Distribution Agreement

In presenting this thesis as a partial fulfillment of the requirements for a degree from Emory University, I hereby grant to Emory University and its agents the non-exclusive license to archive, make accessible, and display my thesis in whole or in part in all forms of media, now or hereafter now, including display on the World Wide Web. I understand that I may select some access restrictions as part of the online submission of this thesis. I retain all ownership rights to the copyright of the thesis. I also retain the right to use in future works (such as articles or books) all or part of this thesis.

Micah Dettweiler April 12, 2017

Inhibition of multidrug-resistant bacteria by Civil War plant medicines

by

Micah Dettweiler

Dr. Cassandra Quave
Adviser

Biology

Dr. Cassandra Quave

Adviser

Dr. Jacobus de Roode

Committee Member

Dr. Jorge E. Vidal

Committee Member

2017

Inhibition of multidrug-resistant bacteria by Civil War plant medicines

By

Micah Dettweiler

Dr. Cassandra Quave

Adviser

An abstract of
a thesis submitted to the Faculty of Emory College of Arts and Sciences
of Emory University in partial fulfillment
of the requirements of the degree of
Bachelor of Sciences with Honors

Biology

2017

Abstract

Inhibition of multidrug-resistant bacteria by Civil War plant medicines
By Micah Dettweiler

A shortage of conventional medicine during the American Civil War (1861-1865) led Confederate physicians to use preparations of native plants as medicines. Francis Porcher, a southern botanist, compiled in 1863 a book of native medicinal plants, including plants used in Native American traditional medicine. In this project, Porcher's book was consulted. Samples from three plant species which he used for the formulation of antiseptics, *Liriodendron tulipifera*, *Aralia spinosa*, and *Quercus alba*, were collected in Lullwater Preserve, Atlanta, Georgia. Chemical extracts of these plant samples were tested for the ability to inhibit growth, biofilm production, and quorum sensing in three species of multidrug-resistant pathogenic bacteria.

Bulk plant specimens were dried and ground to a powder. Chemical extraction for each was performed via sonication in methanol, then each extract was either partitioned or fractionated by column chromatograph to create 19 samples. Samples were dissolved in DMSO (10 mg/mL) and tested at concentrations ranging from 2-256 µg/mL for growth inhibition and biofilm inhibition of *Staphylococcus aureus*, *Klebsiella pneumoniae*, and *Acinetobacter baumannii*. Samples 617B (hexane partition), 619 (bark extract), and 619F2 (tannin fraction) displayed the most growth inhibition of *S. aureus* with $MIC_{90} \leq 256$ µg/mL, and extracts 619, 619F2, and 620 (gall extract) displayed growth inhibition of *K. pneumoniae* and *A. baumannii*. Extracts 616 (leaf extract), 616F1 (non-tannin fraction), 618B (hexane partition), 619F2, 620, and 621 (bark extract) displayed biofilm inhibition of *S. aureus* at sub-MIC_{50} concentrations.

The extracts from the three plants showed activity in growth inhibition, biofilm inhibition, and quorum quenching of drug-resistant bacteria. The data herein suggest that these plant extracts represent promising natural product compositions for antibiotic drug development.

Inhibition of multidrug-resistant bacteria by Civil War plant medicines

By

Micah Dettweiler

Dr. Cassandra Quave

Adviser

A thesis submitted to the Faculty of Emory College of Arts and Sciences
of Emory University in partial fulfillment
of the requirements of the degree of
Bachelor of Sciences with Honors

Biology

2017

Acknowledgements

Dr. Cassandra Quave for advising and mentoring

Dr. E. Jane Bradbury for help identifying and collecting plants

Dr. James Lyles for help with chemistry

Kate Nelson for help with microbiology

Akram Salam and Danielle Cicka for help editing

Thesis committee:

Dr. Jacobus de Roode

Dr. Jorge E. Vidal

Dr. Cassandra Quave

Table of Contents

List of Tables and Figures

Chapter 1: Introduction

This chapter gives background information relevant to this project. The modern problem of antibiotic resistance necessitates a search for new antibiotics and new ways to treat infections. During the American Civil War, plant medicines were used to treat infections; natural products such as these may be sources of new antibiotics.

Antibiotic resistance

Antibiotic resistance in pathogenic microbes poses a significant threat to human health (1); antibiotics are critical not only in treating bacterial diseases but also in enabling surgery and other procedures with high risks of infection (2). Given the great genetic diversity and capacity for evolution present in bacteria, a rise in antibiotic resistance is an inevitable response to antibiotic use (3). In 1940, even before penicillin was widely used, penicillin resistance was observed (4). Any single antibiotic, then, is not a permanent solution but another step in the struggle to survive the bacteria we coexist with.

There are many mechanisms by which antibiotics operate and many mechanisms by which resistance arises and functions. In the example of penicillin, a β-lactam compound disrupts bacterial cell walls, leading to lysis (5). The first observed mechanism of resistance to penicillin was production of β-lactamases; other recorded modes of resistance include intrinsic resistance and penicillin tolerance (5). Most antibiotics follow this general model in that they inhibit an essential bacterial process. Wherever these antibiotics are used, then, there is a strong selective pressure exerted on a bacterial population for the development of resistance.

Several factors complicate the relationship between antibiotics and bacteria. For example, the innate immune system plays a role in fighting infections with or without the use of antibiotics. Further, commensal members of the microbiome may outcompete pathogenic

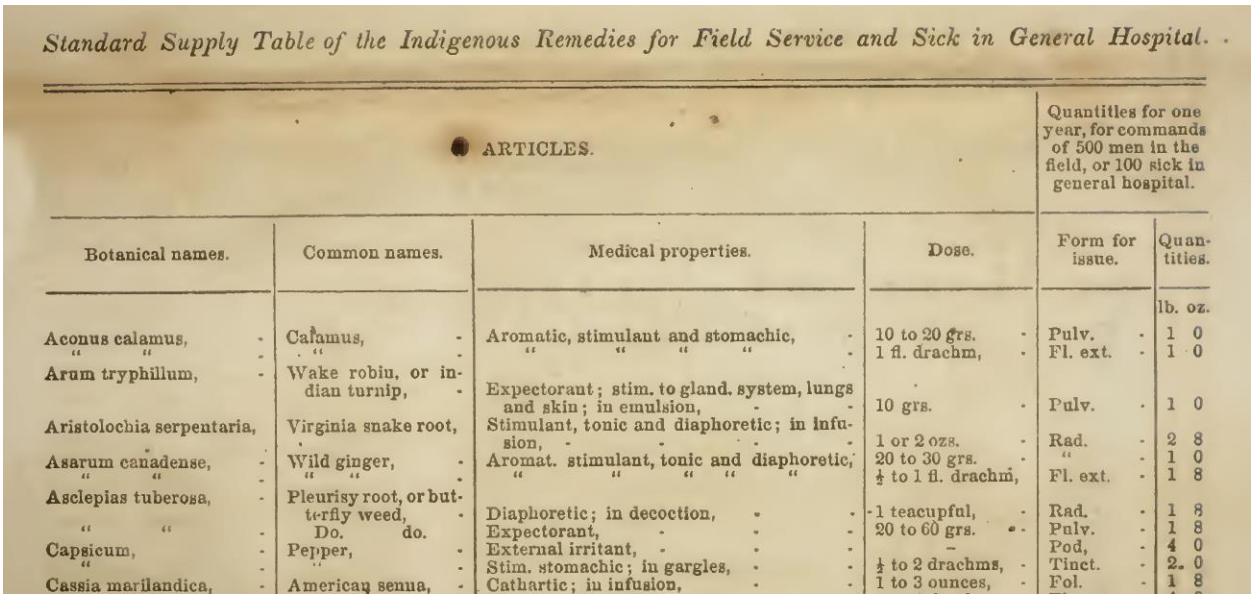
bacteria or may themselves become pathogenic under certain circumstances (6). Relevant to this study, bacterial community effects such as biofilms and quorum sensing produce resistance and virulence not necessarily observed *in vitro* (7, 8). Biofilms are extracellular mixtures of polysaccharides and proteins that can physically protect bacteria populations from antibiotics; biofilms are associated with chronic infections, especially in the case of medical implants (7). Quorum sensing is a system by which toxin production or other pathogenic activity is initiated when extracellular communication indicates achievement of a threshold population of bacteria. Inhibition of quorum sensing, then, is therapeutic but not bactericidal (8). In the absence of new antibiotics, multidrug-resistant infections may in many cases be treatable by administering biofilm inhibitors or quorum quenchers to increase the vulnerability of bacteria to the immune system or conventional antibiotics.

Historical perspective

The natural product compositions investigated in this study are plant extracts used during the American Civil War, a period of history in which infections were treated without the use of modern antibiotics. The accepted definition of antiseptic was “tonic useful to prevent external or internal mortification” (9). During the war, a Union blockade (10) (Fig. 1.1) prevented the Confederacy from importing sufficient amounts of conventional medicines such as quinine, morphine, and chloroform (11).

Figure 1.1. Contemporary cartoon of Union blockade (12)

Francis Porcher, a Confederate botanist, was commissioned to find and catalog plants native to the southern US that could be used as medicines (9). Porcher compiled a book of his findings, including 37 plant species to be used as antiseptics, treating gangrene and other infections (9). From this research, Samuel Moore, the Confederate Surgeon General, published a field guide of native plant medicines to be used by battlefield physicians (Fig. 1.2), including methods of collection, preparation, and administration (13). Infection was a leading cause of death for soldiers in the Civil War and was often treated with amputation (14). It may be hoped that Porcher's work with natural products saved many lives and limbs.



Standard Supply Table of the Indigenous Remedies for Field Service and Sick in General Hospital.

ARTICLES.				Quantities for one year, for commands of 500 men in the field, or 100 sick in general hospital.	
Botanical names.	Common names.	Medical properties.	Dose.	Form for issue.	Quantities.
					lb. oz.
Aconus calamus,	Calamus,	Aromatic, stimulant and stomachic,	10 to 20 grs.	Pulv.	1 0
" "	" "	" " " "	1 fl. drachm,	Fl. ext.	1 0
Arum tryphillum,	Wake robin, or indian turnip,	Expectorant; stim. to gland. system, lungs and skin; in emulsion,	10 grs.	Pulv.	1 0
Aristolochia serpentaria,	Virginia snake root,	Stimulant, tonic and diaphoretic; in infusion,	1 or 2 ozs.	Rad.	2 8
Asarum canadense,	Wild ginger,	Aromat. stimulant, tonic and diaphoretic,	20 to 30 grs.	"	1 0
" "	" "	" " " " "	½ to 1 fl. drachm,	Fl. ext.	1 8
Asclepias tuberosa,	Pleurisy root, or butterfly weed,	Diaphoretic; in decoction,	1 teacupful,	Rad.	1 8
" "	Do. do.	Expectorant,	20 to 60 grs.	Pulv.	1 8
Capsicum,	Pepper,	External irritant,	–	Pod,	4 0
"	"	Stim. stomachic; in gargles,	½ to 2 drachms,	Tinct.	2 0
Cassia marilandica,	American senna,	Cathartic; in infusion,	1 to 3 ounces,	Fol.	1 8

Figure 1.2. Supply table of plant medicines issued by Confederate surgeon general S. P. Moore (13).

Natural products

Natural products—compounds produced by living organisms—are used directly as medicine by an estimated 4 billion people for whom traditional medicine is a primary healthcare source (15). Approximately 25% of modern drugs are derived from natural products used in traditional medicine (16). Plants in particular produce a large variety of secondary metabolites to interact with their environments, and some of these serve to control local microbes by encouraging or inhibiting bacterial growth and/or function.

Many of the plant species Porcher described as antiseptic have not been tested by modern labs for antibiotic activity, especially adjuvant activity and activity against multidrug-resistant bacteria. The aim of this study, then, is to identify sources of novel chemical entities (NCEs) with antibiotic activity. While the majority of drugs on the market today are synthetic, many are still derived from natural products; a review of new drugs from 1980-2005 found that only one

new FDA-approved NCE, sorafenib, was created through combinatorial chemistry (17). Searching natural products for NCEs may be a more effective tactic, especially when systems of traditional medicine or historical pharmacopoeias are available to use as heuristics.

One benefit of natural product extracts as antibiotic agents over single-compound drugs is that they, containing dozens to thousands of compounds, can have multiple mechanisms of activity, making it more difficult for resistance to develop. For example, *Quercus robur* bark was found to exert its quorum quenching activity via two distinct mechanisms (18).

Project goals

In this study, samples of three species from Porcher's book were selected for investigation based on convenience of collection: *Quercus alba* (Fagaceae), *Aralia spinosa* (Araliaceae), and *Liriodendron tulipifera* (Magnoliaceae). The hypothesis is that, given the historic use of these plants as antiseptics, their extracts may inhibit growth, biofilm production, and/or quorum sensing in pathogenic bacteria. Multidrug-resistant bacteria were used in all experiments to examine the potential use of these plant compounds to combat the effects of antibiotic resistance.

