Hexane partition
429C		Ethyl Acetate (EtOAc) partition
429D		Butanol partition
429E		Aqueous partition
430	Fruits	Crude extract
430B		Hexane partition
430C		Ethyl Acetate (EtOAc) partition
430D		Butanol partition
430E		Aqueous partition

Table 3.1. Extract names and descriptions

Fractionation through flash chromatography

Following initial quorum quenching assays, the most active partition, the butanol partition of the fruits (430D) was subjected to fractionation through flash chromatography. Fractionation was performed using a CombiFlash® Rf+ (Teledyne ISCO) flash chromatography system with a RediSep Rf Gold silica column. The dry load column was prepared by binding extract 430D to Celite 545 at a ratio of 1:4. Flash chromatography was run using a three solvent system of (A) hexane, (B) dichloromethane, and (C) methanol. The gradient began with 100% A for 6 column volumes (CV), then went to 100% B over 12 CV and was held for 18.2 CV. The gradient was then changed to 74.5:25.5 B:C over the course of 3.1 CV. These conditions were held for 6.8 CV, following which the gradient changed to 68.8:31.2 B:C over 0.7 CV and was held at these conditions for 7.5 CV. Finally the gradient was adjusted to 100% over 2.2 CV and held for 14.6 CV. The chromatography was monitored at 254 and 280 nm, as well as via ELSD. Tube volumes were combined to create eight fractions: 430D-F1 (tubes 1-5), 430D-F2 (tubes 6-11), 430D-F3 (tubes 12-16), 430D-F4 (tubes 17-24), 430D-F5 (tube 24), 430D-F6 (tubes 25-28), 430D-F7 (tubes 29-31), 430D-F8 (tubes 32-38).

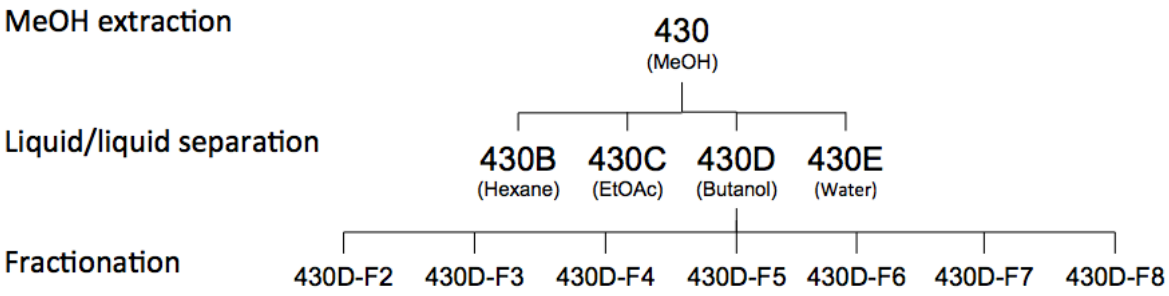


Figure 3.3. Fractionation scheme for *S. terebinthifolius* fruit extract

High Pressure Liquid Chromatography (HPLC)

Chromatograms of 430, 430D, and 430D-F3, F4, and F5 were generated using HPLC in order to gain information about the chemical composition and shared constituents of each extract. An Agilent Eclipse XDB-C18 4.6 x 250 mm, 5-µm analytical column, with a compatible guard column, was used at 40°C. Active sub-fractions were dissolved in DMSO (10 mg mL^{-1}), filtered at 0.2-microns, and a 10 µL injection was eluted at a flow rate of 1.9 mL/min using a gradient system consisting of (A) 0.1% formic acid in H_2O; (B) 0.1% formic acid in acetonitrile (ACN). The mobile phase was 98:2 A:B at time 0 min, 70:30 A:B at 40 min, 2:98 A:B at 63 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. The chromatography was monitored at 217, 254, 320, and 500 nm.

Cell lines

Quorum sensing reporter strains of *S. aureus* representing the four *agr* groups included AH1677, AH430, AH1747, and AH1872 (Table 3.2). All strains were provided by Dr. Horswill of the University of Iowa. AH1677 is an *agr* I reporter from strain AH845 (CA-MRSA USA 300 LAC), AH430 is an *agr* II reporter from SA502A, AH1747 is an *agr* III reporter from strain CA-

MRSA MW2, and AH1872 is an *agr* IV reporter from MN EV(407). All strains contained plasmid pDB59 that served as an *agr* fluorescence reporter and was maintained through culture in media containing chloramphenicol at a concentration of 10 µg/mL. Chloramphenicol, purchased from Sigma-Aldrich, was dissolved in 95% EtOH to a stock concentration of 20 mg/mL and stored at -20°C until it was added to the media. Cultures were grown on Tryptic Soy Agar (TSA), purchased from Teknova, with 10 µg/mL chloramphenicol (TSAcm10 plates).

Designation	Other Characteristics
AH430	SA502a + pDB59 cmR, yfp reporter, agr group II
AH1677	AH845 + pDB59 cmR, yfp reporter, agr group I
AH1747	MW2 + pDB59 cmR, yfp reporter, agr group III
AH1872	MN EV(407) + pDB59 cmR, yfp reporter, agr group IV
NRS385; NR-46071	HA-MRSA, PFT USA500, MLST ST8, SCC mecIV, agr group I, sea+, seb+

Table 3.2. Description of *S. aureus* strains

Minimum Inhibitory Concentration Assay (Growth inhibition)

Minimum inhibitory concentration (MIC) assays were performed to test for growth inhibition of *S. aureus* by each extract. All extracts were dissolved in DMSO to a stock concentration of 10 mg/mL and stored at -20°C. A small volume of each strain taken from frozen stock was plated on Tryptic Soy Agar and allowed to incubate overnight at 37°C. Following incubation, a single colony of each *agr* reporter strain was obtained from the TSA plate and grown overnight in Tryptic Soy Broth (TSB), purchased from Himedia, at 37°C while shaking at 200 rpm in a Biotek Shaker and Incubator. After overnight incubation, when the culture had reached the logarithmic growth phase, it was standardized to 5 x 10^5 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 TSB. MIC assays were performed in sterile 96 well flat bottom

polystyrene plates from Corning. Each extract, from a stock concentration of 10 mg/mL in DMSO as well as a DMSO control, was added to one well at an initial concentration of 512 µg/mL. Two-fold serial dilutions were made to yield a final well volume of 200 µL and concentration of 0.125 µg/mL, testing each extract at 12 concentrations ranging from 512-0.125 µg/mL. Two antibiotic controls, Ampicillin and Vancomycin, were tested at 12 concentrations ranging from 0.03125 to 64 µg/mL. Ampicillin anhydrous was purchased from MP Biomedicals, and Vancomycin hydrochloride from *Streptomyces orientalis* was purchased from Sigma-Aldrich. Vancomycin was dissolved to 10 mg/mL in DIH_2O, and Ampicillin was dissolved to 10 mg/mL in PBS. Plates were incubated at 37°C for 18 hours. After 18 hours, the OD of each well was measured using a Biotek Cytation 3 imaging reader. Percent growth inhibition was calculated with the equation:

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

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

Agr Reporter Assay

S. aureus agr reporter strains AH1677 (*agr I*), AH430 (*agr II*), AH1747 (*agr III*), and AH1872 (*agr IV*) were grown overnight in TSB supplemented with 10 µg/mL chloramphenicol at 37°C while shaking at 200 rpm. Cell density was standardized by OD to 5 x 10^5 CFU/mL and diluted to the necessary concentration in Cation-adjusted BBL™ Mueller Hinton II Broth (CAMHB), purchased from Becton, Dickinson, and Company, with 10 µg/mL chloramphenicol.

For extracts 429B-E and 430B-E, each extract was tested at three sub-growth-inhibitory concentrations. For 430DF2-F8, each fraction was tested at 8 concentrations ranging from 256-2 μg/mL. 96 well black sided, clear bottom, tissue-culture treated polystyrene Costar plates with final well volume of 200 μL were used for all *agr* inhibition assays. Plates were incubated at 37°C while shaking at 260 rpm for 24 hours. After 24 hours, plates were removed and OD and fluorescence were measured by plate reader at an excitation of 493nm and emission of 535nm. Percent inhibition of fluorescence was calculated using a similar formula to the MIC equation.

δ -toxin Assay

In an effort to confirm that the *agr* inhibition observed in the reporter assay led to decreased production of exotoxin, the amount of δ-toxin produced by cultures was quantified after treatment with different extracts. NRS385, a USA500 *agr* I strain of *S. aureus*, was used instead of the previously mentioned strains so that δ-toxin in the supernatant could be more easily quantified using HPLC. Overnight cultures of NRS385 in TSB were standardized to 5 x 10^5 CFU/mL based on OD reads and diluted to the necessary concentration in TSB. The culture was added to 15 mL test tubes containing DMSO, 430, 430D, and 430D-F5 at concentrations of 2, 8, and 32 μg/mL for a culture volume of 1.5 mL. Each concentration of extract and DMSO was tested in quadruplicate alongside four untreated tubes that would serve as a growth control. All extracts were tested at sub-MIC50 concentrations, meaning concentrations that would inhibit less than 50% of growth, as previously determined by MIC assay for NRS385. Tubes were incubated at a 45° angle at 37°C while shaking at 275 rpm for 15 hours. After incubation, the tubes were placed on ice and centrifuged at 13,000 rcf for 5 minutes at a temperature of 4°C. Supernatants were removed and 750 μL of each supernatant was placed in a vial for HPLC

quantification of delta toxin. The remaining volume of supernatant was sterile filtered with a 0.22 µm nylon syringe filter and stored at -20°C until needed for later treatment of cells in the HaCat cytotoxicity assay.

Frozen supernatants were defrosted and transferred to HPLC autosampler vials. Resolution of the de-formylated and formylated δ-toxin peaks was achieved on an Agilent 1260 Infinity system with a Resource PHE 1-mL (GE Healthcare, Sweden) analytical column (M Otto & Gotz, 2000; C. L. Quave, Plano, & Bennett, 2010). δ-toxin was eluted at a flow rate of 2 mL min^{-1} using a two solvent system: (A) 0.1% trifluoracetic acid (TFA) in water and (B) 0.1% TFA in acetonitrile (ACN). The mobile phase was 10% B for 3 min, 90% B for 7.5 min, 100% B for 2 min, and 0% B for 2 min. Peak integration was at 214 nm, with de-formylated and formylated δ-toxin recorded at a retention time of 6.4 and 6.8 minutes, respectively. The de-formylated and formylated δ-toxin peaks were combined to calculate total peak area (Figure 3.4).

