Bacterial biofilm inhibition and antifungal activity of neotropical plants

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Abstract

This thesis examined the antimicrobial activity of select neotropical plants from Costa Rica and traditional Q'eqchi Maya medicines from Belize. In particular the potential for interference with bacterial quorum sensing (QS) and biofilm formation as well as fungal growth were assessed. Of one hundred and twenty six extracts collected from Costa Rica, one third showed significant QS inhibition while 13 species displayed more biofilm inhibitory activities than the positive control allicin. The active species belonged to the Lepidobotryaceae, Melastomataceae, Meliaceae, Sapindaceae, and Simaroubaceae. Twelve Marcgraviaceae species were tested for the same biological activities; of these, three showed similar QS inhibition to that of the positive control *Delisea pulchra* (Greville) Montagne and five with at least 30% biofilm inhibition. Only one species inhibited fungal growth – *Marcgravia nervosa* Triana & Planch. Bioassay-guided isolation of this plant resulted in the identification of the active principle as a naphthoquinone, with a minimum inhibitory concentration (MIC) ranging from 85 to 100 μM against *Saccharomyces cerevisiae.* Similarly, sixty one Q'eqchi' Maya medicinal plant species were evaluated for their antimicrobial activities. Of these, four species showed more QS inhibition than *D. pulchra*, seven with comparable biofilm inhibitory activities that of allicin, and two with similarly antifungal activity to berberine. Two spirostanol saponins were isolated from *Cestrum schlechtendahlii* G.Don, an active antifungal plant. The major saponin showed growth inhibition against *Saccharomyces cerevisiae* and *Fusarium graminearum*, with MICs of 16.5 μM and 132 μM, respectively. Further analyses of this compound using chemical genomics suggested that its antifungal mechanism of action is pleiotropic, affecting multiple targets. Taken together, these findings showed that neotropical plants and traditional Q'eqchi' Maya medicines

contain phytochemicals that interfere with bacterial biofilm formation and quorum sensing as well as fungal growth.

Résumé

Cette thèse a examiné l'activité antimicrobienne de plantes néotropicales du Costa Rica et des médecines traditionnels Q'eqchi' Maya de Belize. En particulier, le potentiel d'interférence avec le quorum sensing (la détection quorum) bactérien et la formation des biofilms, ainsi que la croissance fongique, ont été évalués. De cent vingt-six extraits recueillies au Costa Rica, un tiers ont montrés une inhibition significative de quorum sensing (QS) et 13 espèces ont démontrées une activité d'inhibition de biofilm plus que le contrôle positif allicine. Les espèces actives appartenaient à la Lepidobotryaceae, Melastomataceae, Meliaceae, Sapindaceae et Simaroubaceae. Douze espèces de Marcgraviaceae ont été testées pour les mêmes activités biologiques; de ceux-ci, trois ont montrées une inhibition de QS similaire au contrôle positif *Delisea pulchra* (Greville) Montagne et cinq ont montrées au moins 30% d'inhibition de biofilm. Une seule espèce a inhibé la croissance fongique - *Marcgravia nervosa* Triana & Planch. L'isolation guidée par essai biologique de cette plante a conduit à l'identification du principe actif en naphtoquinone, avec un CMI allant de 85 à 100 µM contre *Saccharomyces cerevisiae*. De même, soixante et une espèces de plantes médicinales Q'eqchi' Maya ont été évaluées pour leurs activités antimicrobiennes. Parmi ceux-ci, quatre espèces ont montrées une inhibition de QS plus que *D. pulchra*, sept ont montrées activité d'inhibition de biofilm comparable à l'allicine, et deux ont montrées une activité antifongique similaire à berbérine. Deux saponins spirostanol ont été isolés de *Cestrum schlechtendahlii* G.Don, une plante avec de l'activité antifongique. La saponine majeure a montré l'inhibition de la croissance de *Saccharomyces cerevisiae* et *Fusarium graminearum*, avec des concentrations minimales inhibitrices (CMIs) de 16,5 µM et 132 µM, respectivement. Une analyse utilisant la génomique chimique a suggéré que son mécanisme d'action antifongique est pléiotropique. Pris ensemble, ces résultats ont montrés que

les plantes néotropicales et les médecines traditionnels Q'eqchi' Maya contiennent des composés phytochimiques qui interfèrent avec la formation de biofilm et le quorum sensing bactérien ainsi que la croissance fongique.