Chapter 2: Literature Review

This chapter gives an overview of literature on the antibiotic-resistant bacteria and plants used in this study.

Bacteria used

The bacteria studied in this project—*S. aureus*, *A. baumannii*, *K. pneumoniae,* and *P. aeruginosa*—are frequently associated with infected wounds and may therefore be among the species targeted by Civil War antiseptics. In our modern context, resistant strains of these bacteria are the cause of difficult or impossible-to-treat infections in hospitals and in combat wounds. Multidrug-resistant strains of each of the bacteria described below were used in this study.

S. aureus is a gram-positive bacterium. It generally grows on the skin or in the respiratory tract and as such can cause respiratory infections and a variety of skin conditions including boils, atopic dermatitis, and scalded skin syndrome. When skin or mucous membranes break, local populations of *S. aureus* can infect the bloodstream; *S. aureus* is in fact among the largest causes of bacteremia in developed countries (19). Methicillin-resistant *S. aureus* (MRSA) was the strain used in this study.

A. baumannii is a gram-negative bacterium generally encountered in nosocomial infections, in wounds, in the blood, and in the respiratory system (20). Many of these infections have been found in soldiers deployed in Iraq and Afghanistan in recent decades (21). Multidrug-resistant infections of *A. baumannii* can have mortality rates as high as 35% (20).

K. pneumoniae is a gram-negative nitrogen-fixing soil bacteria and has shown benefits as an endophyte to agricultural productivity (22). In humans, it is normally found on the skin and in the digestive system (23). *K. pneumoniae* is largely associated with deadly pneumoniae in

immuno-compromised individuals, but it also can infect the urinary tract and wound sites. Carbapenem-resistant *K. pneumoniae* (the strain used in this study) is listed as one of the CDC's three most urgent antibiotic resistant threats (1).

P. aeruginosa is a versatile gram-negative bacterium found in soil and water and on skin. *P. aeruginosa* is frequently found on cockroaches in hospitals and human habitations, leading to speculation that insect agents may aid in the pervasiveness of this bacterium (24). *P. aeruginosa* infections can develop in a variety of tissues and are generally accompanied by inflammation and sepsis. These infections can be deadly if developed in vital organs and in immuno-compromised individuals (25).

Plants studied

The three species used in this study—*Q. alba*, *A. spinosa*, and *L. tulipifera*—were all recognized as medicinal (Table 2.1) and used by American pharmacists in the 19th century (26). Interestingly, a 1947 survey of higher plants found no significant antibacterial activity in both *A. spinosa* and *L. tulipifera* (27).

Species	Plant part	Use	Reference
Q. alba	bark	infusion for tuberculosis	(28)
Q. alba	bark	liniment	(29)
A. spinosa	leaves, stems, and bark (along with *Podophyllum peltatum* rhizomes)	salve for skin cancer	(30)
A. spinosa	root bark	cathartic, diaphoretic, emetic, treatment for rheumatism	(31)
A. spinosa	bark, roots, berries	treatment for boils, toothache, snakebite	(32)
L. tulipifera	bark	treatment for fevers, diarrhea, rheumatism, and snakebites received in dreams	(33)

Table 2.1 Traditional uses of *Q. alba*, *A. spinosa*, and *L. tulipifera*.

Figure 2.1. *Quercus alba* vouchers deposited at Emory University Herbarium

Figure 2.2. *Aralia spinosa* vouchers deposited at Emory University Herbarium

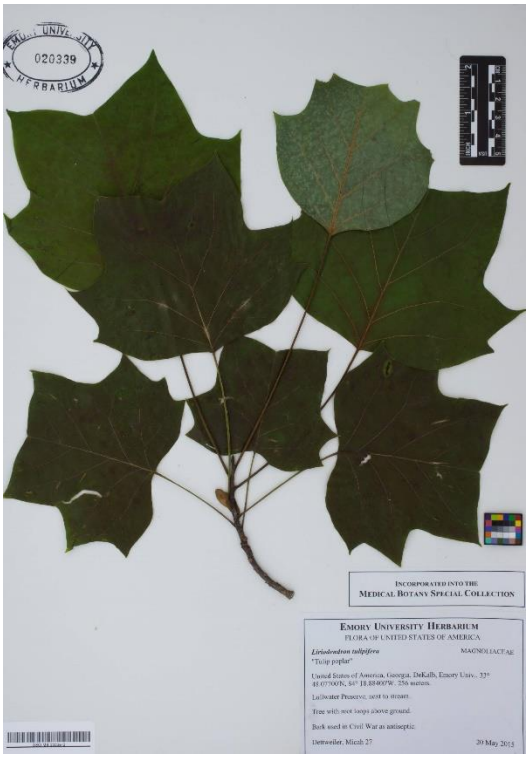


Figure 2.3. *Liriodendron tulipifera* voucher deposited at Emory University Herbarium

Species	Plant part	Activity observed	Reference
Q. alba	bark	antioxidant	(34)
Q. alba	leaves	anthelmintic	(35)
Q. alba	leaves	promote growth of soil gymnamobae	(36)
Q. alba	pollen	antifungal	(37)
Q. robur	bark	antibacterial	(18, 38)
Q. infectoria	galls, seeds	antibacterial and biofilm inhibition	(39–48)
Q. incana	leaves	antioxidant, antifungal, antibacterial	(49)
Q. coccifera	fruit	antibacterial	(50)
Q. cerris	leaves, stems, fruits	biofilm inhibition	(51)
Q. dilatata	aerial parts	antibacterial	(52)
Q. leucotrichophora	bark	antibacterial	(53)
Q. castaneifolia	fruit	antibacterial	(54)
Q. ilex	leaves, bark	antifungal, antibacterial	(55–57)
Q. virginiana	leaves	biofilm and quorum sensing inhibition	(58, 59)
A. elata	whole plant	antioxidant, anti-inflammatory	(60)
A. nudicaulis	rhizomes	antibacterial	(61)
A. cachemirica	aerial parts	antibacterial, anti-inflammatory, biofilm inhibition	(62)
A. continentalis	roots	antibacterial	(63)
L. tulipifera	leaves	antioxidant	(64)
L. tulipifera	bark, root bark, stem, leaves	anti-cancer	(64–71)
L. tulipifera	leaves, heartwood	antimicrobial	(71, 72)

L. tulipifera	leaves, twigs	antifibrotic	(73)
L. tulipifera	bark, leaves	antimalarial	(74)
L. tulipifera* x *chinense	bark	biofilm and quorum sensing inhibition of MRSA	(75)
L. chinense		antibacterial	(76)

Table 2.2 Activity reported in literature of *Quercus* spp., *Aralia* spp., and *Liriodendron* spp.

L. chinense and *A. elata* are both used in Chinese traditional medicine and as such, these species and their relatives (including *L. tulipifera* and *A. spinosa*) have been the subject of several studies for various medicinal characteristics (Table 2.2).

Plant chemistry

Quercus alba (Fig. 2.1)

Much of the research into oak chemistry (*Q. alba* and *Q. robur* specifically) is focused on the use of oak wood as a material for barrels in wine-making (77). *Q. alba* wood contains 3-10% non-cellulosic compounds, including carbohydrates, acids, and phenols (77). Porcher noted that *Q. alba* has lower tannin and gallic acid content than other oak species (9). In wine-making, aromatic oak phenols contribute to taste and ellagitannins produce astringency (77). Ellagitannins have been found to inhibit fungal growth, allowing *Q. alba* heartwood to be decay-resistant (78). Tannins are frequently bioactive, binding to and inhibiting a variety of enzymes (78). Tannins, therefore, are relevant to human health both as anti-nutritive compounds (preventing absorption of nutrients, especially proteins) and as antibacterials. Tannic acid, for example, has been found to inhibit bacterial growth in food and water (79).

Oak galls are formed in response to attacks by gall wasps and typically have high tannin levels (50-70% in *Q. infectoria*), as well as gallic acid and ellagic acid (80).

Aralia spinosa (Fig. 2.2)

A. spinosa leaf and stem extracts contain tannins, alkaloids, and saponins (81). Alkaloids are the active components of many natural products used as medicine, including antimalarials such as quinine and antibiotics such as metronidazole (82). Some saponins are cytotoxic, creating pores in cell membranes, which can lead to lysis in animal cells, but saponins are also reported to stimulate the immune system (83).

Liriodendron tulipifera (Fig. 2.3)

L. tulipifera has been found to contain sesquiterpene lactones, alkaloids, and various sugars (64). Sesquiterpene lactones are present in leaves and flower heads of a variety of plant species and have been known to cause toxic reactions in animals, especially in the gastrointestinal tract (84). The antiplasmodial activity of *L. tulipifera* has been traced to six aporphine alkaloids present in the bark and two sesquiterpene lactones present in the leaves (74).

Chapter 3: Materials and Methods

This chapter describes the methods of this study. Plant material was collected, extracted, and fractionated, then tested in bioassays for inhibition in bacteria of growth, biofilm production, and quorum sensing. Human cytotoxicity assays and HPLC-FTMS were also carried out.

Plant material

Samples of *Liriodendron tulipifera* L., *Aralia spinosa* L., and *Quercus alba* L. were identified and collected according to established methods (85) in May 2015 from Lullwater Preserve, Atlanta, Georgia (Fig 3.1). Leaves were gathered manually and a handsaw was used to cut segments of roots and branches for bark collection.

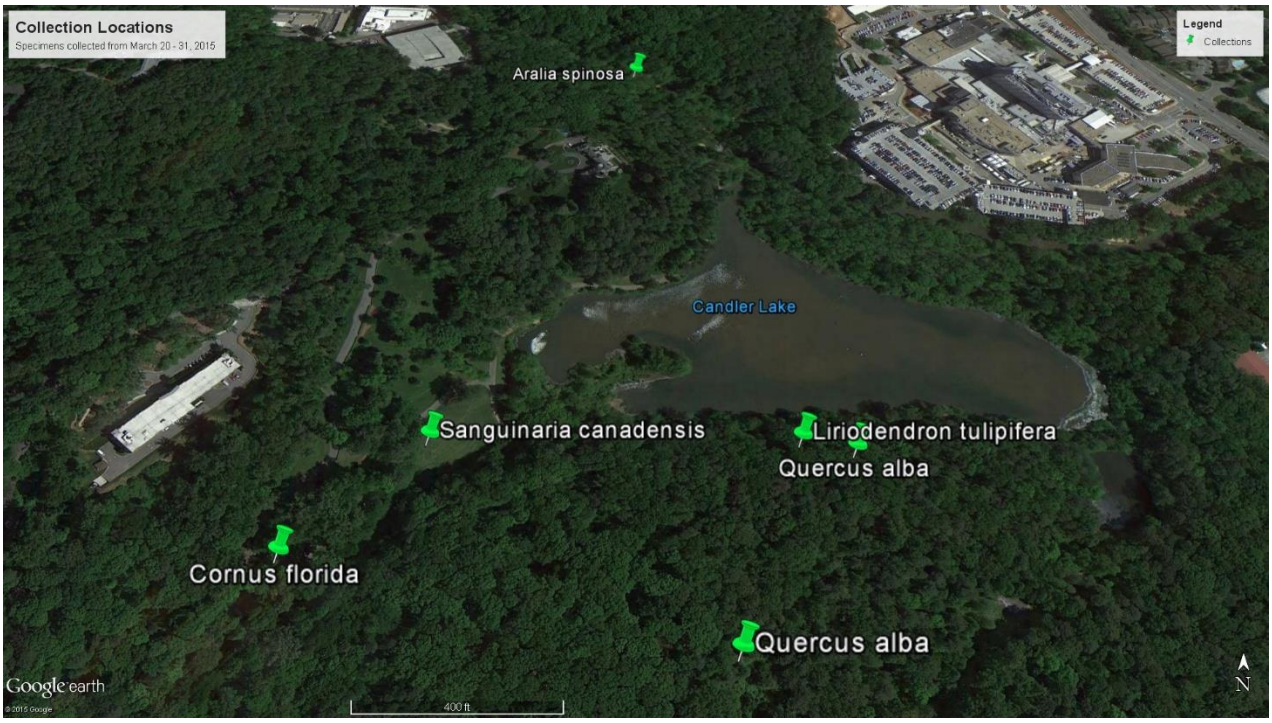


Figure 3.1. Collection locations of plant samples.

Vouchers were deposited in the Emory University Herbarium in Atlanta. Samples were dried and ground into powder (Table 3.1).