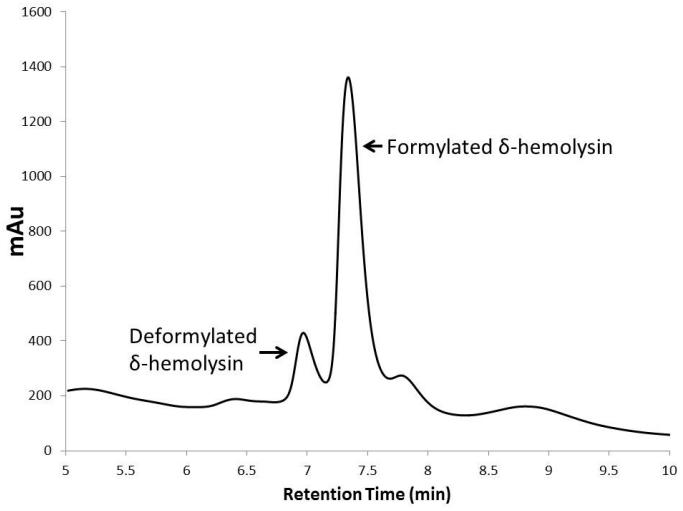


Figure 3.4. HPLC chromatogram of δ-toxin

Growth Curve Assay

S. aureus agr I reporter strain AH1677 was grown overnight in TSB supplemented with 10 μg/mL chloramphenicol at 37°C while shaking at 250 rpm. Cell density was standardized by OD to 5 x 10^5 CFU/mL and diluted to the necessary concentration in Cation-adjusted BBL™ Mueller Hinton II Broth (CAMHB) with 10 μg/mL chloramphenicol. Standardized liquid culture was placed into 50mL glass tissue culture flasks at a 10:1 flask:volume ratio. Cultures were treated with 32 μg/mL 430D-F5 or DMSO in triplicate. An untreated growth control was also prepared in triplicate. Flasks were incubated at 37°C while shaking at 250 rpm. Growth and fluorescence were measured hourly by placing 100μL aliquots of each culture in a 96 well black sided, clear bottom, tissue-culture treated polystyrene plate. Growth was measured using OD_{600} values, and fluorescence was measured at an excitation of 493nm and emission of 535nm.

HaCat cytotoxicity assay

Human immortalized keratinocytes (HaCat cell line) provided by Dr. Pollack at Emory University School of Medicine were maintained in Dulbecco's modified Eagle's medium with glucose at 4.5 g/L and L-glutamine. Media was supplemented with 10% heat-inactivated fetal bovine serum and a solution of Penicillin and 100 μg/mL Streptomycin. Cells were cultured in 75 cm^2 flasks incubated at 37°C with 5% CO_2. A solution of 0.25% trypsin and 0.1% EDTA in HBSS was used to detach cells from the bottom of the flask before splitting and plating.

After cells had reached at least 80% confluence in the flask, defined as the flask bottom being 80% covered with adhered cells, they were seeded to a 96 well tissue culture plate. In order to do this, cells were resuspended, counted with a hemocytometer, and standardized to a concentration of 4x10^4 cells per 200 μL well volume. 200 μL of culture was placed in each well

and the plate was incubated for 48 hours at 37°C with 5% CO_2. After incubation, medium was removed and replaced with fresh medium. Sterile filtered extracts (430, 430D, and 430D-F5) as well as DMSO were added to wells at 256 µg/mL and serial diluted to 0.125 µg/mL. Plates were incubated for 24 hours at 37°C with 5% CO_2. After 24 hours, 20 µL of lysis buffer was added to each well and the plate was incubated for 45 minutes. Plates were centrifuged at 1500 rpm for 4 minutes. Following centrifugation, 50 µL of supernatant from each well was transferred to a new 96 well plate and incubated for 20 minutes while being protected from light. After incubation, 50 µL of Stop Solution was added to all wells. The plate reader was then used to record absorbance at 490 nm.

Mass spectrometry of 430D-F5

Liquid chromatography-Fourier transform mass spectrometry (LC-FTMS) was performed on bioactive fractions using a Thermo Scientific LTQ-FT Ultra MS equipped with a Shimadzu SIL-ACHT and Dionex 3600SD HPLC pump. For chromatography an Agilent Eclipse XDB-C18 4.6 x 250 mm, 5 µm analytical column, with guard column, was used at room temperature. Samples were prepared as previously described and a 50 µL injection was eluted at a flow rate of 1.9 mL min^{-1} using mobile phases of (A) 0.1% formic acid in H_2O; (B) 0.1% formic acid in ACN. The linear gradient had initial conditions of 95:5 A:B at 0 min and held until 3 min, 68:32 A:B at 23 min, 40:55 A:B at 40 min, 0:100 A:B at 75 min and held for 9 min before returning to initial conditions for a 9 min flush. Data was acquired in MS^1 mode scanning from a *m/z* of 150 – 1500 in negative and positive ESI (electrospray ionization) mode and processed with Thermo Scientific Xcalibur 2.2 SP1.48 (San Jose, CA). The capillary temperature was 275.0°C, sheath gas of 40, source voltage 5.00 kV, source current 100.0 µA, and the capillary voltage -19.0 V or

+32.0 V for negative and positive modes, respectively. Putative formulas were determined by performing isotope abundance analysis on the high-resolution mass spectral data with Xcaliber software and reporting the best fitting empirical formula.

Statistical analysis

All assays performed were analyzed using a two-tailed Student's *t*-test with unequal variance as calculated by Microsoft Excel 2010. DMSO treated (vehicle control) cultures were used as a vehicle control and were compared to those treated with extract for all statistical analyses. P-values less than 0.05 were considered statistically significant. All graph error bars shown represent standard deviation. All assays and other experiments were performed in triplicate or quadruplicate.

CHAPTER 4: RESULTS

Extraction and Separation

A series of bioassays was utilized to determine which extract to pursue based on quorum quenching activity and non-biocide activity. Figure 4.1 shows the fractionation scheme of extract 430 (*S. terebinthifolius*) fruits. Percent yield from initial dry plant material is listed under each extract. Of the leaf (429) and fruit (430) extracts, 430 produced greater yield (28.46%), while 429 yielded less product (16.95%). 430D-F5 had a yield of 5.79% from fried plant material, the second highest yield of all of the fractions and the highest yield of the active fractions (430D-F3, F4, and F5)

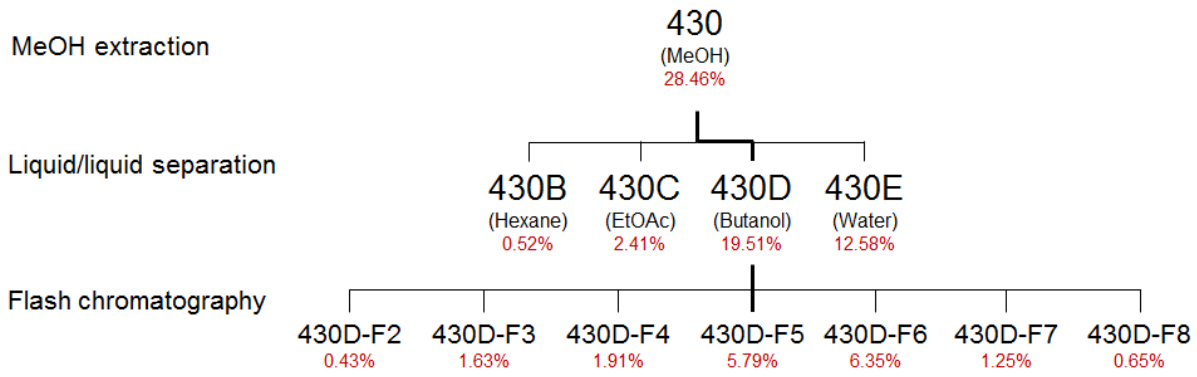


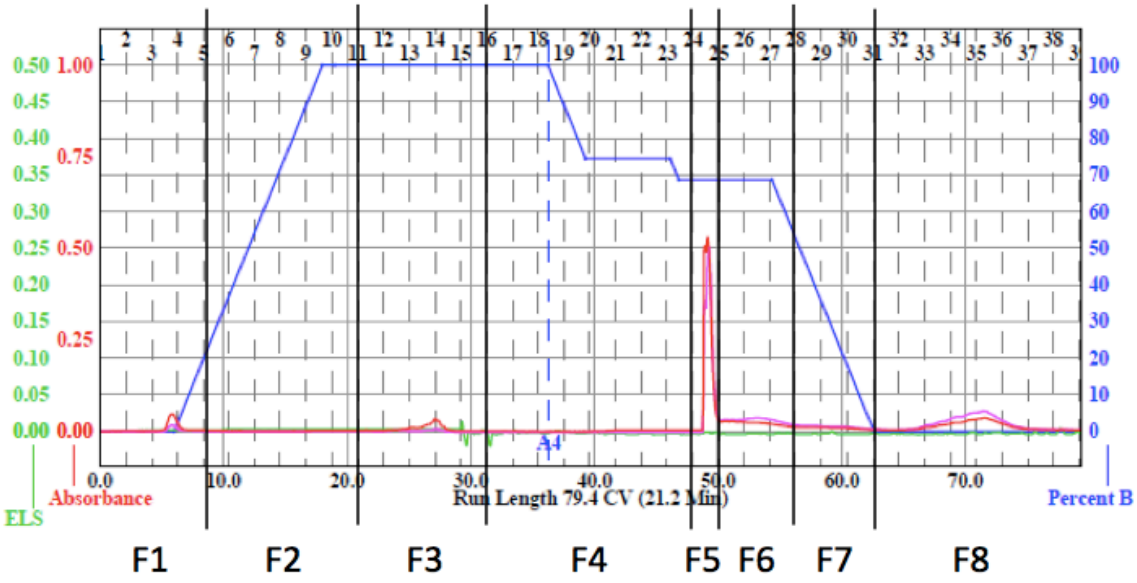
Figure 4.1. 430 Fractionation scheme. Percent yield from dry plant material is shown under each extract and fraction.

Chromatography and Fractionation

The most active 430 partition, 430D was separated into fractions with the use of flash chromatography. Figure 4.2.A shows the flash chromatogram and indicates fractions produced. Pink and red peaks represent different constituents that eluted throughout the flash run. HPLC analysis of the most active partition (430D) and fractions (430D-F3, 430D-F4, and 430D-F5)

revealed common peaks, as can be seen in Figure 4.2.B. Compared to 430D, 430D-F3 showed a larger series of peaks from 55 to 60 minutes, while 430D-F5 showed larger peaks at 46 minutes and 66 minutes.

A.



B

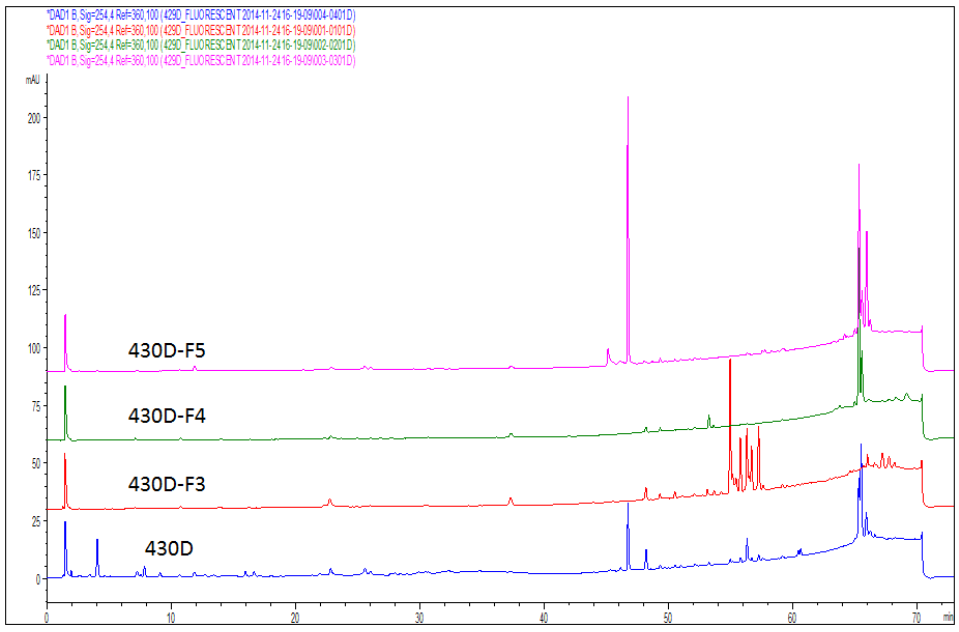


Figure 4.2. (A) Fractions generated through flash chromatography of 430D. Fractions are separated by lines and labeled below the horizontal axis. **(B)** Overlaid HPLC Chromatograms of 430D and the three most active fractions (F3, F4, F5) at 254 nm

Growth inhibition (MICs)

MIC tests suggest that extract 430 and its fractions display little to no growth inhibition of all four strains of *S. aureus* tested (Table 4.1). 430D-F5, which showed the most *agr* inhibition, had a MIC50 of 128 µg/mL for AH430 and 256 µg/mL for AH1872, while MIC90s were not detected in the concentration range tested (0.25-512 µg/mL). For all extracts tested, concentrations associated with *agr* inhibition are well below concentrations necessary for growth inhibition.