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List of Abbreviations

ACN – acetonitrile

AHL – *N*–acyl-homoserine lactone

APCI – Atmospheric Pressure Chemical Ionization

AUC – area under the curve

BITI – Belize Indigenous Training Institute

CFU – colony-forming unit

CC – column chromatography

CMC – carboxymethylcellulose

COSY – Correlation spectroscopy

DAD – Diode Array Detector

DCM – dichloromethane

DEPT – Distortionless Enhancement by Polarization Transfer

dpi – dots per inch

ESI – Electrospray ionization

EtOAc – ethyl acetate

EtOH – ethanol

GYEP – glucose yeast extract peptone

HPLC – High Performance Liquid Chromatography

HMBC – Heteronuclear Multiple Bond Coherence

HMQC –Heteronuclear Multiple Quantum Coherence

HREIMS – High Resolution Electrospray Ionization Mass Spectrometry

HSL – homoserine lactone

IC_{50} – half maximal inhibitory concentration

ID – internal diameter

IR – infrared

LB – Luria–Bertani

LC – Liquid Chromatography

MeOH – methanol

MIC – minimum inhibitory concentration

MS – Mass Spectrometry

NMR – Nuclear Magnetic Resonance

NOESY – Nuclear Overhauser Effect Spectroscopy

OD – optical density

ORF – open reading frame

PCR – polymerase chain reaction

PDA – Photodiode Array Detector

QMHA – Q'eqchi' Maya Healers Association

QS – quorum sensing

QTOF – Quadrupole-time-of-flight

SEM – standard error of the mean

UPLC – Ultra Performance Liquid Chromatography

UV – ultraviolet

rpm – revolutions per minute

TFA – trifluoroacetic acid

TGY – tryptone glucose yeast extract

TLC – Thin Layer Chromatography

TMS – tetramethylsilane

YPD – yeast extract peptone dextrose

Chapter 1 – Introduction and Literature review

1.1 General introduction

The focus of this thesis was to examine the antimicrobial activity of select neotropical plants and to identify novel phytochemicals that can interfere with the formation of bacterial biofilm and fungal growth. In particular, two targeted discovery methods were used to select potential plant species: a taxonomic study of neotropical families and a study of ethnobotanical materials. In collaboration with the Universidad Nacional Autonoma (UNA) of Costa Rica and the Q'eqchi' Maya Healers Association (QMHA) of Belize, prominent and rare plant families of the neotropics and traditional medicines were assessed for biofilm inhibitory and antifungal activities using model organisms.

Microbial biofilms develop on many living organisms, such as marine algae and terrestrial plants. They are both beneficial (resident bacteria on plant leaves and roots to prevent unwanted colonization from potential pathogens) and harmful (chronic infections in cystic fibrosis patients) (Davey and O'Toole 2000; Donlan and Costerton, 2002). Many human pathogens are also biofilm formers; these are responsible for many persistent nosocomial (hospital-acquired) infections especially in the immunocompromised population. With the rapid increase in antibiotic resistance to conventional therapies, the need for alternative antimicrobials is of particular interest. Recently, teixobactin (an antimicrobial depsipeptide) was discovered from the bacterium *Eleftheria terrae*; this compound inhibits bacterial cell wall synthesis by binding to lipid II and lipid II components (precursors for peptidoglycan and techoic acid,

respectively) and is the first new antibiotic in 30 years (Ling et al. 2015). According to the Centers for Disease Control and Prevention (CDC), "biofilms account for over 60% of human infections and virtually all chronic, recurrent, and implanted device-associated infections" (CDC 2000). To exacerbate the current antibiotics resistance situation, bacteria and fungi in biofilms are protected by the extracellular polymeric substance matrix and thus much less susceptible to antimicrobial drug treatments. Drug discovery from plants is a well established field (Gurib-Fakim 2006; Heinrich 2000). In this study, the rationale for a study of tropical plants was that the hot and humid environment of the tropics, which favours microbial pathogens growth, may have resulted in the evolution of biofilm inhibitors in plants.

1.2 Background and literature review

Quorum sensing (QS) and biofilm formation

Bacteria and fungi can exist as planktonic cells or as biofilms. A biofilm consists of a 3-dimensional aggregation of microbial communities attached to a substratum and surrounded by an extracellular polymeric substance matrix (Hall-Stoodley et al. 2004; Stoodley et al. 2002). The matrix is self-produced by the microbes and consists principally of exopolysaccharides, as well as smaller amounts of nucleic acids, proteins, and other molecules (Davey and O'Toole 2000; Donlan and Costerton 2002). The microbial communities, referred to as microcolonies, are sessile and irreversibly attached to the substratum or to one another (Donlan and Costerton 2002). A biofilm can be homogenous (one resident species) or heterogeneous (two or more resident species). Biofilms are a naturally-occurring phenomenon and form on virtually any surface that is intermittently exposed to water. These surfaces include pipe linings, plant leaves and roots, body surfaces, and medical devices such as pacemakers and catheters (Donlan and Costerton 2002; Hall-Stoodley et al. 2004).