Plant	Plant Part	Drying Procedure	Grinding Procedure	Extract Number
Liriodendron tulipifera	leaves	drying cabinet	Wiley Mill with 2mm mesh	616
Liriodendron tulipifera	root inner bark	cut into 3x3cm pieces, drying cabinet	Wiley Mill with 2mm mesh	617
Aralia spinosa	leaves	drying cabinet	Wiley Mill with 2mm mesh	618
Quercus alba	bark	cut into 3x3cm pieces, drying cabinet	Wiley Mill with 2mm mesh	619
Quercus alba	branch galls	drying cabinet	coffee grinder	620
Liriodendron tulipifera	branch inner bark	cut into 1x3cm pieces, drying cabinet	coffee grinder	621

Table 3.1. Preparation of Plant Materials

Extraction, partitioning, and fractionation

All ground material (Table 3.1) was sonicated in methanol (1g/10mL). After 20 minutes the sample was filtered sequentially with Whatman filter paper 8 and 2 (coarse and fine), then fresh methanol was added to the plant material for a second round of sonication. The two filtrates for each extraction were combined and dried *in vacuo*. The resulting residue was resuspended in water, freeze dried using an acetone-dry ice bath, and lyophilized. The dried extract was collected and 20 mg of each extract was dissolved in DMSO (10 mg/mL) for biological testing.

Extracts 617 and 618 were suspended in water (1 g/10 mL) and were sequentially partitioned in hexane, ethyl acetate, and butanol, yielding 4 partitions. Extracts 616 and 619 were dissolved in 95% ethanol (1 g/2 mL and 1 g/3 mL, respectively), chromatographed on a Sephadex LH-20 column (25 g, 32x2.5 cm), and sequentially eluted with 95% ethanol (300 mL), 70% acetone (300 mL), and 100% acetone (150 mL) to yield three fractions. All partitions and fractions were dried *in vacuo*, resuspended in water, freeze-dried, and lyophilized before being dissolved in DMSO (10mg/mL) for biological testing.

Bacterial strains and growth conditions

In this study, six strains of *Staphylococcus aureus* (UAMS1, NRS385, AH1747, AH1677, AH430, AH1872), one strain of *Staphylococcus epidermidis* (NRS101), three strains of *Klebsiella pneumoniae* (EU32, EU33, EU34), two strains of *Acinetobacter baumannii* (EU27, EU35), and one strain of *Pseudomonas aeruginosa* (AH071) were used (Table 3.2). To create liquid cultures for all assays, strains were grown overnight in tryptic soy broth (TSB) with constant shaking (230 rpm). All strains were maintained on Tryptic Soy Agar (TSA) and TSB.

Strain	Species	Strain ID	Characteristics	Reference
UAMS1	*Staphylococcus aureus*	ATCC49230	MRSA isolate from human osteomyelitis	(86)
UAMS929	*Staphylococcus aureus*		Biofilm-deficient mutant	(51)
NRS385	*Staphylococcus aureus*	USA500	MRSA, also resistant to ERY, CLIN, TET, SXT, LEV, GM	(87)
AH430	*Staphylococcus aureus*	SA502a + pDB59 cmR	*agr* type II YFP reporter	(8)
AH1677	*Staphylococcus aureus*	AH845 + pDB59 cmR	*agr* type I YFP reporter	(8)
AH1747	*Staphylococcus aureus*	MW2 + pDB59 cmR	*agr* type III YFP reporter	(8)
AH1872	*Staphylococcus aureus*	MN EV(AH407) + pDB59 cmR	*agr* type IV YFP reporter	(8)
NRS101	*Staphylococcus epidermidis*	ATCC35984	biofilm producer	(88)
AH71	*Pseudomonas aeruginosa*	PAO1		
EU27	*Acinetobacter baumannii*	OIFC143	isolate from human thigh wound	(88)
EU35	*Acinetobacter baumannii*	H72721	isolate from human sputum	(88)
EU32	*Klebsiella pneumoniae*	NR-15410	carbapenem resistance from bla_{KPC} gene	(88)
EU33	*Klebsiella pneumoniae*	NR-15411	carbapenem resistance from bla_{KPC} gene	(88)
EU34	*Klebsiella pneumoniae*	NR-15412	carbapenem resistance from bla_{KPC} gene	(88)

Table 3.2. Multidrug-resistant strains utilized.

Growth inhibition assays

Assays were carried out under CLSI M100-S23 guidelines (89). Working culture was created by standardizing liquid culture using a BioTek Cytation3 and inoculating into CAHMB to a concentration of 0.0006 CFU. Working culture was added to extracts and controls in 96-well microtiter plates (Grenier-Bio 655-185) so that each well contained 0.2 mL of liquid. Untreated (no drug) controls and antibiotic controls (ampicillin, kanamycin, and vancomycin for *Staphylococcus* spp. assays, gentamicin and tetracycline for other species, 0.5 to 64 µg/mL) were included for each strain. Extracts and vehicle were tested at a concentration range of 2 to 256 µg/mL, using 2-fold serial dilution. Plates were incubated at 37°C, *S. aureus*, *S. epidermidis*, and *P. aeruginosa* for 18 hours and *A. baumannii* and *K. pneumoniae* for 22 hours. Optical density (OD_{600}) was measured using a BioTek Cytation3 plate reader at initial and final timepoints, to account for extract color. Growth inhibition was calculated using the formula: $\left(1 - \left(\frac{\Delta ODtest}{\overline{\Delta OD}vehicle}\right)\right) * 100$ = % growth inhibition. MIC_{50} was defined as the lowest concentration at which an extract displayed $\geq$ 50% inhibition.

Extracts active against multidrug-resistant *A. baumanii* (EU27) and *K. pneumoniae* (EU 32) were tested for growth inhibition of *S. epidermidis* and additional strains of *A. baumanii* and *K. pneumoniae*.

Biofilm inhibition assays

Biofilm assay methods did not work with *A. baumanii* and *K. pneumoniae*, so the following methods refer only to *S. aureus*. To create working cultures for biofilm assays, liquid culture was inoculated into media (30 g TSB powder, 30 g NaCl, and 5 g dextrose per 1000 ml H_2O) to a concentration of 0.0006 CFU. Then, human plasma (2% of working culture) was added (90). Working culture was added to extracts and controls in 96-well microtiter plates (Falcon 35-1172) so that each well contained 0.2 mL of liquid. Negative control wells contained only working culture, positive control wells contained working culture and 220D-F2 (2 to 256 µg/mL), a known biofilm inhibitor (90). UAMS929, an *S. aureus* strain which cannot form a biofilm, was used as another positive control in *S. aureus* assays. Extracts and vehicle were tested at a concentration range of 2 to 256 µg/mL, using 2-fold serial dilution. All extracts were tested at sub-MIC_{50} concentrations (determined in growth inhibition assays). Plates were incubated at 37°C for 22 hours. Optical density (OD_{600}) was measured using a BioTek Cytation3 plate reader at initial and final timepoints, to account for extract color. Plates were rinsed twice with 1X PBS (200 µL/well) and once with 100% ethanol (200 µL/well), then stained with crystal violet (50 µL/well). Plates were stained for 15 minutes, then rinsed with water and left to air dry. Dry plates were eluted with ethanol and dye was quantified at 595 nm with a BioTek Cytation 3 plate reader. Biofilm inhibition was calculated using the formula: $\left(1-\left(\frac{ODtest}{\overline{OD}vehicle}\right)\right)*100=\%$ biofilm inhibition. $MBIC_{50}$ was defined as the lowest concentration at which an extract displayed $\geq$ 50% inhibition.

Quorum quenching assays

Quorum sensing in *S. aureus* is accomplished through the *agr* gene (8) and was detected in this experiment with reporter strains for *agr* gene types I, II, III, and IV. The *agr* reporter strains used in this experiment required chloramphenicol to maintain the reporter plasmid, so they were grown with 10 µg/mL chloramphenicol in TSA, in TSB, and in assay. Fluorescent reporter strains for *agr* I, *agr* II, *agr* III, and *agr* IV were cultured with extracts (0.5 to 64 µg/mL) in 96-well plates (black sided, clear bottom, tissue culture treated Costar 3603). Plates were incubated at 37°C with shaking (1200 rpm) in a Stuart SI505 incubator (Bibby Scientific, Burlington, NJ) with a humidified chamber. Fluorescence (493 nm excitation, 535 nm emission) and optical density (OD) (600 nm) readings were taken at beginning (0 hours), end (22 hours), and 3 time points in between. Inhibition of quorum sensing was calculated using the growth inhibition formula with the fluorescence readings.

Cytotoxicity assays

Human immortalized keratinocytes (HaCaT cell line) were maintained in Dulbecco's modified Eagle's medium with L-glutamine and 4.5 g/L glucose (Corning, Corning, NY) supplemented with 10% heat-inactivated fetal bovine serum (Seradigm, Randor, PA) and 1X solution of 100 IU Penicillin and 100 µg/mL Streptomycin (Corning, Corning, NY) at 37°C, 5% CO_2 in 75 cm^2 flasks (Greiner Bio-One). Upon reaching suitable confluency (90–95%), cells were detached from the flask bottom for cell splitting and plating using 0.25% trypsin, 0.1% ethylenediaminetetraacetic acid (EDTA) in Hanks' balanced salt solution (HBSS) without Ca^{2+}, Mg^{2+}, or $NaHCO_3$ (Corning, Corning, NY). Toxicity of extracts was evaluated with the LDH cytotoxicity assay (G-Biosciences, St. Louis, MO). Briefly, the cell culture was standardized to 4 x 10^4 cells mL-1 using a hemocytometer and 200 µL added per well in a 96 well tissue culture treated microtiter plate (Falcon 35–3075). Plates were incubated for 48 hours to allow for

seeding, prior to media aspiration. Either media containing extracts or vehicle were serially diluted 2-fold (2-256 µg/mL) and were processed 24 hours later following manufacturer's protocol for chemical induced cytotoxicity. Cytotoxicity was calculated using the growth inhibition formula.

Chemical Analysis

HPLC methods were based on Mämmelä (91). HPLC was run on an Agilent 1260 Infinity system running OpenLab CDS ChemStation (Agilent Technologies, Santa Clara, CA, USA) with an Agilent ZORBAX Eclipse XDB-C18 (250 mm x 4.6 mm, 5 µm) column with compatible guard column at a column temperature of 35°C. DI water with 1% formic acid and methanol with 1% formic acid were designated as mobile phases A and B, respectively, and were run at 1 mL/min following the gradient shown in Table 3.3. Samples 619F2 (oak bark extract tannin fraction) and 620 (oak gall extract) were chosen as the most potent antibacterials of the samples tested and were prepared for HPLC at 10 mg/mL in DI water. Injection volume of samples was 10 µL.

Time (min)	% A	% B
0.00	95	5
9.00	95	5
69.00	0	100
78.00	0	100
78.01	95	5
87.00	95	5

Table 3.3. HPLC solvent gradient. Mobile phase A is 1% formic acid in water and mobile phase B is 1% formic acid in methanol.

Initial HPLC results (Fig. 4.7) showed most compounds eluting in the first half of the method, so the modified gradient shown in Table 3.4 was used for mass spectrometry. Liquid chromatography-Fourier transform mass spectrometry (LC-FTMS) was performed for the extracts using a Shimadzu SIL-ACHT and Dionex 3600SD HPLC pump. 10 µL injections of each extract were run at ambient temperature with the same mobile phases previously described. The Scifinder database was used to help identify the compounds present in 619F2 and 620.

Time (min)	**% A**	**% B**
0.00	95	5
9.00	95	5
85.00	38	62
109.00	0	100
119.00	0	100
119.10	95	5
129.00	95	5

Table 3.4. HPLC-FTMS solvent gradient. A is 1% formic acid in water and B is 1% formic acid in methanol.

Chapter 4: Results

Results follow in the same order as methods. Data for percent yield, growth inhibition, biofilm inhibition, quorum sensing inhibition, cytotoxicity, and HPLC-FTMS is included.

Extract yield

Extraction in methanol yielded six crude extracts. Extract yield was highest (27.1% of dry mass) in sample 620 (*Q. alba* galls). Other crude extracts had yields ranging from 8-11% (Fig. 4.1).