	AH1677 (agr I)		AH430 (agr II)		AH1747 (agr III)		AH1872 (agr IV)	
	IC50	IC90	IC50	IC90	IC50	IC90	IC50	IC90
430	-	-	512	-	-	-	-	-
430D	-	-	-	-	-	-	-	-
430D-F3	512	512	256	512	256	512	512	512
430D-F4	-	-	-	-	-	-	-	-
430D-F5	-	-	128	-	-	-	256	-
Amp	-	-	0.0625	0.25	-	-	2	16
Van	0.5	1	1	1	8	8	8	8

"-" indicates that the MIC was not detected at extract concentrations ranging from 0.25-512 µg/mL. All concentrations in the table are reported in µg/mL.

Table 4.1. Growth inhibition of *S. aureus* by fruit extracts. Values were calculated as percent inhibition of vehicle (DMSO) control growth.

***Agr* reporter assay**

Extracts were tested for *agr* inhibitory (quorum quenching) activity against *S. aureus* strains representing all four *agr* allelic groups. Initially *agr* reporter assays were performed to test quorum-quenching ability of leaf extract and its partitions (429 and 429B-E) and fruit extract and its partitions (430 and 430B-E). Each was tested at a low, middle, and high concentration (values listed in Table 4.2). These concentrations were chosen based on MICs to ensure that the *agr* reporter assays were performed at sub growth inhibitory concentrations. Data were presented

as percent inhibition of *agr* activity of the untreated growth control (Figure 4.3). Partitions showing the greatest amount of *agr* inhibition are 429B, 430B, and 430D. The parent extracts, 429 and 430, also show quorum quenching activity in all four strains. The hexane (B) partitions of both the 429 (leaf) and 430 (fruit) extracts showed inhibitory activity, while inhibitory activity of the butanol (D) partition was only seen in 430. Extracts 430, 430B, and 430D showed considerable activity across all four strains. Activity of 429 and 429B was seen in all strains except AH1872 (*agr* IV). As with the initial MIC tests, minimal growth inhibition was observed.

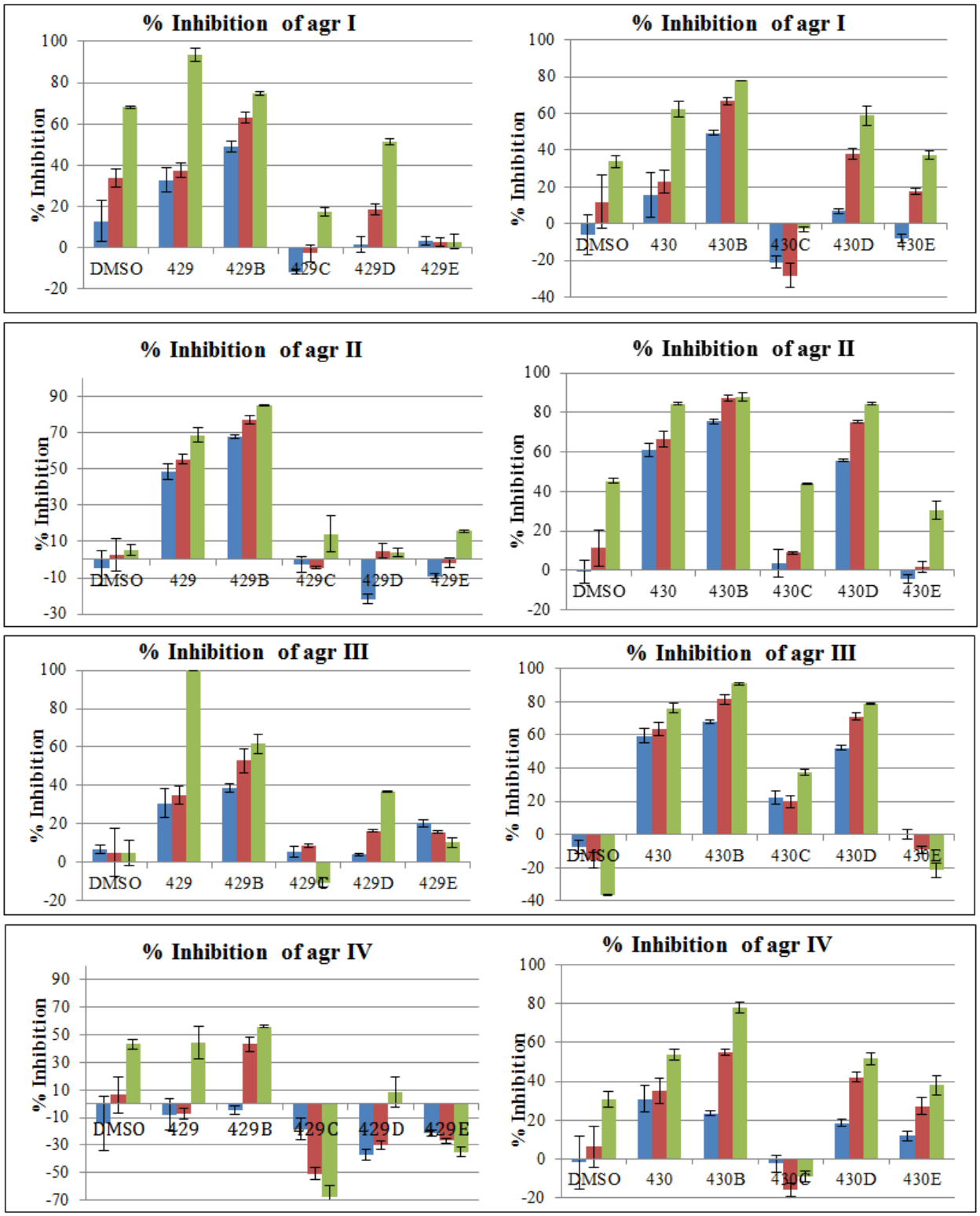


Figure 4.3. Fluorescent reporter strains were used to identify fractions that could inhibit the *agr* system in *S. aureus*. Data are presented as percent inhibition of *agr* activity in the untreated growth control. Each reporter strain was treated with a low (blue), middle (red), and high (green) concentration. Extract concentrations are listed in Table 4.2.

	AH1677 (agr I)			AH430 (agr II)			AH1747 (agr III)			AH1872 (agr IV)		
	low	mid	high	low	mid	high	low	mid	high	low	mid	high
429	32	64	128	16	32	64	32	64	128	32	64	128
429B	32	64	128	32	64	128	32	64	128	32	64	128
429C	2	4	8	1	2	4	1	2	4	1	2	4
429D	8	16	32	4	8	16	8	16	32	8	16	32
429E	32	64	128	32	64	128	32	64	128	32	64	128
430	16	32	64	16	32	64	16	32	64	16	32	64
430B	32	64	128	32	64	128	32	64	128	32	64	128
430C	1	2	4	1	2	4	1	2	4	1	2	4
430D	16	32	64	16	32	64	16	32	64	16	32	64
430E	32	64	128	32	64	128	32	64	128	32	64	128

Table 4.2. *Agr* reporter strains were treated with a low, middle, and high concentration of each extract. These concentrations were chosen based on the growth MIC values for each extract to ensure that each was being tested at sub-growth inhibitory concentrations.

Following the first *agr* reporter assay, 430D was pursued as the preferred partition. While 430B (hexane partition) also demonstrated significant quorum quenching activity, the nature of the hexane partition (oily/resinous in chemical property), as well as low yield, make it substantially more difficult to manipulate and study in the lab. Thus, the active crystalline 430D (butanol partition) was selected for further analysis. After fractionation using flash chromatography, the *agr* fluorescent reporter assay was again used to identify the fractions with the greatest quorum quenching ability. IC_{50} and IC_{90} values for each extract fraction are reported in Table 4.3. Extracts 430 and 430D were tested and continued to show considerable *agr* inhibition. Of the 430D fractions, 430D-F3, 430D-F4, and 430D-F5 appeared to exhibit the greatest quorum quenching activity when tested at concentrations ranging from 2-256 μg/mL. Extracts 430, 430D, 430D-F3, 430D-F4, and 430D-F5 had low IC_{50} values ranging from 2-8 μg/mL for *agr* types I, II, and III. These extracts showed higher IC_{50} values for *agr* type IV, ranging from 32-128 μg/mL. For *agr* type IV, 430D-F5 showed the lowest IC_{50} of 32 μg/mL. Growth was measured simultaneously with fluorescence using an OD_{600} read. Of the fractions tested, only 430D-F8 showed greater than 50% growth inhibition for all strains. Apart from

430D-F8, the only other growth inhibition detected that was greater than 50% was in *agr* type III treated with 256 µg/mL of 430D.

	agr I (AH1677)				agr II (AH430)				agr III (AH1747)				agr IV (AH1872)			
	agr activity		growth		agr activity		growth		agr activity		growth		agr activity		growth	
	IC50	IC90	IC50	IC90	IC50	IC90	IC50	IC90	IC50	IC90	IC50	IC90	IC50	IC90	IC50	IC90
430	2	-	-	-	8	-	-	-	2	128	-	-	128	-	-	-
430D	4	-	-	-	4	-	-	-	2	64	256	-	128	-	-	-
430D-F2	4	-	-	-	-	-	-	-	2	-	-	-	-	-	-	-
430D-F3	2	128	-	-	8	64	-	-	2	128	-	-	128	-	-	-
430D-F4	2	-	-	-	4	-	-	-	4	-	-	-	64	-	-	-
430D-F5	2	-	-	-	4	-	-	-	4	128	-	-	32	-	-	-
430D-F6	4	-	-	-	64	-	-	-	128	-	-	-	-	-	-	-
430D-F7	4	-	-	-	-	-	-	-	128	-	-	-	-	-	-	-
430D-F8	256	-	128	-	32	-	256	-	16	128	32	256	16	-	32	-

Table 4.3. Fluorescent *agr* reporter strains were used to identify fractions of 430D (the butanol partition of *S. terebinthifolius* fruits) that exhibit quorum quenching activity. This table summarizes concentrations of each extract needed to inhibit 50% and 90% of *agr* activity of DMSO treated controls after incubation for 24 hrs. "-" indicates that the IC_{50} or IC_{90} were not detected at concentrations ranging from 2-256 µg/mL. All concentrations in the table are reported in µg/mL.

Figure 4.4 depicts growth and *agr* activity of the most active fractions presented as percent inhibition of OD_{600} or fluorescence of DMSO control treated cells. 430, 430D, 430D-F3, 430D-F4, and 430D-F5 all showed considerable quorum quenching activity with minimal to no growth inhibition. Less quorum quenching of *agr* IV was observed compared to other *agr* types, while similar levels of quorum quenching activity was observed for *agr* groups I, II, and III.