Many species are capable of converting from the planktonic to the biofilm mode of growth and vice versa (Figure 1.1). Environmental cues such as nutrient limitations, pH, and oxygen availability are believed to be the contributing factors to initiation of biofilm formation and these vary from species to species (Davey and O'Toole 2000). The biofilm mode of growth provides many advantages over the planktonic state. These advantages include protection from environmental stressors such as desiccation and antimicrobials, metabolic cooperation, increased genetic diversity via horizontal gene transfer, and cell-to-cell communication (Davey and O'Toole, 2000; Donlan and Costerton 2002; Hall-Stoodley et al. 2004).

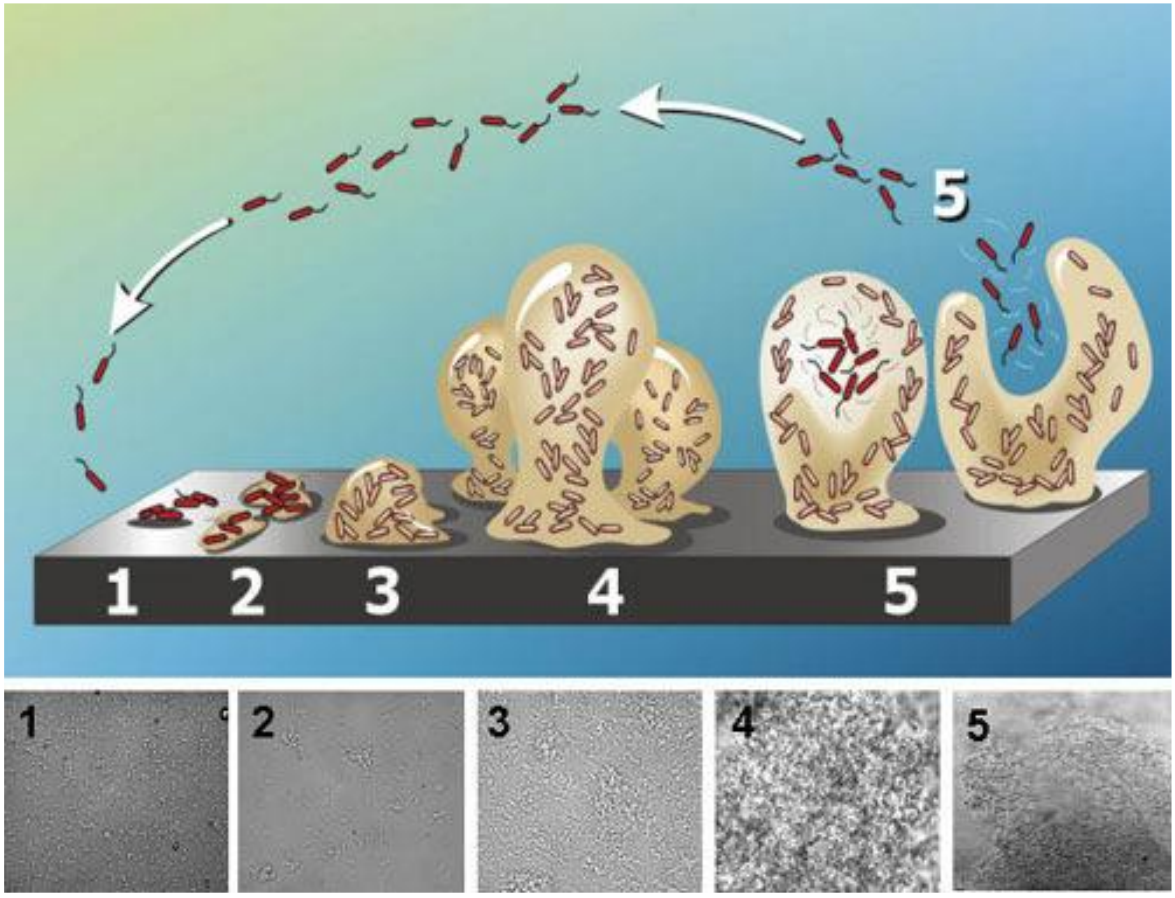

Figure 1.1 The conversion between microbial planktonic and biofilm growths. Top panel – Biofilm formation is depicted as a five-stage process: 1) initial attachment of planktonic cells to substratum, 2) production of extracellular polymeric substance (EPS) matrix to facilitate adherence, 3) development of early biofilm, 4) mature biofilm with distinct architecture, and 5) dispersal of biofilm and conversion back to planktonic growth. Bottom panels – Growth of a *Pseudomonas aeruginosa* biofilm on a glass substratum (Figure from Stoodley et al. 2002, used with permission).

The problem associated with biofilms is that many pathogens are also biofilm formers. These include *Pseudomonas aeruginosa* in cystic fibrosis (Singh et al. 2000), *Campylobacter jejeuni* in food poisoning (Kalmakoff et al. 2006), and staphylococci and *Candida albicans* in device-related infections (Donlan and Costerton 2002; Mukherjee and Chandra 2004). Furthermore, bacteria in biofilms have been reported to be up to 1000 times less susceptible to antimicrobials than their planktonic equivalents (Gilbert et al. 1997). The mechanisms of resistance in biofilms are still under heavy debate (Davey and O'Toole 2000; Donlan and Costerton 2002). The matrix is thought to delay penetration and to contain enzymes responsible for the inactivation of antimicrobials (Davey and O'Toole 2000). Recently, a study by Mah et al. (2003) showed that periplasmic glucans in *Pseudomonas aeruginosa* biofilm bound to and prevented tobramycin from entering the cells. Resistor cells are also believed to form by slowing down their growth rate and entering a viable but nonculturable (VBNC) state where they are resistant to antibiotics (Davey and O'Toole 2000). This highlights the need to search for novel antimicrobials that are effective against biofilms.