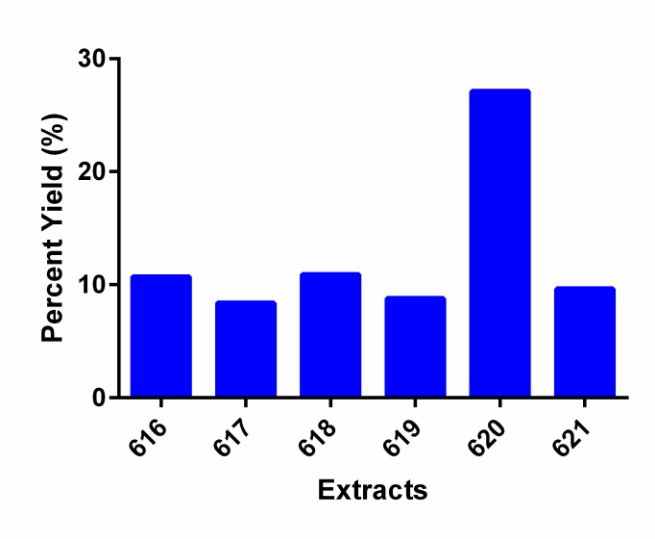


Figure 4.1. Percent Yield of Methanolic Extracts

Masses of partitions and fractions of crude extracts varied from < 0.1% to 4% relative to dry plant matter (Table 4.1). The non-tannin fraction of *L. tulipifera* leaves (616F1) was more than 10 times as massive as the tannin fraction (Table 4.1), suggesting that tannin content is not high in *L. tulipifera* leaves. The tannin and non-tannin fractions of *Q. alba* bark were similar in mass.

Sample	Percent Yield (%)
616F1	1.8072
616F2	0.1521
616F3	0.0151
617B	2.3514
617C	0.8224
617D	2.5848
617E	2.3637
618B	0.6011
618C	2.6888
618D	3.8983
618E	3.0991
619F1	4.0942
619F2	3.4340
619F3	0.0117

Table 4.1. Percent Yield of Partitions and Fractions. Percentages relative to dry plant matter.

Growth inhibition

All 19 extracts were tested for growth inhibition of *S. aureus*, *A. baumannii*, *K. pneumoniae*, and *P. aeruginosa* (Table 4.2). Samples from *L. tulipifera* and *Q. alba* were shown to be most active in inhibition of *S. aureus* growth. *Q. alba* samples 619, 619F2, and 620 displayed inhibition of *A. baumannii* and *K. pneumoniae*.

	Species	**S. aureus**	**S. aureus**	**A. baumannii**	**K. pneumoniae**	**P. aeruginosa**
Sample	Strain	UAMS1	NRS385	EU27	EU32	AH71
616	MIC_{50}	-	256	-	-	-
	MIC_{90}	-	-	-	-	-
616F1	MIC_{50}	256	256	-	-	-
	MIC_{90}	-	-	-	-	-
616F2	MIC_{50}	-	-	-	-	-
	MIC_{90}	-	-	-	-	-
617	MIC_{50}	128	256	-	-	-
	MIC_{90}	-	-	-	-	-
617B	MIC_{50}	64	128	-	-	-
	MIC_{90}	256	256	-	-	-
617C	MIC_{50}	128	128	-	-	256
	MIC_{90}	-	-	-	-	-
617D	MIC_{50}	-	-	-	-	-
	MIC_{90}	-	-	-	-	-
617E	MIC_{50}	-	256	-	-	-
	MIC_{90}	-	-	-	-	-
618	MIC_{50}	-	256	-	-	-
	MIC_{90}	-	-	-	-	-
618B	MIC_{50}	128	128	-	-	-
	MIC_{90}	-	-	-	-	-
618C	MIC_{50}	-	128	-	-	-
	MIC_{90}	-	-	-	-	-
618D	MIC_{50}	-	-	-	-	-
	MIC_{90}	-	-	-	-	-
618E	MIC_{50}	-	-	-	-	-
	MIC_{90}	-	-	-	-	-
619	MIC_{50}	128	256	64	128	-
	MIC_{90}	256	256	-	-	-

619F1	MIC_{50}	-	256	-	-	-
	MIC_{90}	-	-	-	-	-
619F2	MIC_{50}	64	128	32	64	128
	MIC_{90}	128	128	-	-	-
619W	MIC_{50}	-	NT	-	-	-
	MIC_{90}	-	NT	-	-	-
620	MIC_{50}	128	-	32	32	64
	MIC_{90}	-	-	-	-	256
620W	MIC_{50}	64	NT	32	-	NT
	MIC_{90}	-	NT	-	-	NT
621	MIC_{50}	-	256	-	-	-
	MIC_{90}	-	-	-	-	-
Amp	MIC_{50}	-	-	NT	-	NT
	MIC_{90}	-	-	NT	-	NT
Kan	MIC_{50}	2	-	NT	NT	NT
	MIC_{90}	4	-	NT	NT	NT
Van	MIC_{50}	4	8	NT	NT	NT
	MIC_{90}	8	8	NT	NT	NT
Gent	MIC_{50}	NT	NT	64	0.5	0.5
	MIC_{90}	NT	NT	-	0.5	0.5
Tet	MIC_{50}	NT	NT	2	4	NT
	MIC_{90}	NT	NT	4	8	NT

Table 4.2. Growth inhibition of 4 multidrug-resistant bacteria by Civil War samples. MIC values are expressed as concentration (μg/mL). NT indicates 'not tested'.

Extracts which displayed strong activity against *S. aureus*, *A. baumannii*, *K. pneumoniae*, and *P. aeruginosa* (619, 619F2, and 620) were tested for growth inhibition of *S. epidermidis* and different strains of *K. pneumoniae*. No samples were found to be active against the additional *K. pneumoniae* strains tested (Table 4.3). *Q. alba* samples 619 and 619F2 were found to inhibit growth of *S. epidermidis*.

	Species	**K. pneumoniae**			**S. epidermidis**
Sample	Strain	EU33	EU34	EU36	NRS101
619	MIC_{50}	-	-	-	256
	MIC_{90}	-	-	-	-
619F2	MIC_{50}	-	-	-	64
	MIC_{90}	-	-	-	-
620	MIC_{50}	-	-	-	-
	MIC_{90}	-	-	-	-
Amp	MIC_{50}	-	-	-	64
	MIC_{90}	-	-	-	64
Kan	MIC_{50}	NT	NT	NT	-
	MIC_{90}	NT	NT	NT	-
Van	MIC_{50}	NT	NT	NT	16
	MIC_{90}	NT	NT	NT	16
Gent	MIC_{50}	16	2	64	NT
	MIC_{90}	32	2	64	NT
Tet	MIC_{50}	1	4	2	NT
	MIC_{90}	4	4	4	NT

Table 4.3. Growth inhibition of additional strains by *Q. alba* samples 619, 619F2, and 620. MIC values expressed as concentration (μg/mL). NT indicates 'not tested'.

Biofilm inhibition

Samples from all species tested inhibited *S. aureus* biofilms (Table 4.4). Figure 4.2 shows biofilm inhibition across serial dilutions of the most active samples. Some samples, such as 616F1 and 618B, displayed little growth inhibition activity against *S. aureus* but strongly inhibited biofilm formation.

Extract	$MBIC_{50}$	$MBIC_{90}$
616	256	-
616F1	32	-
616F2	128	256
617	2	-
617B	8	-
617C	-	-
617D	-	-
617E	-	-
618	-	-
618B	2	32
618C	-	-
618D	256	256
618E	-	-
619	-	-
619F1	256	-
619F2	1	8
619W	32	32
620	4	16
620W	16	-
621	64	256
220D-F2	8	16

Table 4.4. Inhibition of UAMS1 *S. aureus* biofilm formation by Civil War samples. MBIC values expressed as concentrations (μg/mL) that inhibit 50% and 90% of biofilm.

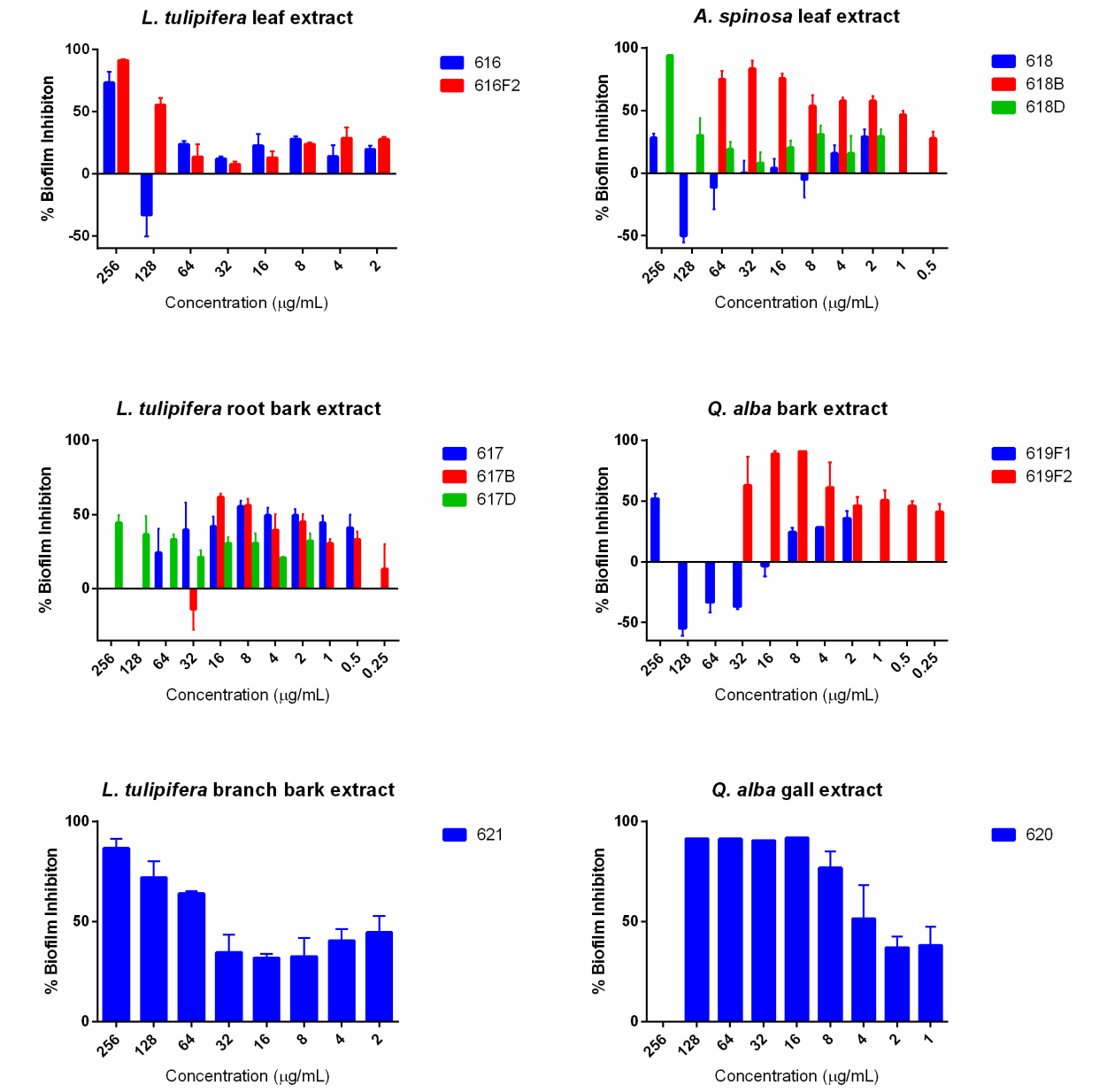


Figure 4.2. Biofilm inhibition of *S. aureus* by Civil War samples. Extracts tested at sub-MIC_{50} concentration. Percent biofilm inhibition calculated as inhibition compared to vehicle control.

Quorum sensing inhibition

Transcription of *S. aureus* Agr types I, II, and III was inhibited by various Civil War samples (Table 4.5). *L. tulipifera* samples 617B and 617C, *A. spinosa* sample 618C, and *Q. alba* sample 619F1 exhibited the most activity in these assays, mostly against *agr* III. No samples demonstrated inhibition of *agr* IV transcription. Figures 4.3 and 4.4 show results from quorum sensing inhibition screens and dilutions, respectively.

Sample	AH430 *agr* II	AH1677 *agr* I	AH1747 *agr* III	AH1872 *agr* IV
616	-	-	-	-
616F1	-	-	32	-
616F2	-	-	-	-
617	-	-	-	-
617B	-	-	-	-
617C	-	32	16	-
617D	-	-	-	-
617E	-	-	-	-
618	-	-	-	-
618B	-	-	-	-
618C	8	-	32	-
618D	-	-	-	-
618E	-	-	-	-
619	-	-	-	-
619F1	-	-	16	-
619F2	64	-	-	-
619W	-	-	-	-
620	-	-	-	-
621	-	-	32	-
224CF2	2	64	8	64

Table 4.5. Inhibition of *S. aureus* quorum sensing by Civil War samples. Reported here are $FLIC_{50}$ values, concentrations at which 50% of fluorescence is inhibited.