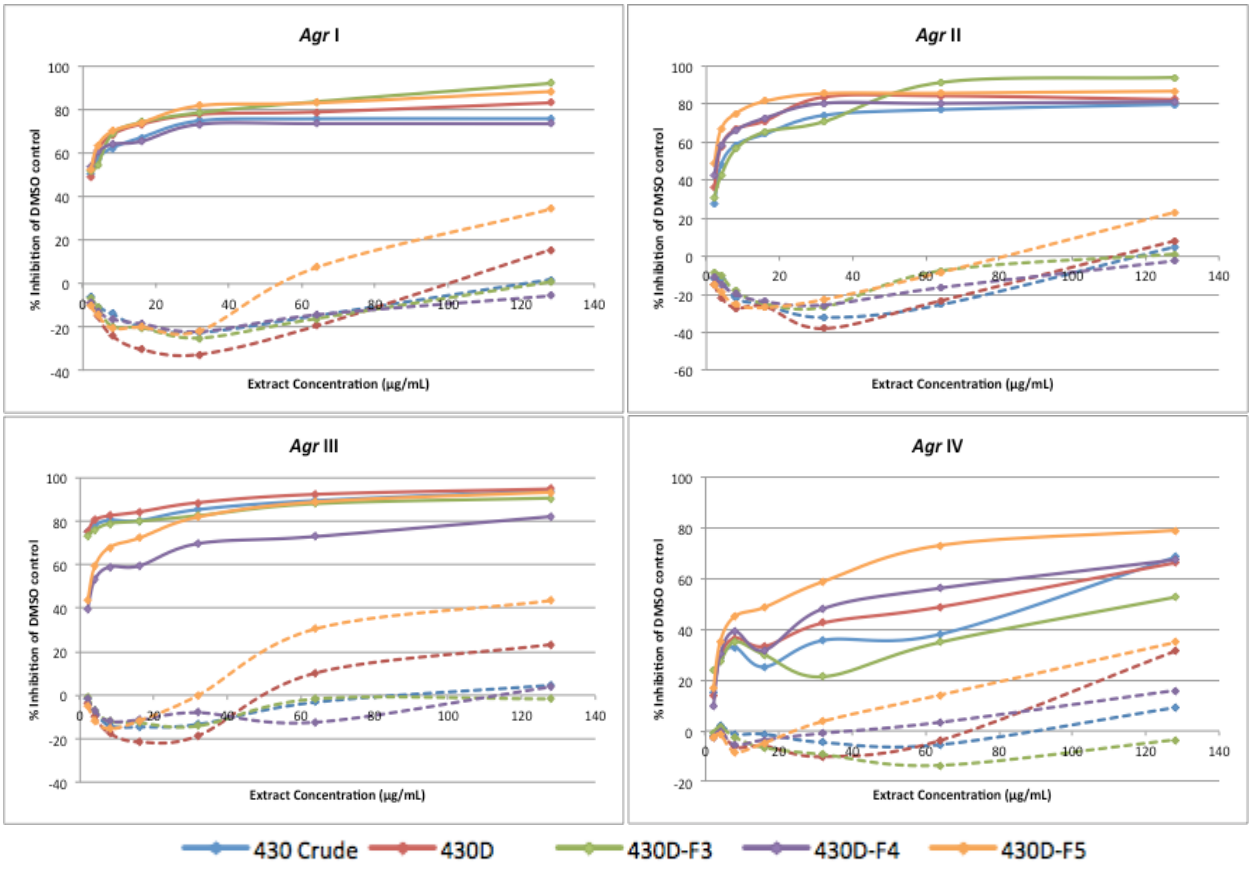
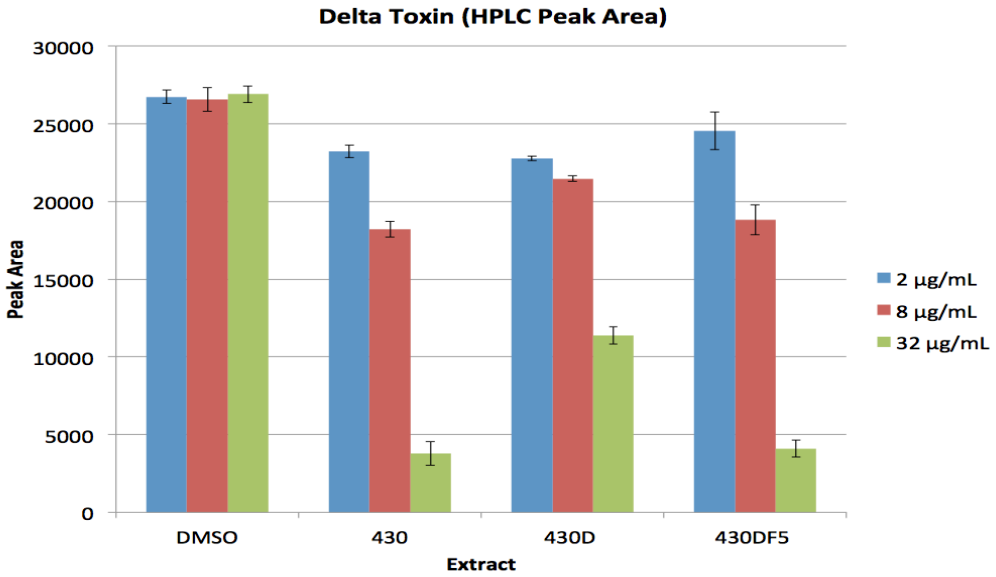


Figure 4.4. Fluorescent reporter strains were used to identify fractions of 430D that exhibit quorum quenching activity. Data are represented as percent inhibition of *agr* activity or growth of the vehicle (DMSO) control at 24 hours. The solid lines represent *agr* inhibition, measured by fluorescence, and the dashed line represents growth inhibition, measured using OD_{600}.

δ-toxin assay

HPLC was used to quantify δ-toxin present in the bacterial supernatant of cells treated with 430, 430D, and 430D-F5 as well as a DMSO control (Figure 4.5). Peak area was calculated by combining the peak areas for the two forms of δ-toxin present (de-formylated and formylated). Figure 4.6 shows an overlay of the δ-toxin chromatogram of untreated supernatant (control) and samples of 430D-F5 treated bacterial supernatants at the three concentrations tested. The peak represents the amount of de-formylated and formylated δ-toxin present in the supernatant. At a concentration of 32 µg/mL, all extracts tested showed considerable inhibition of δ-toxin

production, with 430D showing slightly less inhibitory activity. All results were statistically significant ($p < 0.05$).



* All p-values were <0.001 except 430DF5 (p = 0.029).

Figure 4.5. HPLC was used to quantify δ-toxin present in the bacterial supernatant of cells treated with 430, 430D, and 430D-F5 as well as a DMSO control through calculation of HPLC peak area.

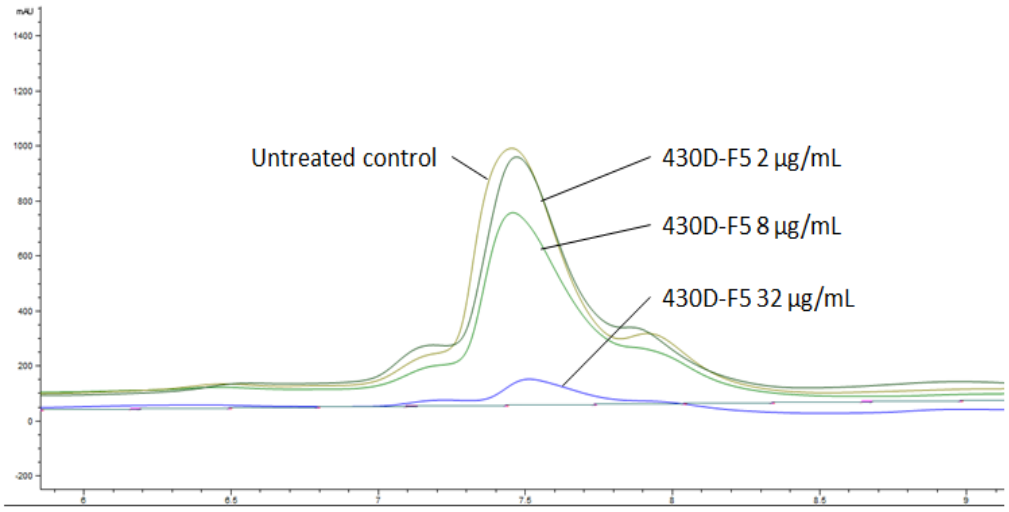
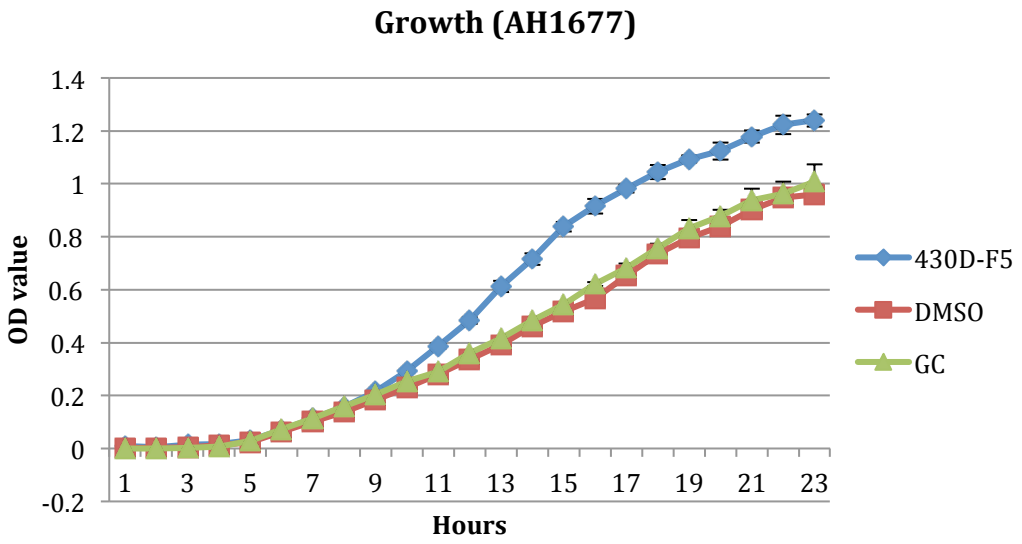
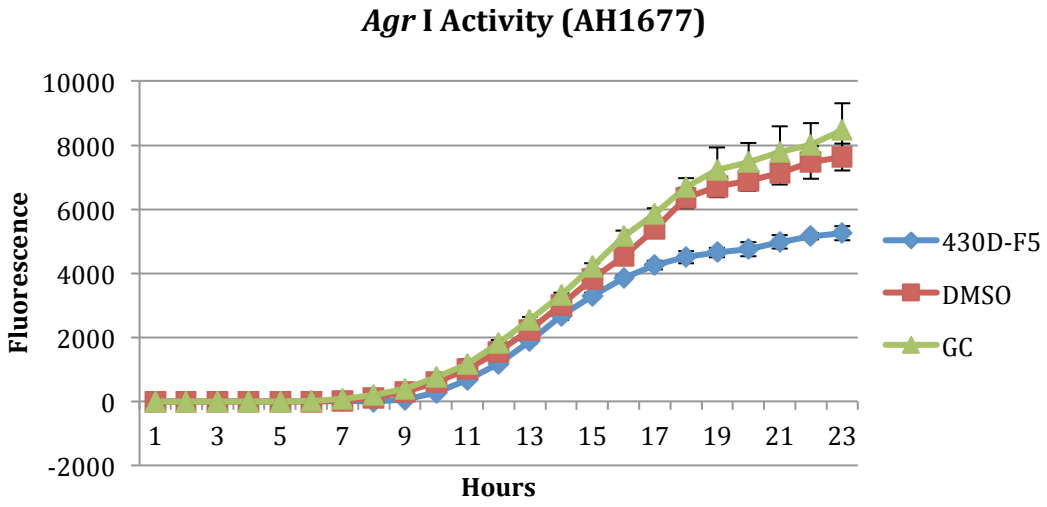


Figure 4.6. HPLC chromatogram of δ-toxin present in 430D-F5 treated bacterial supernatant.