There are four developmental stages in the formation of a homogenous (single species) biofilm (Figure 1.1). These stages include: the initial attachment to a surface, the formation of microcolonies, the development of the extracellular polymeric matrix, and the detachment of the biofilm (Costerton 1999; Davey and O'Toole 2000; Stoodley et al. 2002). Each stage requires the expression of specific genes. In order to coordinate and regulate gene expression between

microcolonies, bacteria and fungi use a cell-to-cell communication system known as quorum sensing (QS). QS involves signal molecules called autoinducers that are released by the microbes themselves (Figure 1.2). Gram positive bacteria use autoinducing peptides (AIPs) for signalling and gram negative bacteria have lipid-based molecules known as *N*–acyl–homoserine lactones (AHLs) (Davey and O'Toole 2000; Davies et al. 1998; Donlan and Costerton 2002; Hall-Stoodley et al. 2004; Raffa et al. 2005). In fungi such as *Candida albicans*, the quorum sensing molecules are farnesol and tyrosol (Alem et al. 2006; Sudoh et al. 1993). Once populations reach a specific density or threshold, expression of certain genes such as virulence factors can occur. In most bacterial species, QS is crucial to each stage of biofilm formation. In particular, genes responsible for surface and cell attachment as well as the synthesis of matrix components are under QS regulation (Mack et al. 2004 and references therein). *P. aeruginosa* mutants lacking AHL synthesis are unable to form the proper biofilm architecture (Davey and O'Toole; Davies et al. 1998). In addition to autoinducer production, the expression of many other QS-regulated genes such as adhesion proteins is also required for biofilm formation and biofilm dispersal (Mack et al. 2004 and references therein; O'Toole and Kolter 1998). QS allows microbial cells to remain dynamic and respond to changes in the environment. Typically, in Gram-negative bacteria such as *Pseudomonas* sp. and *Vibrio* sp., the induction of QS systems promotes biofilm formation (Aswathanarayan and Rai 2015 and references therein; Ganin et al. 2015 and references therein). In Gram-positive bacteria, this relationship is more complex; for *Bacillus subtilis*, activation of QS-associated genes triggers surface attachment and subsequent development of a biofilm (Singh et al. 2015) where as in *Staphyloccocus aureus*, repression of the *agr* QS system is actually required for biofilm formation (Ganin et al. 2015 and references therein).