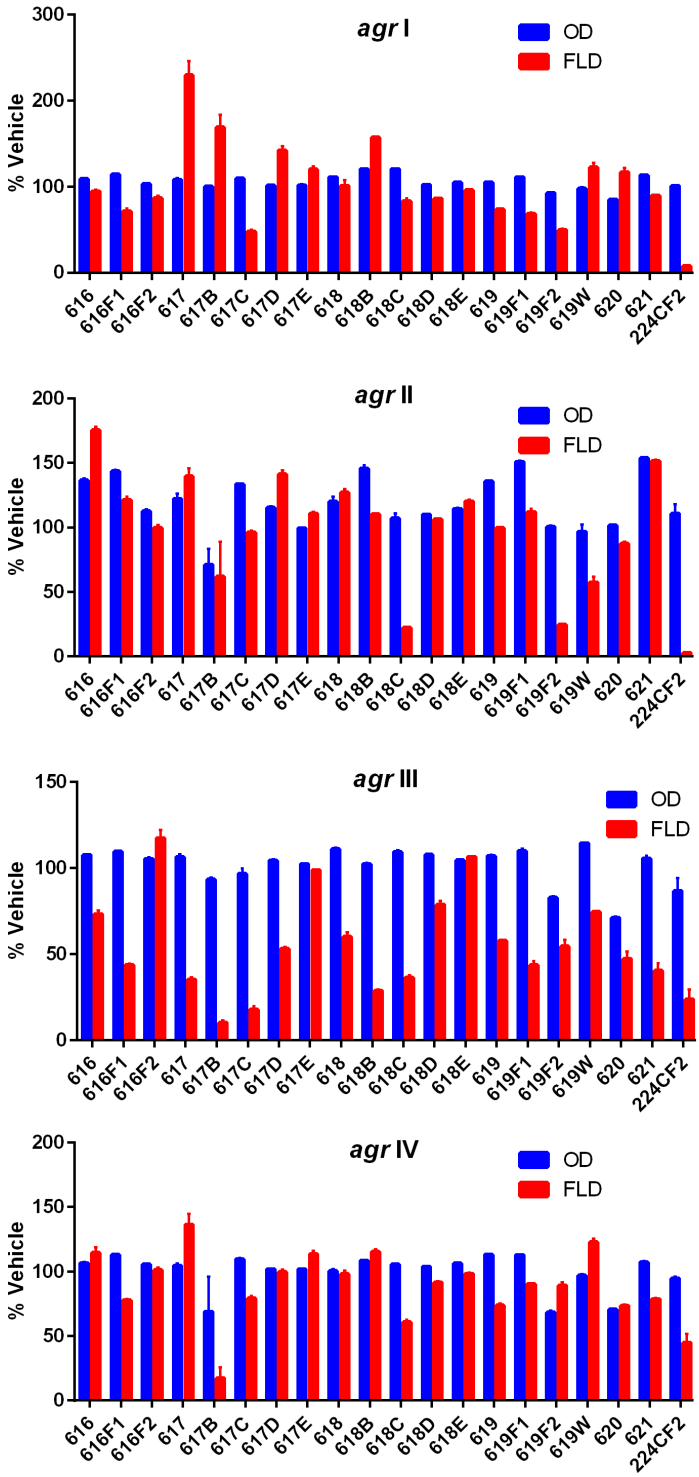


Figure 4.3. Quorum sensing inhibition by samples at 64 µg/mL. OD represents *S. aureus* growth and FLD represents expression of the *agr* gene.

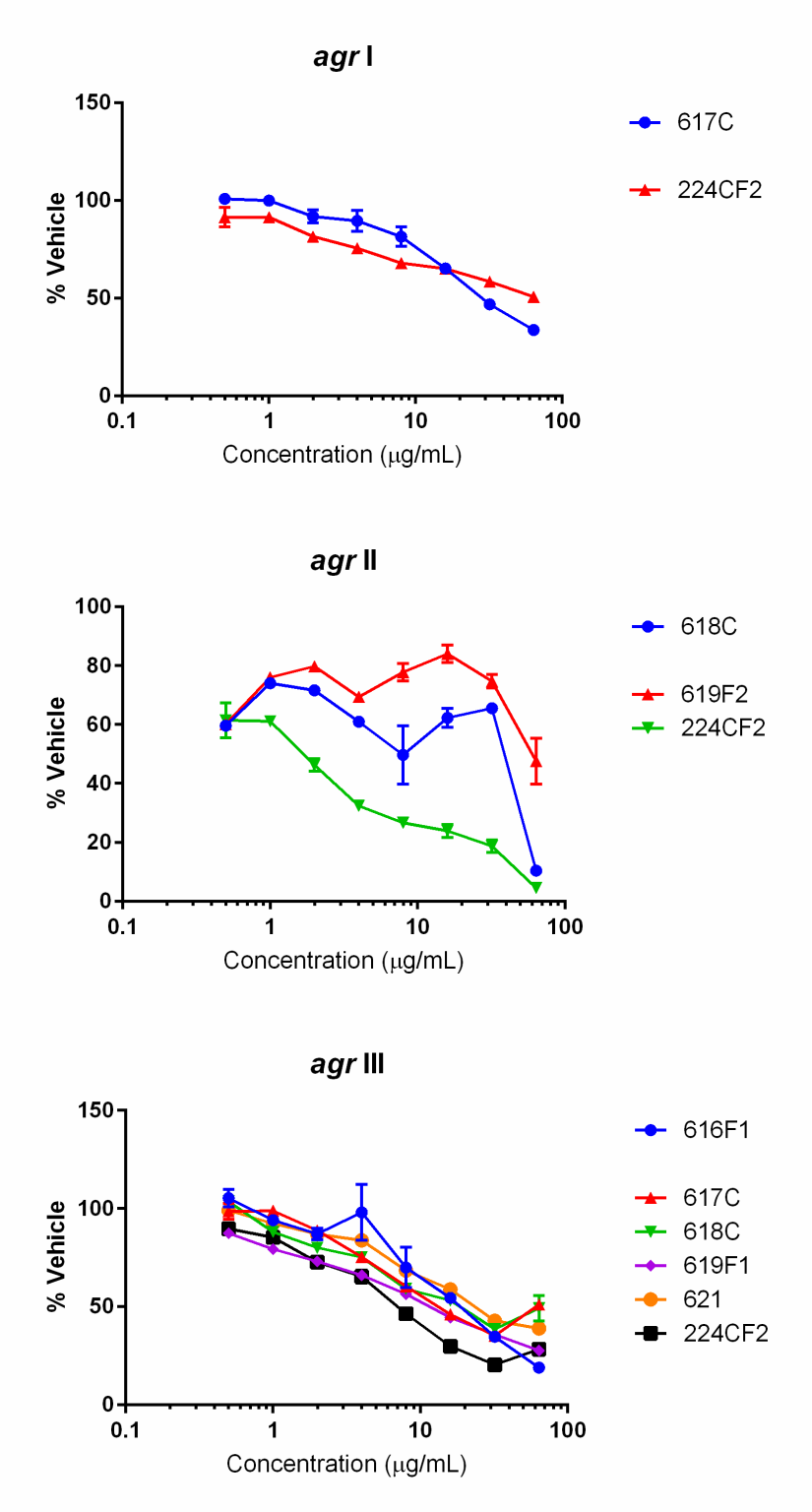


Figure 4.4. Quorum sensing inhibition by active samples from 0.5 to 64 µg/mL. Only 224CF2, the control, showed activity against *agr* IV (Fig. 4.3).

Cytotoxicity

Of the 19 extracts studied, 13 were recognized (via the experiments previously described) to have potential antibiotic activity and were tested with human keratinocytes to determine cytotoxicity. *L. tulipifera* root bark samples (617, 617B, and 617C) displayed high levels of cytotoxicity (Table 4.6). *Q. alba* samples displayed no significant cytotoxicity at test concentrations (2 to 256 μg/mL) Figure 4.6 shows cytotoxicity across serial dilutions of the most toxic samples.

HaCaT cells	**IC50**	**IC90**
616	256	256
616F1	256	-
617	16	-
617B	-	-
617C	16	-
618	256	-
618B	256	-
618C	128	-
619	-	-
619F1	-	-
619F2	-	-
620	-	-
621	-	-

Table 4.6. Growth inhibition of human keratinocytes by *L. tulipifera*, *A. spinosa*, and *Q. alba* samples. MIC_{50} is the minimum concentration required to inhibit 50% of growth and was calculated relative to the growth control.

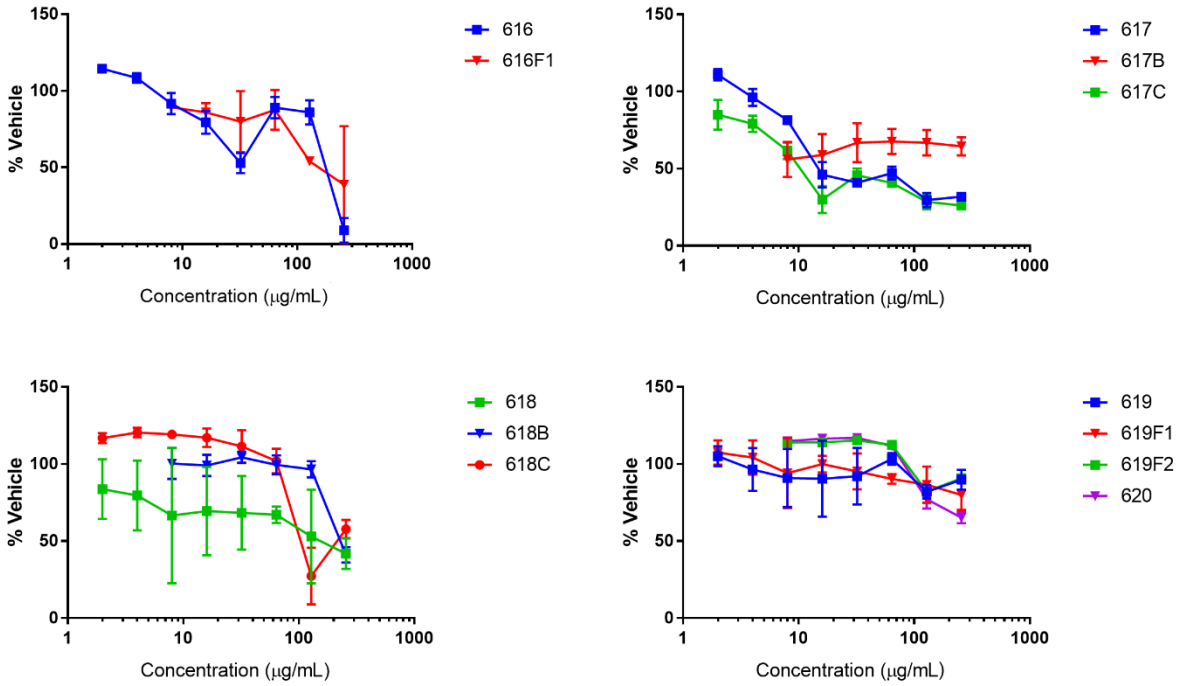
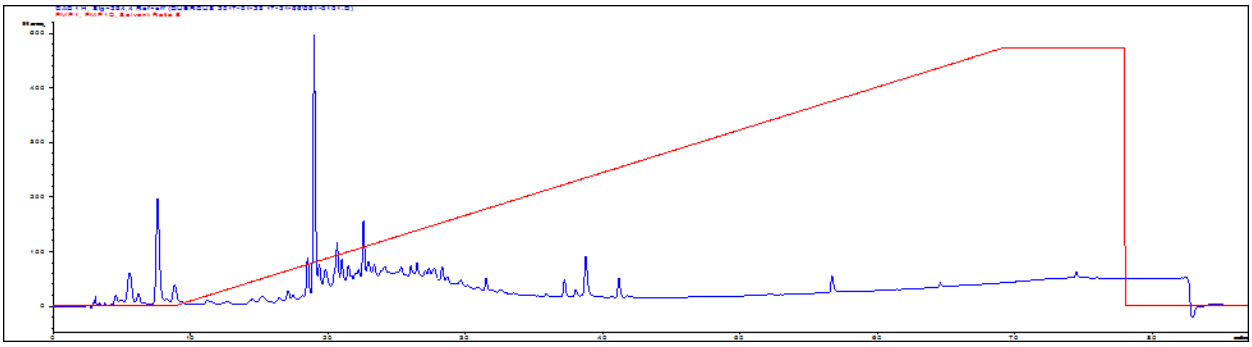


Figure 4.6. Cytotoxicity of Civil War samples. Percent keratinocyte survival is relative to vehicle control.

Chemical analysis

Q. alba samples 619F2 and 620 were selected for chemical analysis because of their strong antibacterial activity both in growth inhibition and in adjuvant assays and because of their lack of toxicity towards human cells. Initial HPLC indicated a wealth of compounds near the beginning of the run (Fig. 4.7), so the gradient for HPLC-FTMS was adjusted to achieve greater separation in that region. HPLC-FTMS revealed that 619F2 and 620 have few compounds in common (Fig. 4.8).