Growth curve analysis

Growth and fluorescence were measured hourly over the course of 23 hours for untreated AH1677 cultures as well as those treated with DMSO and 32 µg/mL 430D-F5 (Figure 4.7). Cultures treated with 430D-F5 exhibited increased growth, but decreased fluorescence compared to the DMSO and untreated controls. The DMSO and untreated controls exhibited similar amounts of growth and fluorescence.





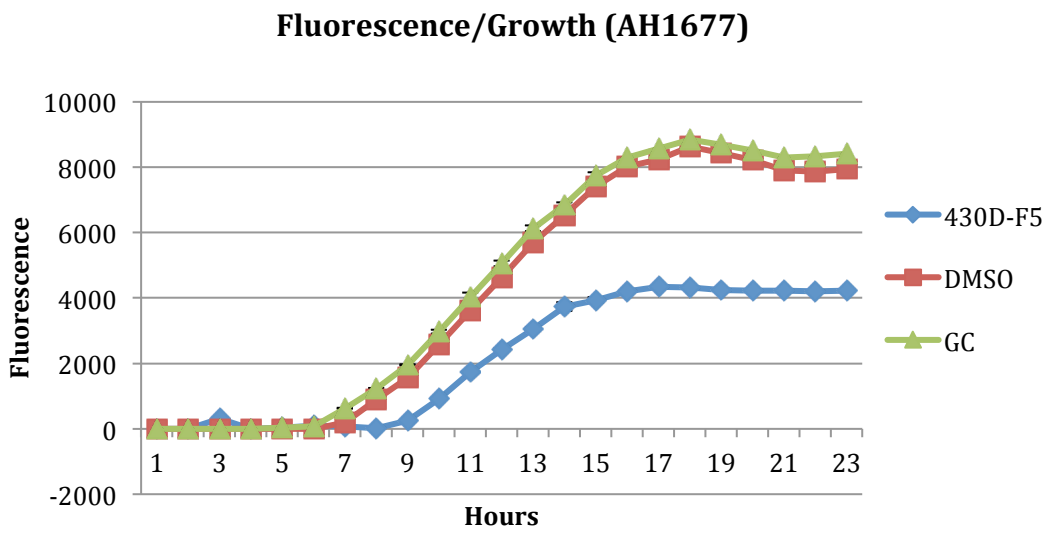


Figure 4.7. Growth and fluorescence of AH1677 (*agr* I reporter strain) over time. Data in the third graph is represented as fluorescence divided by growth in order to account for culture density.

HaCat cytotoxicity assay

All extracts tested showed minimal to no cytotoxicity at concentrations capable of quorum quenching activity of all four *agr* types. Cytotoxicity of greater than 50% was only observed for extracts 430D and 430D-F5. In addition, the DMSO vehicle control resulted in minimal to no cell death.

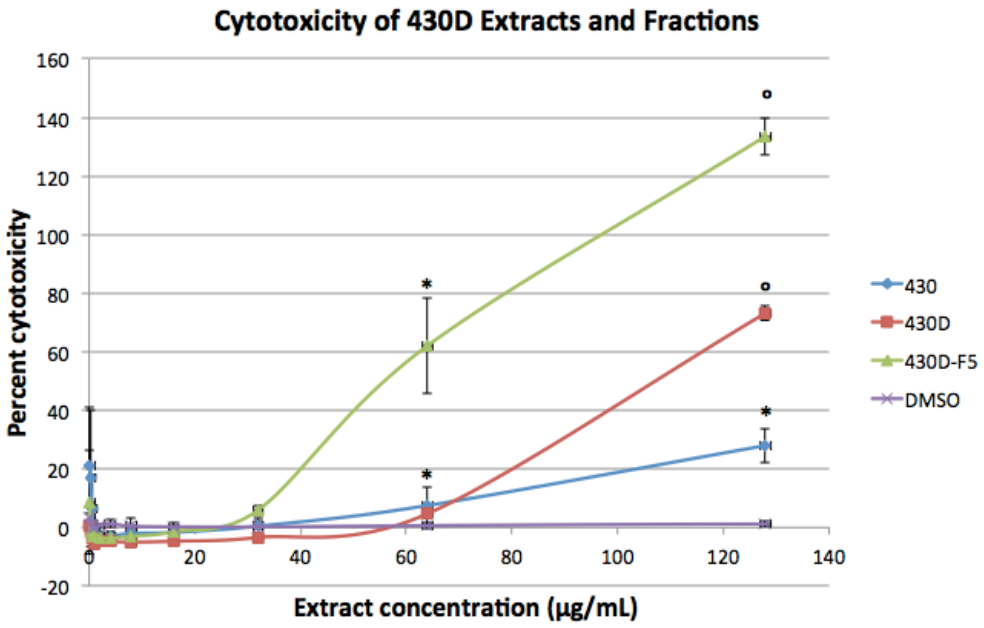


Figure 4.8 HaCat cytotoxicity assay. The cytotoxicity of each extract is shown as a percentage of maximum cytotoxicity as determined by the positive control treated with a lysing agent. ($p < 0.05$: *, $p<0.001$: °)

Mass spectrometry of 430D-F5

Extract 430D-F5 was examined using Liquid chromatography-Fourier transform mass spectrometry. This process revealed 47 possible compounds present in 430D-F5. Table 4.4 lists chromatography retention times and putative molecular formulas of these constituents.

RT (min)	Formula	RT (min)	Formula	RT (min)	Formula	RT (min)	Formula	RT (min)	Formula	RT (min)	Formula
29.16	$C_{30}H_{19}O_{10}$	47.62	$C_{30}H_{46}O_3$	53.65	$C_{29}H_{60}O_{13}N_{20}$	57.95	$C_{20}H_{47}O_9N_6$	66.47	$C_{38}H_{52}O$	71.67	$C_{44}H_{62}O$
29.22	$C_{30}H_{17}O_{10}$	47.92	$C_{30}H_{48}O_{15}N_2$	54.25	$C_{111}H_{47}O_{14}N_{20}$	59.03	$C_{18}H_45O_8N_9$	66.91	$C_{44}H_{62}O$	72.28	$C_{56}H_{95}O_8N$
32.69	$C_{30}H_{23}O_{10}$ $C_{27}H_{27}O_{12}$	48.66	$C_{23}H_{50}O_8$	54.27	$C_{30}H_{45}O_4$ $C_{27}H_{49}O_6$	59.66	$C_{59}H_{48}O_5$	67.77	$C_{30}H_{45}O_3$	72.28	$C_{30}H_{45}O_3$
32.75	$C_{17}H_{19}O_{12}N_9$	49.85	$C_{30}H_{48}O_{15}N_2$	54.65	$C_{26}H_{47}O_8N$	59.88	$C_{20}H_{47}O_{10}N_6$	67.89	$C_{44}H_{62}O$	72.72	$C_{56}H_{95}O_8N$
35.96	$C_{30}H_{45}O_4$ $C_{31}H_{49}O_3$	50.22	$C_{30}H_{46}O_3$	54.65	$C_{27}H_{33}O_4$	60.4	$C_{30}H_{44}O_2$	68.83	$C_{44}H_{62}O$	72.78	$C_{30}H_{45}O_3$
36.02	$C_{30}H_{45}O_5$	50.6	$C_{27}H_{47}O_{13}N$	56.46	$C_{29}H_{29}ON_2$	60.8	$C_{30}H_{45}O_4$ $C_{27}H_{49}O_6$	69.67	$C_{44}H_{62}O$	73.18	$C_{30}H_{47}O_3$ $C_{31}H_{51}O_2$
46.23	$C_{16}H_{19}O_{10}$	51.31	$C_{30}H_{45}O_4$ $C_{27}H_{49}O_6$	57.76	$C_{17}H_{43}O_8N_9$	61.14	$C_{30}H_{44}O_2$	70.03	$C_{44}H_{62}O$	73.55	$C_{44}H_{62}O$
47.06	$C_{23}H_{50}O_7$	52.63	$C_{30}H_{57}O_{15}N_3$	57.91	$C_{57}H_{98}O_{11}$	61.81	$C_{34}H_{59}O_9N$	70.75	$C_{44}H_{62}O$		

Table 4.4 Probable molecular formulas of 430D-F5 constituents as determined by mass spectrometry

CHAPTER 5: DISCUSSION

Quorum quenching activity of *Schinus terebinthifolius* in Methicillin resistant *S. aureus*

Based on experimental data, it is likely that there is at least one bioactive compound present in the fruit of *Schinus terebinthifolius* that is capable of inhibiting quorum sensing in methicillin resistant *Staphylococcus aureus* without inhibiting growth. These quorum quenching compounds could be used as an alternative to antibiotics or an adjuvant therapy in combination with antibiotics to improve treatment outcomes.

Initially, an MIC assay was performed for crude extracts of *S. terebinthifolius* leaves and fruits as well as extract partitions prepared using liquid/liquid separations. This initial MIC was performed in order to determine concentrations of each extract that caused little to no growth inhibition of the *S. aureus agr* reporter strains. The same process of MIC testing was employed to determine sub-inhibitory concentrations at which to test 430D fractions in the second round of *agr* reporter assays (Table 4.1). It was necessary to test for *agr* inhibition at sub-growth inhibitory concentrations for two reasons. First, if a given extract inhibited growth of *S. aureus,* it would result in a decrease in fluorescence. This decrease in fluorescence would make it appear that the *agr* system was being inhibited when in reality it was not, thus yielding a false positive result. Second, it is not a goal of this study to identify compounds that inhibit bacterial growth or kill microorganisms. Rather, it is the goal to identify compounds that inhibit the *agr* pathway, which is not essential for growth in *S. aureus*.

Fluorescent reporter assays were performed to test for inhibition of the *agr* system. Four fluorescent reporter strains, representing the four *agr* groups, were treated with each extract at a range of concentrations. Each strain contained a YFP (yellow fluorescent protein) reporter associated with the *agr* system. Fluorescence, measured using a plate reader, corresponded to *agr*

activity. When crude extracts of leaves (429) and fruits (430) as well as extract partitions were tested at sub-growth inhibitory concentrations, multiple extracts caused inhibition of the *agr* system with minimal impact on growth. Both crude extracts of 429 (leaves) and 430 (fruits) showed quorum quenching activity. 429 inhibited *agr* groups I, II, and III, while 430 appeared to show some inhibition of all four *agr* groups (Figure 4.3). Of the 429 partitions, 429B, the active hexane partition, showed the most quorum quenching activity across all four *agr* strains tested. 429D showed limited inhibition of *agr* I and *agr* III. This data suggests that compounds in the leaves (429) with the most quorum quenching activity are likely nonpolar molecules present in the hexane partition. Of the 430 partitons, both 430B, the hexane partition, and 430D, the butanol partition showed considerable quorum quenching activity of all four *agr* groups. The most quorum quenching activity, roughly 80% inhibition, was observed in the *agr* II and *agr* III strains. Similar levels of inhibition were observed when these strains were treated with 430, 430B, and 430D. Data from this series of assays suggests that quorum quenching compounds found in the fruits are likely nonpolar molecules, found in the hexane partition, or fairly polar molecules, found in the butanol partition. We chose to pursue 430D for further chemical and microbiological analysis rather than 429B due to 430D's high level of quorum quenching activity, high yield from dried plant material, and ease in further chemical characterization. 429B, the lipophilic partition of the leaves, poses a challenge for chemical analysis due to its high levels of fats and waxes.

Based on data gathered from the first set of *agr* reporter assays, 430D (the butanol partition of fruits) was chosen as the partition to pursue in further bioassays. Flash chromatography was used to produce seven fractions of 430D. Initially, eight fractions were produced, but upon drying it was discovered that 430D-F1 contained no product. Following

determination of growth MICs (Table 4.1), a second series of *agr* reporter assays were performed. Quorum quenching activity continued to be seen in 430 and 430D. It was necessary to test the crude extract and the butanol partition to ensure that bioactivity was not lost throughout the bioassay guided fractionation process. Of the fractions tested, 430D-F3, 430D-F4, and 430D-F5 appeared to have the most quorum quenching activity (Table 4.3). It is common for neighboring fractions, such as these, to demonstrate similar activity as they may contain some of the same or similar compounds. 430D-F3, F4, and F5 all showed considerable quorum quenching activity while having little to no impact on growth (Figure 4.4). All three fractions had very low IC_{50} concentrations (the concentration needed for 50% inhibition of the *agr* system compared to the DMSO control) for *agr* groups I, II, and III (Table 4.3). Although at least 50% *agr* inhibiton was observed in all four *agr* groups, much higher concentrations of all three extracts were needed for the same effect in the *agr* IV strain. Due to its high levels of *agr* inhibition across all four strains and ability to be produced in fairly high yield through flash chromatography, 430D-F5 was further investigated through a series of bioassays (Table 4.1).

A δ-toxin assay was performed to further confirm inhibition of the *agr* pathway. δ-toxin, also known as δ-hemolysin, is a toxin produced by the *agr* system. For this reason, a decrease in δ-toxin production would further confirm *agr* inhibition at the level of translation. When combined with data gathered from the reporter assays that show inhibition of *agr* at the transcription level, inhibition of translational inhibition supports the quorum quenching activity of 430D-F5. Extracts 430, 430D, and 430D-F5 were tested in order to track bioactivity through the separation and fractionation process. All three extracts showed inhibition of δ-toxin production compared to the DMSO control (Figure 4.5). Extract 430D showed less quorum quenching activity than 430 and 430D-F5 at the highest concentration tested. This slight loss of

bioactivity in the 430D partition may be the result of the removal of other bioactive compounds in the crude extract, such as bioactive compounds found in 430B (hexane partition). Quorum quenching activity increased in 430D-F5, because it contains a higher concentration of bioactive compounds found in 430D.

Next, a growth assay was performed in an effort to determine any effect that 430D-F5 may have on *S. aureus* growth over time and confirm that the extract is a true quorum quencher. The experiment was modeled after the design of the δ-toxin assay with cultures at a 10:1 flask:volume ratio. While the growth curves and *agr* activity of DMSO treated and growth control cultures appeared almost identical over time, the cultures treated with 430D-F5 appeared to grow more quickly (Figure 4.7). This may be a consequence of inhibiting the *agr* system, which plays an important role in sensing of bacterial density. Without the ability to determine cell density through quorum sensing and production of AIPs by the *agr* system, it may take higher cell density and a longer period of time to transition from exponential into stationary growth. An uninhibited growth curve also indicates that *agr* inhibition observed in the reporter assays and delta toxin assay is the result of quorum quenching activity of 430D-F5 rather than just delayed growth.

Following the bioassay guided fractionation process and identification of the most active fractions, a cytotoxicity assay was performed to determine if fractions that caused quorum quenching in *S. aureus* were harmful to human cells. Human keratinocytes were utilized in this assay as they are the primary cell type that forms the epidermis. It is important to investigate any possible damage that the extracts may cause to skin cells, as skin and soft tissue infections caused by *S. aureus* are the focus of this study. The three extracts tested (430, 430D, and 430D-F5) showed little to no cytotoxicity at concentrations associated with quorum quenching activity

in all four *agr* groups (Figure 4.8). Cytotoxicity seemed to increase throughout the fractionation process with 430D-F5 showing the most cytotoxicity, followed by 430D, and 430, which showed minimal cytotoxicity. Minimal damage to skin cells seen in this assay is consistent with the widespread use of *S. terebinthifolius* as a topical treatment of skin and soft tissue infections. Although cytotoxicity seems to increase throughout the fractionation process, this does not mean that the compounds responsible for quorum-quenching are also cytotoxic. Through mass spectroscopy, 430D-F5 was found to contain 47 possible compounds (Table 4.4). Different compounds may be responsible for quorum quenching and cytotoxicity. In order to determine which compound or compounds are responsible for each, it is necessary to continue with the process of bioassay guided fractionation and cytotoxicity testing. With more information about the role of different compounds, it may be possible to enhance quorum quenching activity while decreasing cytotoxic effects.

Quenching without killing: a unique approach to drug development

This study began with the goal of investigating the anti-infective properties of *Schinus terebinthifolius,* a traditional medicine used in treatment of SSTIs and wounds. Specifically, we investigated bioactive compounds in *Schinus terebinthifolius* that are able to inhibit the *agr* system in *Staphylococcus aureus* without killing or inhibiting growth. The *agr* system was chosen as a target, because it is necessary for virulence and quorum sensing but not growth. As the global regulator of virulence in *S. aureus,* the *agr* system is responsible for initial infection of host cells, evasion of the host immune system, quorum sensing, and the destruction of tissues with toxins and other enzymes (Novick, 2003). Through inhibition of the *agr* system with the use of small molecule inhibitors isolated from natural products, *S. aureus* virulence could be

decreased, thus limiting the severity of disease. With a weakened pathogen, it may be possible for the host immune system to fight the *S. aureus* infection with minimal to no use of antimicrobial therapies.

By inhibiting virulence without inhibiting growth, thus limiting direct selective pressure, it may be possible to lessen the risk of developing drug resistance. In a similar study focusing on the identification of small molecule inhibitors of the *agr* system, no resistance was observed after prolonged treatment of multiple generations of *S. aureus* both *in vivo* and *in vitro*. When *S. aureus* was treated with antibiotics for the same number of generations, development of resistance was noted (Sully et al., 2014). This finding lends support to the use of small molecule *agr* inhibitors as an alternative or adjuvant to conventional antibiotics.

The specificity of *agr* inhibitors would also reduce the negative effects of treatment on other host bacteria that may play an important role in the microbiome but do not encode the *agr* system. Although inhibition of the *agr* pathway would not result in growth inhibition, lack of quorum sensing ability may result in a delayed or attenuated infection that could ultimately be cleared by the host's immune system (Quave, Plano, & Bennett, 2011).

Although a quorum quenching approach comes with the possibility of limiting drug resistance and decreasing negative side effects that are associated with antibiotics, the approach may have some limitations. For example, ideally the quorum quenching approach would rely on the host immune system to clear an *S. aureus* infection that has been weakened by a small molecule *agr* inhibitor. This approach may not be successful in immunocompromised individuals. If the infection cannot be cleared and begins to negatively impact the health of the patient, it may be necessary to supplement therapy with antibiotics. Most likely, small molecule

agr inhibitors will not be broadly effective as a stand-alone therapy, rather they will find most utility as antibiotic adjuvants used in co-therapy.

With an increase in the development of antimicrobial resistance, it has become common practice for multi-drug therapy to be used in the treatment of a variety of infectious diseases. Bacteria can develop resistance through three main mechanisms. These include modifying the active site of the drug target in the cell, producing enzymes that can deactivate the antibiotic, and development of efflux pumps that remove the antimicrobial agent from the bacteria (Hemaiswarya, Kruthiventi, & Doble, 2008). The use of multiple drugs with different targets results in more effective therapies through targeting a variety of disease processes and a decreased risk of drug resistance (Wagner, 2011).

Synergistic chemistry: treating infectious diseases with natural products

Synergism is defined as multiple drugs having greater effect when used together than they would when used alone (Williamson, 2001). In other words, the effect of a combination of compounds is greater than the sum of its parts. Positive effects of such synergism can manifest as an increase in therapeutic effect of a given drug or a decrease in negative side effects (Williamson, 2001). Natural botanical products are of particular interest in the investigation of synergism. The synergism of plant secondary metabolites can be seen in botanical antibiotic defense mechanisms. Plants are able to combat a variety of infections in their natural environment even though individual secondary metabolites often have limited potency (Hemaiswarya et al., 2008). Synergism in plants has been utilized for centuries in traditional medicine. As stated by Williamson, “this concept, that a whole or partially purified extract of a plant offers advantages over a single isolated ingredient, underpins the philosophy of herbal

medicine" (Williamson, 2001). The benefits of synergistic activity in natural products can be achieved through uses of whole or partially purified plants or plant extracts, a combination of different plants, and more recently, a combination of plant compounds and modern pharmaceuticals, such as antibiotics. Some mechanisms believed to be responsible for synergistic effects include improvement in solubility and bioavailability of active constituents, ability to attack multiple targets, a decrease in negative side effects, and targeting of resistance mechanisms (Wagner, 2011).

It is likely that synergy plays a role in the bioactivity of *Schinus terebinthifolius.* This can be observed throughout the bioassay guided fractionation process and is a possible explanation for the loss of activity at different stages of separation and fractionation (Williamson, 2001). Along these lines, synergy may be responsible for the loss and subsequent regaining of quorum quenching in the δ-toxin assay (Figure 4.5). In the assay, activity appears to be lost in the purification of 430 to 430D (p-value = 7.5×10^{-6}) and regained in the 430D-F5 fraction. Some activity may have been lost in 430D due to the elimination of other bioactive compounds, such as those that were present in the 430B partition, which previously showed quorum quenching activity in initial *agr* reporter assays (Figure 4.3). Activity likely increased in the 430D-F5 fraction due to an increase in the concentration of bioactive compounds still present in 430D, as can be seen in the overlay of the 430D and 430D-F5 HPLC chromatograms (Figure 4.2). Although synergy can be viewed as an explanation for loss of bioactivity in the process of natural products drug discovery, it may not fully explain the phenomenon. Other factors that may be at play include the presence of unstable bioactive compounds or elimination or decrease in the concentration of other quorum quenching compounds (Junio et al., 2011; Williamson, 2001). The difficulty in determining the effects of possible synergism between bioactive compounds in

natural products is a limitation of bioassay guided fractionation (Junio et al., 2011). However, with the development of new methods such as checkerboard assays, which allow for the testing of compounds together at a range of concentrations, the healthcare industry will be able to gain a greater understanding of the synergistic effects of different therapeutics (Hemaiswarya et al., 2008).

Today's drug development research must find a compromise between the "silver bullet" approach of modern pharmacology and the "herbal shotgun" approach of traditional medicine (Duke & Bodenschutz-Godwin, 1999). In the past, much of allopathic medicine has looked down upon herbal medicine in favor of a "magic bullet" single compound approach to treating disease. This approach has its benefits, such as easier regulation, more reliable dosages, and ease of creating controlled and consistant laboratory studies and clinical trials (Junio et al., 2011). However, with increased research into synergy and the common practice of multi-drug therapy, the "shotgun" approach of some herbal remedies has been taken more seriously as an alternative to monotherapy. Although critics of herbal medicine sometimes argue that the concentration of active compounds in whole or partially purified plant extracts are too low to have valid clinical effects, recent research about synergistic and cumulative effects of long term treatment with herbal medicines has provided a counter-argument to such criticisms (Williamson, 2001).