619F2



620

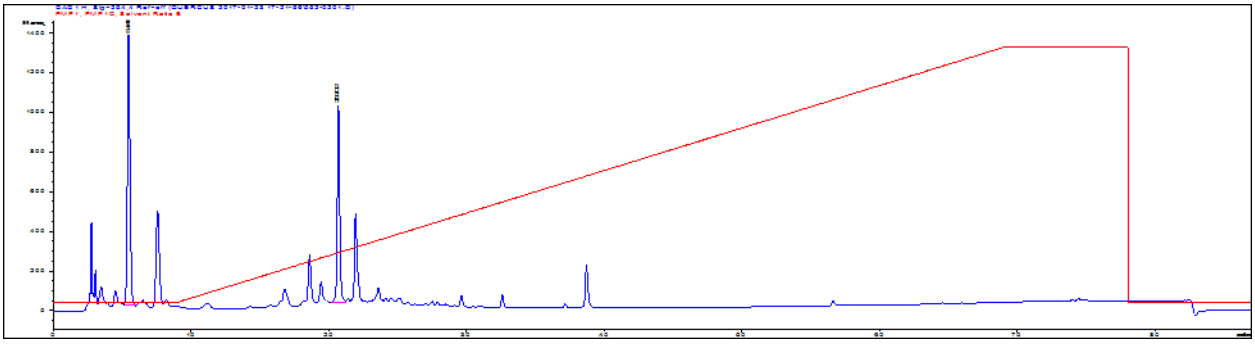


Figure 4.7. HPLC of 619F2 and 620. Red line is % B (methanol).

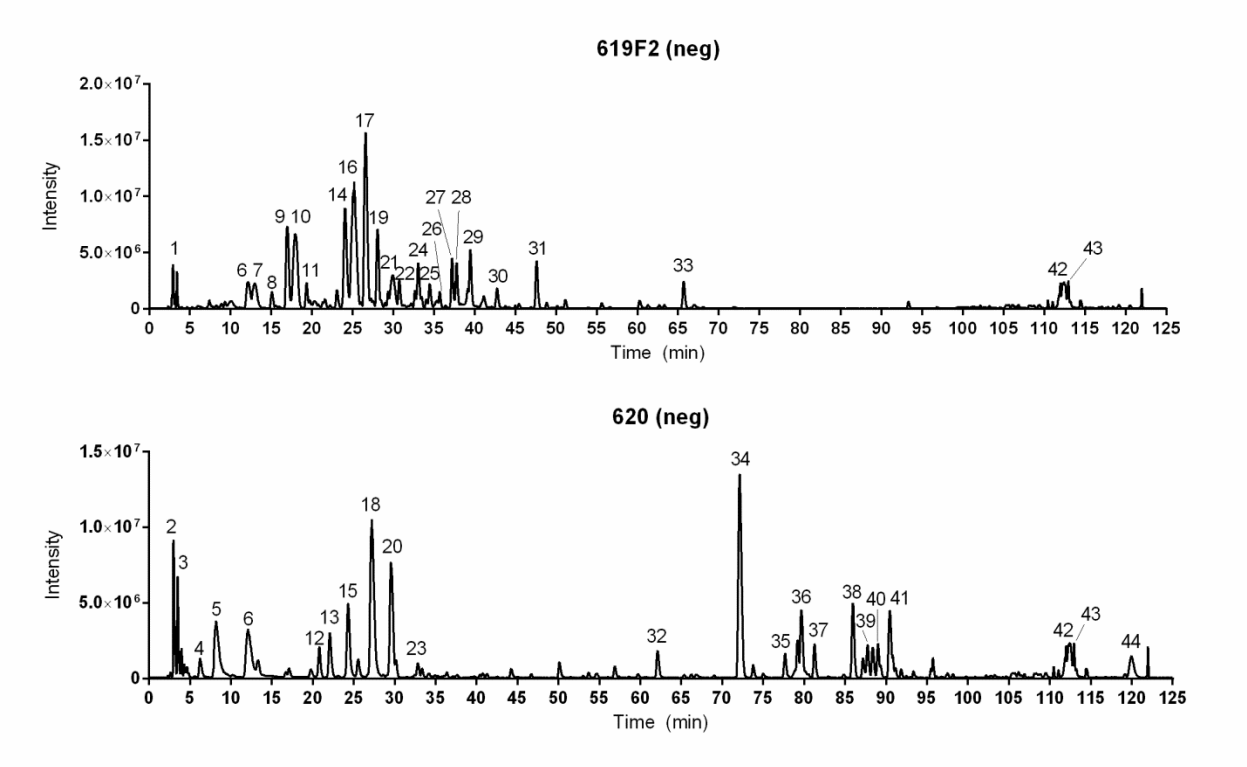


Figure 4.8. MS chromatograms of 619F2 and 620. Peaks in common are 6, 42, and 43.

Analysis of HPLC-FTMS revealed 24 peaks in 619F2 (Table 4.7) and 23 peaks in 620 (Table 4.8) at > 1 % area. All but 9 peaks were putatively matched with known *Quercus* spp. compounds. The three compounds found in both 619F2 and 620 are castalagin/vescalagin, α-amirone, and an unknown compound with molecular weight ~368.

Peak #	Compound	Formula	CAS Number
1	Tribenzo [b,f,h][1,4]dioxecin, D-myo-inositol deriv.	$C_{26}H_{30}O_{13}$	107693-13-0
6	Castalagin/Vescalagin	$C_{41}H_{26}O_{26}$	24312-00-3
7	Procyanidin C isomers	$C_{45}H_{38}O_{18}$	37064-30-5
8	Stenophynin A	$C_{49}H_{36}O_{27}$	105440-40-3
9	Tiliroside	$C_{30}H_{26}O_{13}$	20316-62-5
10	Tiliroside	$C_{30}H_{26}O_{13}$	20316-62-5
11	Tiliroside	$C_{30}H_{26}O_{13}$	20316-62-5
14	Acutissimin isomers	$C_{56}H_{38}O_{31}$	108906-66-7
16	Cinnamtannin isomers	$C_{60}H_{50}O_{24}$	86631-38-1
17	Cinnamtannin isomers	$C_{60}H_{50}O_{24}$	86631-38-1
19	Procyanidin C isomers	$C_{45}H_{38}O_{18}$	37064-30-5
21	Procyanidin B isomers	$C_{30}H_{26}O_{12}$	29106-49-8
22	Benzoic acid, 3,4,5-trihydroxy-, (1 R, 2 S)-1-carboxy-2-[[[(2 E)-3-(3,4-dihydroxyphenyl)-1-oxo-2-propenyl]oxy]methyl]-1,2-ethanediyl ester (9CI)	$C_{27}H_{22}O_{16}$	263839-26-5
24	Procyanidin B isomers	$C_{30}H_{26}O_{12}$	29106-49-8
25	Procyanidin B isomers	$C_{30}H_{26}O_{12}$	29106-49-8
26	Procyanidin C isomers	$C_{45}H_{38}O_{18}$	37064-30-5
27	Procyanidin B O-gallate isomers	$C_{37}H_{30}O_{16}$	73086-04-1
28	Procyanidin B O-gallate isomers	$C_{37}H_{30}O_{16}$	73086-04-1
29	Procyanidin C isomers	$C_{45}H_{38}O_{18}$	37064-30-5
30	Unknown		
31	Castalin (nona-O-methyl,triacetate)	$C_{42}H_{44}O_{21}$	19153-71-0
33	Isocryptomerin	$C_{31}H_{20}O_{10}$	20931-58-2
42	α-Amirone	$C_{30}H_{48}O$	638-96-0
43	Unknown		

Table 4.7. MS table for 619F2. Peak number corresponds with peak numbers in Fig 4.8.

Peak #	Compound	Formula	CAS Number
2	Castalin/Vescalin	$C_{27}H_{20}O_{18}$	19086-75-0
3	Castalin/Vescalin	$C_{27}H_{20}O_{18}$	19086-75-0
4	Grandinin	$C_{46}H_{34}O_{30}$	115166-32-0
5	Castalagin/Vescalagin	$C_{41}H_{26}O_{26}$	24312-00-3
6	Castalagin/Vescalagin	$C_{41}H_{26}O_{26}$	24312-00-3
12	Pedunculagin	$C_{34}H_{24}O_{22}$	7045-42-3
13	Castalin/Vescalin	$C_{27}H_{20}O_{18}$	19086-75-0
15	Castacrenin isomers	$C_{27}H_{18}O_{17}$	173450-72-1
18	Castacrenin isomers	$C_{27}H_{18}O_{17}$	173450-72-1
20	Castacrenin isomers	$C_{27}H_{18}O_{17}$	173450-72-1
23	Unknown		
32	Quisqualin B	$C_{40}H_{26}O_{25}$	192209-61-3
34	Unknown		
35	Quercotriterpenoside isomers	$C_{43}H_{62}O_{15}$	1638430-06-3
36	Unknown		
37	Unknown		
38	Unknown		
39	Olean-12-ene-23,28-dioic acid, 2,3,19,24-tetrahydroxy-,28-D-glucopyranosyl ester	$C_{36}H_{56}O_{13}$	503178-94-7
40	Unknown		
41	Unknown		
42	α-Amirone	$C_{30}H_{48}O$	638-96-0
43	Unknown		
44	Menaquinone 7	$C_{46}H_{66}O_2$	2124-56-4

Table 4.8. MS table for 620. Peak number corresponds with peak numbers in Fig 4.8.

Chapter 5: Discussion

This chapter discusses the implications of results for each of the three species tested. Extracts 616F1, 618B, 618C, 619F2, and 620 are recommended for future study on the basis of their antibiotic activity.

Implications of results

Samples of *L. tulipifera*, *A. spinosa*, and *Q. alba* displayed inhibitory activity against bacteria that cause skin and tissue infections, substantiating their use as antiseptics during the American Civil War. These medicinal plants may be useful in modern medicine as treatments for antibiotic-resistant bacteria. Of particular interest are 618B and 620 as *S. aureus* biofilm inhibitors and 619, 619F2, and 620 as growth inhibitors of carbapenem-resistant *Klebsiella pneumoniae*.

While a 1947 survey of antibacterial properties of plants found no activity in *A. spinosa* and *L. tulipifera* (27), the positive results in this experiment are explainable. The previous study used water extracts whereas this experiment used methanol extracts (27); *L. tulipifera* bark was historically prepared for treatment by dissolving in ethanol (9), which produces an extraction profile similar to methanol (92). Other possible sources of variation include collection date and location, assay method, and extract concentration tested. Additionally, given the variability in how different laboratories may perform one type of extraction, results can vary between related studies. This has occurred many times in the literature. For example, out of two studies of the inhibition of mycobacteria by *Aralia nudicaulis* root (a traditional Native American cure), one reported moderate antibacterial effects while the other reported little activity (61, 93).

Quercus alba

Porcher, in his report, recommended the entire genus *Quercus* as a source of antiseptics (9). This activity is confirmed not only by the results of the experiments reported herein, but also by multiple other studies showing antibiotic effects by *Quercus* spp. extracts (40, 50–55). A European herbal remedy referred to as *Quercus cortex* (originating from *Q. robur*, *Q. petrea*, and *Q. pubescens* bark) has shown weak antibacterial and quorum sensing effects (94). Acorn extract from a variety of oaks has shown inhibition of both gram-positive and gram-negative bacteria (95).

However, the activity of various *Quercus* spp. extracts is far from uniform. For example, the *Q. alba* gall extract (620) in this study inhibited growth of drug-resistant *K. pneumoniae* whereas a study of *Q. infectoria* galls found no significant inhibition of drug-resistant *K. pneumoniae* (40).

Antibacterial activity in oak extracts is frequently attributed to tannins (45), compounds that typically interfere with biological processes by binding to proteins (79). In *Quercus*, tannin content is typically highest in galls, with a reported 70% tannin content in *Q. infectoria* galls (45). In this experiment, higher activity in 620 (gall crude extract) over 619 (bark crude extract) and 619F2 (bark tannin fraction) over 619F1 (bark non-tannin fraction) suggests that *Q. alba*'s growth inhibitory activity is due to tannins. However, quorum sensing inhibition by 619F1 suggests that non-tannin compounds contribute to the antibacterial activity of crude oak extract, the medicine used in the Civil War.

HPLC-FTMS analysis of 619F2 and 620 confirmed the existence of a variety of tannins in both samples (Tables 4.7 and 4.8). Of particular interest are isomers castalagin and vescalagin, ellagitannins found in both 619F2 and 620, as well as related ellagitannins pedunculagin and

grandinin. Ellagitannins have been reported to have antibiotic activity against antibiotic-resistant *S. aureus* (96). While only three MS peaks were found in common between 619F2 and 620, some compounds in one are related to compounds in the other, e.g. castalin in 620 and nona-O-methyl,triacetate-castalin in 619F2.

Tannins have been shown to inhibit growth in a wide range of bacteria, fungi, and viruses. Suggested mechanisms of action include inactivation of microbial enzymes, inhibition of membrane transport, and sequestering essential metal ions in complexes (79). Tannins may also act as biofilm inhibitors by binding to matrix proteins (97). However, tannins have also been found to bind with digestive enzymes and nutrients such as proteins and starches, and as such are generally considered as anti-nutritive; a variety of animals have shown gastrointestinal distress and decreased growth when fed on high-tannin diets (79). Because of this nondiscriminatory binding, external applications of *Q. alba* extracts may be preferable to internal applications; Porcher recommended that powdered oak bark be applied in a wash for gangrene and a poultice for wounds (9).

Leaves of several *Quercus* species (*Q. cerris, Q. ilex, Q. virginiana, Q. incana*) have shown antibacterial properties, including biofilm and quorum sensing inhibition (49, 51, 55, 58). One future research direction could be to compare the antibacterial properties of *Q. alba* leaves with the activity identified in bark and gall extracts.

Aralia spinosa

While *A. spinosa* has several reported uses in traditional medicine (30–32), it has not frequently been studied for medicinal properties. The most notable results of this experiment for *A. spinosa* are significant biofilm inhibition by 618B (leaf hexane partition) and quorum sensing inhibition by 618C (leaf ethyl acetate partition). The presence of these adjuvant properties rather than simple growth inhibition activity in *A. spinosa* leaves may explain the 1947 report of no significant antibiotic activity in *A. spinosa* (27).

Other *Aralia* species have exhibited antibacterial activity in roots (61, 63) and aerial parts (flowers, leaves, and stems) (62), including biofilm inhibition by *A. cachemirica* (62). In his book, Porcher also ascribed antiseptic activity to *A. racemosa* (9).

Liriodendron tulipifera

L. tulipifera has been widely studied and its various parts have exhibited a variety of medicinal effects including antibacterial (71, 72), anti-malarial (74), and anti-cancer (68, 69) activity. The other *Liriodendron* species, *L. chinense*, is used in Chinese traditional medicine and has been shown to have antibacterial effects (76). Additionally, extract from a hybrid of *L. tulipifera* and *L. chinense* has been shown to exhibit inhibition of biofilm production and quorum sensing (75).

In the experiments reported herein, *L. tulipifera* extracts have demonstrated activity in the inhibition of growth, biofilm production, and quorum sensing. However, the root bark extract (617), which is generally more potent than the leaf extract (616) and branch bark extract (621), displayed significant cytotoxicity. It may therefore be ill-suited for medicinal use, or at least dose-limited. A study of *L. tulipifera* for antiplasmodial activity also found high cytotoxicity in active fractions but it has been suggested that, given the use of *L. tulipifera* in traditional

medicine, toxicity may not be problematic *in vivo* at therapeutic doses (74). Porcher's book recommends root bark as the medicinal part of *L. tulipifera* to be harvested (9); perhaps preparation techniques or dosage made the potency/toxicity trade-off worthwhile in the Civil War context. Interestingly, Porcher also suggested *L. tulipifera* bark as a substitute for *Cinchona* bark in malaria treatment, an application supported by recent research (74).

Perhaps the most notable *L. tulipifera* sample with low toxicity is 616F1 (leaf non-tannin fraction), which displayed little growth inhibition but significant biofilm and quorum sensing inhibition— an adjuvant effect similar to the *A. spinosa* samples tested.

Future directions

Further study would focus on bioassay-guided fractionation, a recursive process of fractionation and bioassay to identify individual active compounds and synergistic relationships. Of the samples tested, 616F1, 618B, 618C, 619F2, and 620 exhibit the most promise for antibiotic NCEs and are good candidates for this process. Specifically, the HPLC methods developed for 619F2 and 620 could be used to produce further fractions.

In vivo testing of the antibacterial properties of samples active *in vitro* is the next step in applying this research. Given the potential of some of these samples as adjuvants rather than direct antibiotics, they may be tested as adjuvants of existing antibiotics for the potentiation of antibiotic activity.

Finally, given the activity seen in the extracts tested in this study, it may be worthwhile to investigate the antibacterial properties of other plants recorded as antiseptics in Porcher's book (9) that is the source of this research. In total, 37 plant species were described as antiseptic (9), and it is only the 3 species that happened to be available in bulk at the time and location of collection that were tested in this experiment.

Conclusions

The use of *Q. alba*, *A. spinosa,* and *L. tulipifera* extracts as antiseptics in the Civil War was supported in this study. These traditional medicines were also shown to have a potential modern application as inhibitors of drug-resistant bacteria. *Q. alba* bark (619) and gall (620) extracts exhibited activity as growth inhibitors and, at sub-MIC_{50} levels, as adjuvants inhibiting biofilm formation and quorum sensing in *S. aureus*. HPLC-MS showed that the most active *Q. alba* samples, 619F2 and 620, were composed largely of tannins, both hydrolyzable and condensed; this may cause *Q. alba* extracts to be less viable in internal treatments. *A. spinosa* leaf extracts (618), were found to have adjuvant activity; 618B is a strong inhibitor of biofilm formation and 618C inhibited quorum sensing in *S. aureus*. *L. tulipifera* root bark extracts (617) exhibited cytotoxicity, but leaf (616) and branch bark (621) extracts displayed moderate growth, biofilm, and quorum sensing inhibition of *S. aureus*. The next major step towards applying these results is *in vivo* testing; if antimicrobial activity is robust, further fractionation may then be used to find specific active compounds. As antibiotic-resistant strains of bacteria multiply around the world, it is increasingly important to consider all possible sources of new, and perhaps old, treatments.

References

1. Biggest Threats | Antibiotic/Antimicrobial Resistance | CDC.

2. Zaffiri L, Gardner J, Toledo-Pereyra LH. 2012. History of Antibiotics. From Salvarsan to Cephalosporins. J Invest Surg 25:67–77.

3. Davies J, Davies D. 2010. Origins and Evolution of Antibiotic Resistance. Microbiol Mol Biol Rev 74:417–433.

4. Abraham EP, Chain E. 1940. An Enzyme from Bacteria able to Destroy Penicillin. Nature 146:837–837.

5. Sabath LD, Laverdiere M, Wheeler N, Blazevic D, Wilkinson B. 1977. A new type of penicillin resistance of Staphylococcus aureus. The Lancet 309:443–447.

6. Symptoms and causes - C. difficile infection - Mayo Clinic.

7. Stewart PS, William Costerton J. 2001. Antibiotic resistance of bacteria in biofilms. The Lancet 358:135–138.

8. Quave CL, Lyles JT, Kavanaugh JS, Nelson K, Parlet CP, Crosby HA, Heilmann KP, Horswill AR. 2015. Castanea sativa (European Chestnut) Leaf Extracts Rich in Ursene and Oleanene Derivatives Block Staphylococcus aureus Virulence and Pathogenesis without Detectable Resistance. PLOS ONE 10:e0136486.

9. Porcher FP. 1863. Resources of the Southern Fields and Forests. Evans & Cogswell, Charleston.

10. Randolf S. Milestones: 1861–1865 - Office of the Historian.

11. Foster S. 2014. Medicinal Plants and the Civil War.

12. 2016. Union blockade. Wikipedia.

13. Moore SP. 1863. Standard Supply Table of the Indigenous Remedies for Field Service. Richmond.

14. Parry M. Maimed Men - Life and Limb: The Toll of the American Civil War. Exhibitions.

15. Ekor M. 2014. The growing use of herbal medicines: issues relating to adverse reactions and challenges in monitoring safety. Front Pharmacol 4.

16. WHO | Traditional medicine. WHO.

17. Newman DJ, Cragg GM. 2007. Natural Products as Sources of New Drugs over the Last 25 Years. J Nat Prod 70:461–477.

18. Tolmacheva AA, Rogozhin EA, Deryabin DG. 2014. Antibacterial and quorum sensing regulatory activities of some traditional Eastern-European medicinal plants. Acta Pharm 64:173–186.

19. Rasmussen RV, Fowler VG, Skov R, Bruun NE. 2011. Future challenges and treatment of Staphylococcus aureus bacteremia with emphasis on MRSA. Future Microbiol 6:43–56.

20. Antunes LCS, Visca P, Towner KJ. 2014. Acinetobacter baumannii: evolution of a global pathogen. Pathog Dis 71:292–301.

21. Peleg AY, Seifert H, Paterson DL. 2008. Acinetobacter baumannii: Emergence of a Successful Pathogen. Clin Microbiol Rev 21:538–582.

22. Riggs PJ, Chelius MK, Iniguez AL, Kaeppler SM, Triplett EW, Riggs PJ, Chelius MK, Iniguez AL, Kaeppler SM, Triplett EW. 2001. Enhanced maize productivity by inoculation with diazotrophic bacteria, Enhanced maize productivity by inoculation with diazotrophic bacteria. Funct Plant Biol Funct Plant Biol 28, 28:829, 829–836, 836.

23. 2004. Sherris medical microbiology: an introduction to infectious diseases4th ed. McGraw-Hill, New York.

24. Fotedar R, Banerjee U, Shriniwas. 1993. Vector potential of the German cockroach in dissemination of Pseudomonas aeruginosa. J Hosp Infect 23:55–59.

25. 1994. Pseudomonas aeruginosa: infections and treatment. M. Dekker, New York.

26. Pollock A. 1873. A Botanical Index to All the Medicinal Plants, Barks, Roots, Seeds and Flowers Usually Kept by Druggists. A. Pollock.

27. Hayes LE. 1947. Survey of Higher Plants for Presence of Antibacterial Substances. Bot Gaz 108:408–414.

28. Banks W. 1953. Ethnobotany of the Cherokee Indians. Masters Theses.

29. Speck FG. 1917. Medicine Practices of the Northeastern Algonquians. International Congress of Americanists.

30. Brussell DE. 2004. Araliaceae species used for culinary and medicinal purposes in Niigata-ken, Japan. Econ Bot 58:736–739.

31. Strobel BB. 1826. An Experimental Enquiry Into the Medical Properties of the Aralia Spinosa, Or Prickly Ash. Gray & Ellis.

32. Connor K. 2004. Aralia spinosa L., p. 40–42. *In* .

33. Winston D. 2001. Nwoti—Cherokee Medicine and Ethnobotany. J Am Herbalists Guild 2:45–49.

34. McCune LM, Johns T. 2002. Antioxidant activity in medicinal plants associated with the symptoms of diabetes mellitus used by the Indigenous Peoples of the North American boreal forest. J Ethnopharmacol 82:197–205.

35. Katiki LM, Ferreira JFS, Gonzalez JM, Zajac AM, Lindsay DS, Chagas ACS, Amarante AFT. 2013. Anthelmintic effect of plant extracts containing condensed and hydrolyzable tannins on Caenorhabditis elegans, and their antioxidant capacity. Vet Parasitol 192:218–227.

36. Anderson OR. 2005. Effects of Aqueous Extracts from Leaves and Leaf Litter on the Abundance and Diversity of Soil Gymnamoebae in Laboratory Microcosm Cultures. J Eukaryot Microbiol 52:391–395.

37. Walters D, Meurer-Grimes B, Rovira I. 2001. Antifungal activity of three spermidine conjugates. FEMS Microbiol Lett 201:255–258.

38. Hubert J, Angelis A, Aligiannis N, Rosalia M, Abedini A, Bakiri A, Reynaud R, Nuzillard J-M, Gangloff SC, Skaltsounis A-L, Renault J-H. 2016. In Vitro Dermo-Cosmetic Evaluation of Bark Extracts from Common Temperate Trees. Planta Med 82:1351–1358.

39. Mohammadi-Sichani M, Karbasizadeh V, Dokhaharani SC. 2016. Evaluation of biofilm removal activity of Quercus infectoria galls against Streptococcus mutans. Dent Res J 13:46.

40. Wan Nor Amilah W a. W, Masrah M, Hasmah A, Noor Izani NJ. 2014. In vitro antibacterial activity of Quercus infectoria gall extracts against multidrug resistant bacteria. Trop Biomed 31:680–688.

41. Chusri S, Phatthalung PN, Voravuthikunchai S p. 2012. Anti-biofilm activity of Quercus infectoria G. Olivier against methicillin-resistant Staphylococcus aureus. Lett Appl Microbiol 54:511–517.

42. Basri DF, Tan LS, Shafiei Z, Zin NM. 2012. In Vitro Antibacterial Activity of Galls of Quercus infectoria Olivier against Oral Pathogens. Evid-Based Complement Altern Med ECAM 2012.