In recent years, laboratory and clinical research has added support for the use of natural products in allopathic therapies. Much of the time, secondary metabolites are the main focus of such research (Hemaiswarya et al., 2008). Studies have tested the efficacy of combinations of plant compounds as well as combinations of plant products and synthetic drugs, such as antibiotics. For example, Epigallocatechin gallate, isolated from green tea, is able to work synergistically with existing antibiotics to lower MICs by reducing β-lactamase activity and

weakening the bacterial cell wall (Hemaiswarya et al., 2008). Black pepper (*Piper longum L.*) has also shown synergistic effects by increasing bioavailability of other active constituents. Ayurveda, a system of traditional medicine in India, has taken advantage of this activity through incorporating black pepper into a variety of herbal formulations (Williamson, 2001). Studies such as these help to support the efficacy of herbal medicine as an alternative or compliment to current synthetic drugs used in allopathic medicine.

Embracing natural products as an alternative or compliment to allopathic treatments could improve treatment outcomes and increase global accessibility to healthcare. The synergistic nature of herbal remedies can be used to simultaneously target multiple disease pathways while decreasing the likelihood of development of drug resistance. In addition, embracing natural products as effective cures and encouraging their use can allow for greater access to healthcare globally. As previously stated, limited access to allopathic medicine poses a challenge for over one third of the global population (World Health Organization, 2001). This does not mean however, that these individuals do not have access to some form of healthcare. Many individuals rely on traditional medicine for primary healthcare. In Africa, where access to allopathic medicine is limited, over 80% of individuals rely on traditional medicine as their primary source of medical care (World Health Organization, 2013). In developing countries, where disease burden is high and access to synthetic drugs is limited, medicinal plants can serve as a cheaper, more readily available, and more culturally acceptable alternative. One example, is the investigation into the use of whole leaf tea of *Artemisia annua* as an alternative or compliment to artemisinin, a drug originally isolated from the plant. *Artemisia annua* has been used in Traditional Chinese Medicine (TCM) to treat fevers, now known to be attributed to infectious diseases, such as malaria (Weathers, Towler, Hassanali, Lutgen, & Engeu, 2014).

While artemisinin is expensive and becoming less effective due to the development of drug resistance, use of less expensive and more readily available whole plant leaves seems like a beneficial alternative. *A. annua,* which has considerable bioactivity due to synergism of low concentrations of many active compounds, could be easily grown and prepared by individuals in low resource settings (Weathers et al., 2014). This same concept may also be applied to *Schinus terebinthifolius* due to its wide ranging bioactivity and ability to grow quickly in a variety of environments.

Data from this study suggest that compounds in *S. terebinthifolius* have the ability to inhibit quorum quenching in methicillin resistant *S. aureus.* Although the butanol partition of the fruits have been pursued as the most active quorum quencher based on bioassay guided fractionation, this does not mean that other parts of the plant lack anti-infective activity. All parts of the plant have been used in traditional medicine in Brazil for a variety of ailments including arthritis, bleeding, and, of greatest interest to this study, wound care and infection control. Results lend support to the traditional use of *S. terebinthifolius* fruits to treat wounds and ulcers in which infection control played a likely role (M. Bolson et al., 2014; Martius, 1854). Although not pursued further in this study, leaf extracts 429 and 429B also showed some quorum quenching activity (Figure 4.3). This data also provides support for the traditional use of leaves to treat infections including respiratory infections, urinary tract infections, wounds, and ulcers (Braga et al., 2007; de Lima et al., 2006; Gomes et al., 2013; Martius, 1854; Moreira, 1862). Although it cannot be determined for certain if the leaves and fruits were traditionally used to treat *S. aureus* infections, many of the maladies described, such as skin infections, urinary tract infections, and respiratory infections have been associated with *S. aureus* (Lowy, 1998). In addition, cytotoxicity assays show that the bioactive fraction has minimal negative effects on

human keratinocytes, a major component of the epidermis. This low toxicity supports the use of the plant as a safe topical treatment for skin and soft tissue infections and is consistent with reported traditional uses.

Future directions

Future directions of this study may include, but are not limited to, further chemical analysis and isolation of active constituents identified throughout the bioassay guided fractionation process, resistance studies, further safety and cytotoxicity testing, and studies of efficacy in animal models. Specific active compounds will be identified through the continued process of bioassay guided fractionation and flash chromatography of 430D-F5. As active constituents become more apparent, we will conduct resistance studies similar to those performed by Sully et al (Sully et al., 2014). Efficacy and safety in animal models will also be assessed to gather more information about activity as well as any possible negative side effects or toxicity in animals. If the compounds isolated from *S. terebinthifolius* fruits prove to be a safe and effective adjuvant therapy for MRSA infections, other plant parts used in traditional medicine, such as bark and stems, will also be tested through a similar process of bioassay guided fractionation.

Conclusion

Low cytotoxicity as well as considerable quorum quenching activity at low doses both corroborate the traditional medical uses of *Schinus terebinthifolius* to treat infections and provide support for further investigation of the plant as an alternative treatment or adjuvant therapy for invasive methicillin resistant *S. aureus* infections. Further research will need to be performed to

identify the chemical constituents responsible for observed quorum quenching activity. With the decreased effectiveness of some commonly used pharmaceuticals, in particular antimicrobial agents, it is necessary to identify and develop alternative or adjuvant therapies. With increased knowledge of chemical synergy and bioactivity of natural products, plants are increasingly being investigated as leads for new drugs. The efficacy of natural products has been demonstrated in traditional medical practices, where they have been used for centuries to safely and effectively treat a variety of ailments. Through a combination of ethnobotanical, biological, and chemical knowledge, we can develop more effective therapies that can be successfully utilized in allopathic medicine.

References

Alves, L. A., Freires Ide, A., de Souza, T. M., & de Castro, R. D. (2012). In vitro activity of Schinus terebinthifolius (Brazilian pepper tree) on Candida tropicalis growth and cell wall formation. *Acta Odontol Latinoam, 25*(3), 287-292.

Barbieri, D. S., Tonial, F., Lopez, P. V., Sales Maia, B. H., Santos, G. D., Ribas, M. O., . . . Vicente, V. A. (2014). Antiadherent activity of Schinus terebinthifolius and Croton urucurana extracts on in vitro biofilm formation of Candida albicans and Streptococcus mutans. *Arch Oral Biol, 59*(9), 887-896. doi: 10.1016/j.archoralbio.2014.05.006

Bendaoud, H., Romdhane, M., Souchard, J. P., Cazaux, S., & Bouajila, J. (2010). Chemical composition and anticancer and antioxidant activities of Schinus molle L. and Schinus terebinthifolius Raddi berries essential oils. *J Food Sci, 75*(6), C466-472. doi: 10.1111/j.1750-3841.2010.01711.x

Berkes, F. (2012). *Sacred Ecology* (3 ed.). New York: Routledge.

Berman, K. (2013). Paronychia. *Medline Plus.* from http://www.nlm.nih.gov/medlineplus/ency/article/001444.htm

Berube, B. J., & Bubeck Wardenburg, J. (2013). Staphylococcus aureus alpha-toxin: nearly a century of intrigue. *Toxins (Basel), 5*(6), 1140-1166.

Biodiversity Heritage Library (2015). http://www.biodiversitylibrary.org/

Boles, B. R., & Horswill, A. R. (2008). Agr-mediated dispersal of Staphylococcus aureus biofilms. *PLoS Pathog, 4*(4), e1000052. doi: 10.1371/journal.ppat.1000052

Bolson, M., Hefler, S., Chaves, E., Gasparotto, A., & Cardozo, E. (2014). Ethno-medicinal study of plants used for treatment of human ailments, with residents of the surrounding region of forest fragments of Parana, Brazil. *Journal of Ethnopharmacology*.

Braga, F. G., Bouzada, M. L., Fabri, R. L., de, O. M. M., Moreira, F. O., Scio, E., & Coimbra, E. S. (2007). Antileishmanial and antifungal activity of plants used in traditional medicine in Brazil. *J Ethnopharmacol, 111*(2), 396-402. doi: 10.1016/j.jep.2006.12.006

Brandao, M. G., Zanetti, N. N., Oliveira, P., Grael, C. F., Santos, A. C., & Monte-Mor, R. L. (2008). Brazilian medicinal plants described by 19th century European naturalists and in the Official Pharmacopoeia. *J Ethnopharmacol, 120*(2), 141-148. doi: 10.1016/j.jep.2008.08.004

Burton, R. (1868). *Viagens aos planaltos do Brasil - Tomo I: Do Rio de Janeiro a Morro Velho*.

Carlini, E. A., Duarte-Almeida, J. M., & Tabach, R. (2013). Assessment of the toxicity of the Brazilian pepper trees Schinus terebinthifolius Raddi (Aroeira-da-praia) and Myracrodruon urundeuva Allemao (Aroeira-do-sertao). *Phytother Res, 27*(5), 692-698. doi: 10.1002/ptr.4767

Carvalho, M., Melo, A., Aragao, C., Raffin, F., & Moura, T. (2013). Schinus terebinthifolius Raddi: chemical composition, biological properties and toxicity. *Revista Brasileira de Plantas Medicinais, 15*(1).

Centers for Disease Control and Prevention. (2013a). Antibiotic Resistance Threats in the United States. Atlanta.

Centers for Disease Control and Prevention. (2013b). MRSA Tracking. from http://www.cdc.gov/mrsa/tracking/

Chernoviz, P. L. N. (1864). *Formulario ou Guia Medica* (6 ed.). Paris.

Cosgrove, S. E. (2006). The Relationship between Antimicrobial Resistance and Patient Outcomes: Mortality, Length of Hospital Stay, and Health Care Costs. *Clinical Infectious Diseases, 42*.

Cseke, L., Kirakosyan, A., Kaufman, P., Warber, S., Duke, J., & Brielmann, H. (2006). *Natural Products from Plants* (2 ed.). Florida: Taylor & Francis.

Da Costa, J. C. (1903). *Modern Surgery* (4 ed.).

de Lima, M. R., de Souza Luna, J., dos Santos, A. F., de Andrade, M. C., Sant'Ana, A. E., Genet, J. P., . . . Moreau, N. (2006). Anti-bacterial activity of some Brazilian medicinal plants. *J Ethnopharmacol, 105*(1-2), 137-147. doi: 10.1016/j.jep.2005.10.026

dos Santos, O. J., Barros-Filho, A. K., Malafaia, O., Ribas-Filho, J. M., Santos, R. H., & Santos, R. A. (2012). Schinus terebinthifolius Raddi (Anacardiaceae) in the healing process of gastrorraphy in rats. *Arq Bras Cir Dig, 25*(3), 140-146.

Duke, J. A., & Bodenschutz-Godwin, M. J. (1999). The synergy principle in plants, pathogens, insects, herbivores, and humans. In P. B. Kaufmann, L. Cseke, S. Warber, J. A. Duke & H. L. Brielmann (Eds.), *Natural Products from Plants* (pp. 183-205). New York: CRC Press.

Edwards-Jones, V. (2013). Alternative Antimicrobial Approaches to Fighting Multidrug-Resistant Infections. In M. Rai & K. Kon (Eds.), *Fighting Multidrug Resistance with Herbal Extracts, Essential Oils, and Their Components* (pp. 1-9). San Diego: Elsevier.

El-Massry, K. F., El-Ghorab, A. H., Shaaban, H. A., & Shibamoto, T. (2009). Chemical compositions and antioxidant/antimicrobial activities of various samples prepared from Schinus terebinthifolius leaves cultivated in Egypt. *J Agric Food Chem, 57*(12), 5265-5270. doi: 10.1021/jf900638c

Estevao, L. R., Mendonca Fde, S., Baratella-Evencio, L., Simoes, R. S., Barros, M. E., Arantes, R. M., . . . Evencio-Neto, J. (2013). Effects of aroeira (Schinus terebinthifoliu Raddi) oil on cutaneous wound healing in rats. *Acta Cir Bras, 28*(3), 202-209.