43. Vermani A, Navneet, Prabhat. 2009. Screening of Quercus infectoria gall extracts as anti-bacterial agents against dental pathogens. Indian J Dent Res 20:337.

44. Priya PS, Sasikumar JM, Gowsigan G. 2009. Antibacterial activity of methanol extract of Ruta chalapensis (L), Quercus infectoria (Oliver) and Canthium parviflorum (Lam). Anc Sci Life 29:28–31.

45. Chusri S, Voravuthikunchai SP. 2009. Detailed studies on Quercus infectoria Olivier (nutgalls) as an alternative treatment for methicillin-resistant Staphylococcus aureus infections. J Appl Microbiol 106:89–96.

46. Voravuthikunchai SP, Suwalak S. 2008. Antibacterial activities of semipurified fractions of Quercus infectoria against enterohemorrhagic Escherichia coli O157:H7 and its verocytotoxin production. J Food Prot 71:1223–1227.

47. Chusri S, Voravuthikunchai SP. 2008. Quercus infectoria: a candidate for the control of methicillin-resistant Staphylococcus aureus infections. Phytother Res PTR 22:560–562.

48. Voravuthikunchai S, Lortheeranuwat A, Jeeju W, Sririrak T, Phongpaichit S, Supawita T. 2004. Effective medicinal plants against enterohaemorrhagic Escherichia coli O157:H7. J Ethnopharmacol 94:49–54.

49. Sarwar R, Farooq U, Khan A, Naz S, Khan S, Khan A, Rauf A, Bahadar H, Uddin R. 2015. Evaluation of Antioxidant, Free Radical Scavenging, and Antimicrobial Activity of Quercus incana Roxb. Front Pharmacol 6.

50. Judaki A, Panahi J, Havasian MR, Tajbakhsh P, Roozegar MA. 2014. Study of the inhibitory effect of Quercus Coccifera's aqueous extract on Staphylococcus aureus and Pseudomonas aeruginosa In vitro. Bioinformation 10:689–692.

51. Hobby GH, Quave CL, Nelson K, Compadre CM, Beenken KE, Smeltzer MS. 2012. Quercus cerris extracts limit Staphylococcus aureus biofilm formation. J Ethnopharmacol 144:812–815.

52. Jamil M, ul Haq I, Mirza B, Qayyum M. 2012. Isolation of antibacterial compounds from Quercus dilatata L. through bioassay guided fractionation. Ann Clin Microbiol Antimicrob 11:11.

53. Sati SC, Sati N, Sati OP, Biswas D, Chauhan BS. 2012. Analysis and antimicrobial activity of volatile constituents from Quercus leucotrichophora (Fagaceae) bark. Nat Prod Res 26:869–872.

54. Bahador N, Baserisalehi M. 2011. The effect of Quercus castaneifolia extract on pathogenic enteric bacteria. Anaerobe 17:358–360.

55. Karioti A, Sokovic M, Ciric A, Koukoulitsa C, Bilia AR, Skaltsa H. 2011. Antimicrobial Properties of Quercus ilex L. Proanthocyanidin Dimers and Simple Phenolics: Evaluation of Their Synergistic Activity with Conventional Antimicrobials and Prediction of Their Pharmacokinetic Profile. J Agric Food Chem 59:6412–6422.

56. Berahou A, Auhmani A, Fdil N, Benharref A, Jana M, Gadhi CA. 2007. Antibacterial activity of Quercus ilex bark's extracts. J Ethnopharmacol 112:426–429.

57. Güllüce M, Adigüzel A, Oğütçü H, Sengül M, Karaman I, Sahin F. 2004. Antimicrobial effects of Quercus ilex L. extract. Phytother Res PTR 18:208–211.

58. Adonizio A, Kong K-F, Mathee K. 2008. Inhibition of Quorum Sensing-Controlled Virulence Factor Production in Pseudomonas aeruginosa by South Florida Plant Extracts. Antimicrob Agents Chemother 52:198–203.

59. Adonizio AL, Downum K, Bennett BC, Mathee K. 2006. Anti-quorum sensing activity of medicinal plants in southern Florida. J Ethnopharmacol 105:427–435.

60. Lee C-H, Kang H. 2015. Aralia elata (Miquel) Seemann Suppresses Inflammatory Responses in Macrophage Cell by Regulation of NF-kappa B Signalling. Trop J Pharm Res 14:423–429.

61. Li H, Doucet B, Flewelling AJ, Jean S, Webster D, Robichaud GA, Johnson JA, Gray CA. 2015. Antimycobacterial Natural Products from Endophytes of the Medicinal Plant Aralia nudicaulis. Nat Prod Commun 10:1641–1642.

62. Ali F, Sangwan PL, Koul S, Pandey A, Bani S, Abdullah ST, Sharma PR, Kitchlu S, Khan IA. 2012. 4-epi-Pimaric acid: a phytomolecule as a potent antibacterial and anti-biofilm agent for oral cavity pathogens. Eur J Clin Microbiol Infect Dis 31:149–159.

63. Jeong S-I, Han W-S, Yun Y-H, Kim K-J. 2006. Continentalic acid from Aralia continentalis shows activity against methicillin-resistant Staphylococcus aureus. Phytother Res PTR 20:511–514.

64. Kang Y-F, Liu C-M, Kao C-L, Chen C-Y. 2014. Antioxidant and Anticancer Constituents from the Leaves of Liriodendron tulipifera. Molecules 19:4234–4245.

65. Chen J-H, Lin S-S, Wang W-X, Yuan S-T, Shi J-S, Jia A-Q. 2015. The extract, LXB-1, from the barks of Liriodendron x hybrid, induced apoptosis via Akt, JNK and ERK1/2 pathways in A549 lung cancer cells. Z Naturforschung Sect C- J Biosci 70:305–311.

66. Chiu C-C, Chou H-L, Wu P-F, Chen H-L, Wang H-M, Chen C-Y. 2012. Bio-Functional Constituents from the Stems of Liriodendron tulipifera. Molecules 17:4357–4372.

67. Chen J-H, Yang G-X, Ding Q, Xia T-S, She J, Jia A-Q. 2013. In vitro tumor cytotoxic activities of extracts from three Liriodendron plants. Pak J Pharm Sci 26:233–237.

68. Moon MK, Oh HM, Kwon B-M, Baek N, Kim S-H, Kim JS, Kim DK. 2007. Farnesyl protein transferase and tumor cell growth inhibitory activities of lipiferolide isolated fromLiriodendron tulipifera. Arch Pharm Res 30:299.

69. Doskotch RW, El-Feraly FS. 1969. Antitumor Agents II: Tulipinolide, a New Germacranolide Sesquiterpene, and Costunolide. Two Cytotoxic Substances from Liriodendron tulipifera L. J Pharm Sci 58:877–880.

70. Fang Z, Jung KH, Yan HH, Kim SJ, Son MK, Rumman M, Lee H, Kim KW, Yoo H-D, Hong S-S. 2015. CD-200 induces apoptosis and inhibits Bcr-Abl signaling in imatinib-resistant chronic myeloid leukemia with T315I mutation. Int J Oncol 47:253–261.

71. Miller SL, Villanueva HE, Palazzo MC, Wright BS, Setzer WN. 2009. Seasonal variation and bioactivity in the leaf oil of Liriodendron tulipifera growing in Huntsville, Alabama. Nat Prod Commun 4:839–843.

72. Hufford CD, Funderburk MJ, Morgan JM, Robertson LW. 1975. Two Antimicrobial Alkaloids from Heartwood of Liriodendron tulipifera L. J Pharm Sci 64:789–792.

73. Jeong EJ, Kim N-H, Heo J-D, Lee KY, Rho J-R, Kim YC, Sung SH. 2015. Antifibrotic Compounds from *Liriodendron tulipifera* Attenuating HSC-T6 Proliferation and TNF-α Production in RAW264.7 Cells. Biol Pharm Bull 38:228–234.

74. Graziose R, Rathinasabapathy T, Lategan C, Poulev A, Smith PJ, Grace M, Lila MA, Raskin I. 2011. Antiplasmodial activity of aporphine alkaloids and sesquiterpene lactones from Liriodendron tulipifera L. J Ethnopharmacol 133:26–30.

75. Tan X, Yang D, Yang G, Chen J, Dong W, Shi J, Jia A. 2015. The investigation of inhibiting quorum sensing and methicillin-resistant Staphylococcus aureus biofilm formation from Liriodendron hybrid. Pak J Pharm Sci 28:903–908.

76. Zeng C, Liu Z. 2009. Bacteriostatic Activity and Stability of Extracts from Liriodendron chinense. Hubei Agric Sci 1222–1224.

77. Glabasnia A, Hofmann T. 2006. Sensory-directed identification of taste-active ellagitannins in American (Quercus alba L.) and European oak wood (Quercus robur L.) and quantitative analysis in bourbon whiskey and oak-matured red wines. J Agric Food Chem 54:3380–3390.

78. Hart JH. 1972. Inhibition of Wood-Rotting Fungi by Ellagitannins in the Heartwood of Quercus alba. Phytopathology 62:620.

79. Chung KT, Wong TY, Wei CI, Huang YW, Lin Y. 1998. Tannins and human health: a review. Crit Rev Food Sci Nutr 38:421–464.

80. Basri DF, Fan SH. 2005. The potential of aqueous and acetone extracts of galls of Quercus infectoria as antibacterial agents. Indian J Pharmacol 37:26.

81. Worthley EG, Schott CD. 1967. Bioassay and chemical studies on some dicotyledonous plants. Toxicon 5:73–78.

82. Cushnie TPT, Cushnie B, Lamb AJ. 2014. Alkaloids: An overview of their antibacterial, antibiotic-enhancing and antivirulence activities. Int J Antimicrob Agents 44:377–386.

83. Francis G, Kerem Z, Makkar HPS, Becker K. 2002. The biological action of saponins in animal systems: a review. Br J Nutr 88:587.

84. Sesquiterpene Lactones and their toxicity to livestock. Cornell Univ Dep Anim Sci.

85. Bean AM J. 2006. Collecting and Preserving Plant Specimens - A Manual. Queensland Herbarium.

86. Staphylococcus aureus subsp. aureus Rosenbach ATCC ® 49230™.

87. McDougal LK, Steward CD, Killgore GE, Chaitram JM, McAllister SK, Tenover FC. 2003. Pulsed-Field Gel Electrophoresis Typing of Oxacillin-Resistant Staphylococcus aureus Isolates from the United States: Establishing a National Database. J Clin Microbiol 41:5113–5120.

88. BEI Resources.

89. Cockerill FR. 2013. Performance standards for antimicrobial susceptibility testing: twenty-third informational supplement ; M100 - S23. CLSI, Wayne, PA.

90. Quave CL, Estévez-Carmona M, Compadre CM, Hobby G, Hendrickson H, Beenken KE, Smeltzer MS. 2012. Ellagic acid derivatives from Rubus ulmifolius inhibit Staphylococcus aureus biofilm formation and improve response to antibiotics. PloS One 7:e28737.

91. Mämmelä P, Savolainen H, Lindroos L, Kangas J, Vartiainen T. 2000. Analysis of oak tannins by liquid chromatography-electrospray ionisation mass spectrometry. J Chromatogr A 891:75–83.

92. Xu B j., Chang S k. c. 2007. A Comparative Study on Phenolic Profiles and Antioxidant Activities of Legumes as Affected by Extraction Solvents. J Food Sci 72:S159–S166.

93. Webster D, Lee TDG, Moore J, Manning T, Kunimoto D, LeBlanc D, Johnson JA, Gray CA. 2010. Antimycobacterial screening of traditional medicinal plants using the microplate resazurin assay. Can J Microbiol 56:487.

94. Deryabin DG, Tolmacheva AA. 2015. Antibacterial and Anti-Quorum Sensing Molecular Composition Derived from Quercus cortex (Oak bark) Extract. Molecules 20:17093–17108.

95. Mohebi R, Ghafourian S, Sekawi Z, Khosravi A, Galehdari EA, Hushmandfar R, Ranjbar R, Maleki A, Mohammadzadeh M, Rahbar M, Sadeghifard N. 2011. In vitro and in vivo antibacterial activity of acorn herbal extract against some Gram-negative and Gram-positive bacteria. Roum Arch Microbiol Immunol 70:149–152.

96. Lipińska L, Klewicka E, Sójka M. 2014. The structure, occurrence and biological activity of ellagitannins: a general review. Acta Sci Pol Technol Aliment 13:289–299.

97. Akiyama H, Fujii K, Yamasaki O, Oono T, Iwatsuki K. 2001. Antibacterial action of several tannins against Staphylococcus aureus. J Antimicrob Chemother 48:487–491.