Fedel-Miyasato, L. E., Formagio, A. S., Auharek, S. A., Kassuya, C. A., Navarro, S. D., Cunha-Laura, A. L., . . . Oliveira, R. J. (2014). Antigenotoxic and antimutagenic effects of Schinus terebinthifolius Raddi in Allium cepa and Swiss mice: a comparative study. *Genet Mol Res, 13*(2), 3411-3425. doi: 10.4238/2014.April.30.2

Feuereisen, M. M., Hoppe, J., Zimmermann, B. F., Weber, F., Schulze-Kaysers, N., & Schieber, A. (2014). Characterization of Phenolic Compounds in Brazilian Pepper (Schinus terebinthifolius Raddi) Exocarp. *J Agric Food Chem*. doi: 10.1021/jf500977d

Geiger, J., Pratt, P., Wheeler, G., & Williams, D. (2011). Hybrid Vigor for the Invasive Exotic Brazilian Peppertree (Schinus terebinthifolius Raddi., Anacardiaceae) in Florida. *International Journal of Plant Sciences, 172*(5), 655-663.

Gomes, F. S., Procopio, T. F., Napoleao, T. H., Coelho, L. C., & Paiva, P. M. (2013). Antimicrobial lectin from Schinus terebinthifolius leaf. *J Appl Microbiol, 114*(3), 672-679. doi: 10.1111/jam.12086

Gundidza, M., Gweru, N., Magwa, M., Mmbengwa, V., & Samie, A. (2009). The chemical composition and biological activities of essential oil from the fresh leaves of Schinus terebinthifolius from Zimbabwe. *African Journal od Biotechnology, 8*(24), 7164-7169.

Gurib-Fakim, A. (2006). Medicinal plants: traditions of yesterday and drugs of tomorrow. *Mol Aspects Med, 27*(1), 1-93. doi: 10.1016/j.mam.2005.07.008

Harborne, J. (1993). *Introduction to Ecological Biochemistry*. London: Elsevier.

Hemaiswarya, S., Kruthiventi, A. K., & Doble, M. (2008). Synergism between natural products and antibiotics against infectious diseases. *Phytomedicine, 15*.

Houghton, P. (2001). Old Yet New - Pharmaceuticals from Plants. *Journal of Chemical Education, 78*(2).

Internet Archive. (2015). https://archive.org/index.php

Jain, M. K., Yu, B. Z., Rogers, J. M., Smith, A. E., Boger, E. T., Ostrander, R. L., & Rheingold, A. L. (1995). Specific competitive inhibitor of secreted phospholipase A2 from berries of Schinus terebinthifolius. *Phytochemistry, 39*(3), 537-547.

Jarraud, S., Mougel, C., Thioulouse, J., Lina, G., Meugnier, H., Forey, F., . . . Vandenesch, F. (2002). Relationships between Staphylococcus aureus genetic background, virulence factors, agr groups (alleles), and human disease. *Infect Immun, 70*(2), 631-641.

Jennings, J., & Bowser, B. (2008). *Drink, Power, and Society in the Andes*. Gainesville: University Press of Florida.

Junio, H. A., Sy-Cordero, A. A., Ettefagh, K. A., Burns, J. T., Micko, K. T., Graf, T. N., . . . Cech, N. B. (2011). Synergy directed fractionation of botanical medicines: A case study with Goldenseal (Hydrastis canadensis). *Journal of Natural Products, 74*(7).

Kramer, F. (1957). The Pepper Tree, Schinus Molle L. *Economic Botany, 11*(4), 322-326.

Lipinski, L. C., Wouk, A. F., da Silva, N. L., Perotto, D., & Ollhoff, R. D. (2012). Effects of 3 topical plant extracts on wound healing in beef cattle. *Afr J Tradit Complement Altern Med, 9*(4), 542-547.

Lowy, F. D. (1998). Staphylococcus aureus infections. *N Engl J Med, 339*(8), 520-532. doi: 10.1056/NEJM199808203390806

Martius, K. F. P. v. (1854). *Systema de Materia Medica Vegetal Brasileira*. Rio de Janeiro.

Mayville, P., Ji, G., Beavis, R., Yang, H., Goger, M., Novick, R. P., & Muir, T. W. (1999). Structure-activity analysis of synthetic autoinducing thiolactone peptides from Staphylococcus aureus responsible for virulence. *Proc Natl Acad Sci U S A, 96*(4), 1218-1223.

McDonald, L. C. (2006). Trends in Antimicrobial Resistance in Health Care Associated Pathogens and Effect on Treatment. *Clinical Infectious Diseases, 42*.

Melo, M. C., Gadelha, D. N., Oliveira, T. K., & Brandt, C. T. (2014). Alcohol extract of Schinu sterebinthifolius raddi (anacardiaceae) as a local antimicrobial agent in severe autogenously fecal peritonitis in rats. *Acta Cir Bras, 29 Suppl 1*, 52-56.

Moreira, N. (1862). *Diccionario de Plantas Medicinaes Brasileiras*. Rio de Janeiro.

Morton, J. F. (1978). Brazilian Pepper - Its Impact on People, Animals and the Environment. *Economic Botany, 32*(4), 353-359. doi: Doi 10.1007/Bf02907927

Moura-Costa, G. F., Nocchi, S. R., Ceole, L. F., de Mello, J. C., Nakamura, C. V., Dias Filho, B. P., . . . Ueda-Nakamura, T. (2012). Antimicrobial activity of plants used as medicinals on an indigenous reserve in Rio das Cobras, Parana, Brazil. *J Ethnopharmacol, 143*(2), 631-638. doi: 10.1016/j.jep.2012.07.016

Novick, R. P. (2003). Autoinduction and signal transduction in the regulation of staphylococcal virulence. *Molecular Microbiology, 48*(6), 1429-1449.

Otto, M., & Gotz, F. (2000). Analysis of quorum sensing activity in staphylococci by RP-HPLC of staphylococcal delta-toxin. *BioTechniques, 28*(6), 1088-1096.

Otto, M., Sussmuth, R., Vuong, C., Jung, G., & Gotz, F. (1999). Inhibition of virulence factor expression in Staphylococcus aureus by the Staphylococcus epidermidis agr pheromone and derivatives. *FEBS Lett, 450*(3), 257-262.

Panetta, F., & McKee, J. (1997). Recruitment of the invasive ornamental, Schinus terebinthifolius, is dependent upon frugivores. *Australian Journal of Ecology, 22*, 432-438.

Pang, Y. Y., Schwartz, J., Thoendel, M., Ackermann, L. W., Horswill, A. R., & Nauseef, W. M. (2010). agr-Dependent interactions of Staphylococcus aureus USA300 with human polymorphonuclear neutrophils. *J Innate Immun, 2*(6), 546-559. doi: 10.1159/000319855

Pereira, E. M., Gomes, R. T., Freire, N. R., Aguiar, E. G., Brandao, M., & Santos, V. R. (2011). In vitro antimicrobial activity of Brazilian medicinal plant extracts against pathogenic microorganisms of interest to dentistry. *Planta Med, 77*(4), 401-404. doi: 10.1055/s-0030-1250354

Piso, W. (1648). *Historia naturalis Brasiliae : auspicio et beneficio illustriss*. Lugdun. Batavorum: Apud Franciscum Hackium, et Amstelodami, apud Lud. Elzevirium.

Quave, & Horswill. (2014). Flipping the switch: tools for detecting small molecule inhibitors of staphylococcal virulence. *Frontiers in microbiology, 6*.

Quave, & Pieroni. (2015). A reservoir of ethnobotanical knolwedge informs resilient food security and health strategies in the Balkans. *Nature Plants, 1*.

Quave, Plano, & Bennett. (2011). Quorum sensing inhibitors of Staphylococcus aureus from Italian medicinal plants. *Planta Med, 77*(2), 188-195. doi: 10.1055/s-0030-1250145

Schlossberg, D. (2008). *Clinical Infectious Disease*. New York: Cambridge University Press.

Schmourlo, G., Mendonca-Filho, R. R., Alviano, C. S., & Costa, S. S. (2005). Screening of antifungal agents using ethanol precipitation and bioautography of medicinal and food plants. *J Ethnopharmacol, 96*(3), 563-568. doi: 10.1016/j.jep.2004.10.007

Spaulding, A. R., Salgado-Pabon, W., Kohler, P. L., Horswill, A. R., Leung, D. Y., & Schlievert, P. M. (2013). Staphylococcal and streptococcal superantigen exotoxins. *Clin Microbiol Rev, 26*(3), 422-447. doi: 10.1128/CMR.00104-12

Sully, E. K., Malachowa, N., Elmore, B. O., Alexander, S. M., Femling, J. K., Gray, B. M., . . . Gresham, H. D. (2014). Selective Chemical Inhibition of agr Quorum Sensing in Staphylococcus aureus Promotes Host Defense with Minimal Impact on Resistance. *PLoS Pathog, 10*(6).

Surridge, C. (2015). Plant science's human factor. *Nature Plants, 1*.

Vieira, D. R., Amaral, F. M., Maciel, M. C., Nascimento, F. R., Liberio, S. A., & Rodrigues, V. P. (2014). Plant species used in dental diseases: Ethnopharmacology aspects and antimicrobial activity evaluation. *J Ethnopharmacol*. doi: 10.1016/j.jep.2014.07.021

Vivekananda, J., Salgado, C., & Millenbaugh, N. J. (2014). DNA aptamers as a novel approach to neutralize Staphylococcus aureus alpha-toxin. *Biochem Biophys Res Commun, 444*(3), 433-438. doi: 10.1016/j.bbrc.2014.01.076

Vuong, C., Saenz, H. L., Gotz, F., & Otto, M. (2000). Impact of the agr quorum-sensing system on adherence to polystyrene in Staphylococcus aureus. *J Infect Dis, 182*(6), 1688-1693. doi: 10.1086/317606

Wagner, H. (2011). Synergy research: Approaching a new generation of phytopharmaceuticals. *Fitoterapia, 82*.

Weathers, P. J., Towler, M., Hassanali, A., Lutgen, P., & Engeu, P. O. (2014). Dried-leaf Artemisia annua: A practical malaria therapeutic for developing countries. *World Journal of Pharmacology, 3*(4), 39-55.

Williams, D. A., Muchugu, E., Overholt, W. A., & Cuda, J. P. (2007). Colonization patterns of the invasive Brazilian peppertree, Schinus terebinthifolius, in Florida. *Heredity (Edinb), 98*(5), 284-293. doi: 10.1038/sj.hdy.6800936

Williamson, E. M. (2001). Synergy and other interactions in phytomedicines. *Phytomedicine, 8*(5), 401-409.

World Health Organization. (2001). Legal Status of Traditional Medicine and Complementary/Alternative Medicine: A Worldwide Review.

World Health Organization. (2003). WHO guidelines on good agricultural and collection practices (GACP) for medicinal plants. Geneva.

World Health Organization. (2013). WHO Traditional Medicine Strategy 2014-2023.

World Health Organization. (2014a). Antimicrobial Resistance: Global Report on Surveillance. Geneva.

World Health Organization. (2014b). Antimicrobial Resistane. from http://www.who.int/mediacentre/factsheets/fs194/en/

Wunderlin, R. P., & Hansen, B. F. (2003). *Guide to the Vascular Plants of Florida*: University Press of Florida.