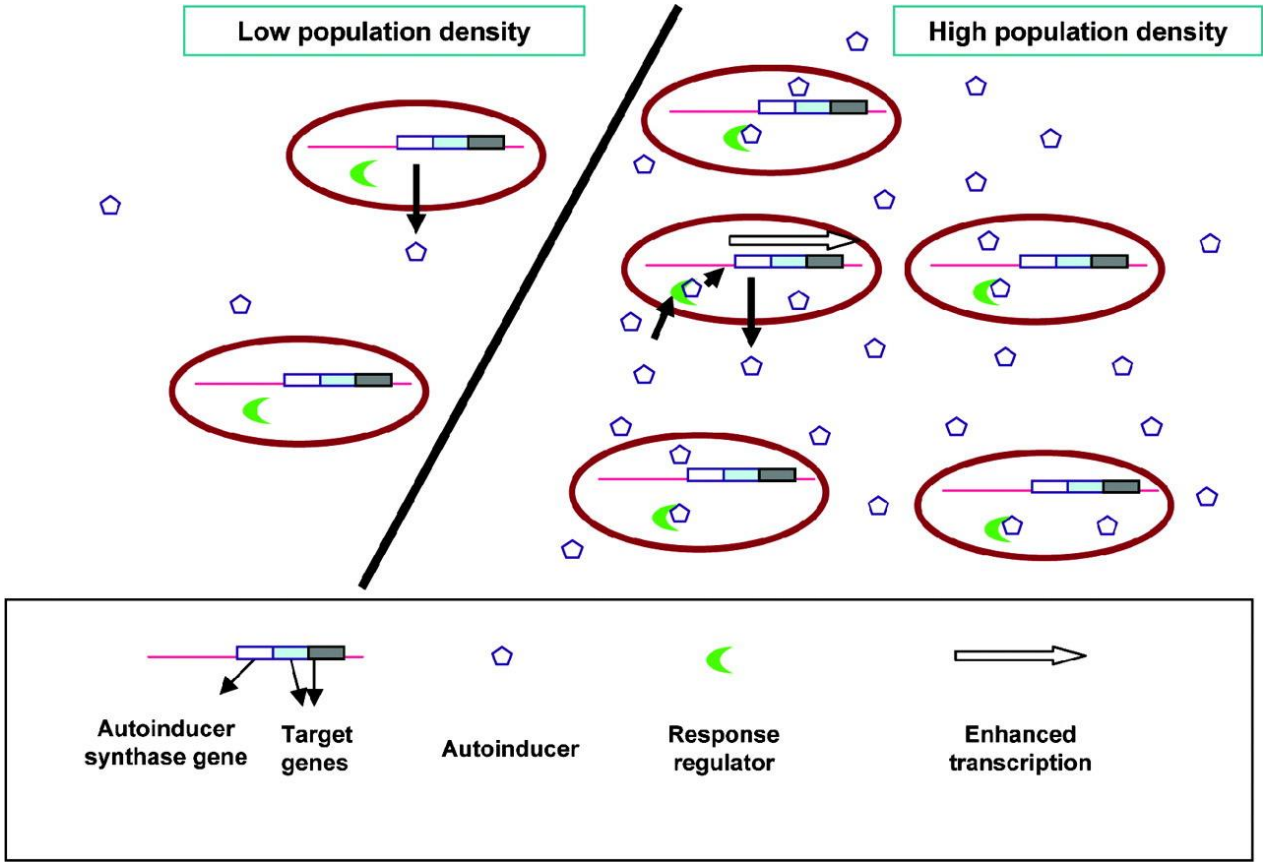

Figure 1.2 Schematic representation of quorum sensing (QS). Autoinducers are constantly produced by the microbes. At low population density, autoinducer concentration is low in the surrounding environment. At high population density, once a threshold concentration is reached, response regulators can be activated to initiate the expression of QS-regulated genes, many of which are responsible for the development of a biofilm (Figure from González and Keshavan 2006, used with permission).

Inhibition of quorum sensing (QS) and biofilm formation by plants

Since the discovery of quorum sensing, much attention has been devoted to finding autoinducer mimics as a possible solution to the biofilm problem. Some of these compounds have been reported in algae and higher plants. The marine red alga *Delisea pulchra* (Greville) Montagne synthesizes halogenated furanones (Figure 1.3) that disrupt QS in bacteria species by mimicking the AHL signal (Givskov et al. 1996; Raffa et al. 2005; Shiner et al. 2005). Similarly,

the green alga *Chlamydomonas reinhardtii* P.A. Dang also produces compounds that interfere with bacterial QS regulation (Teplitski et al. 2004). Among terrestrial plants, pea, soybean, rice, tomato, vanilla, clove, horseradish, grapefruit, and garlic also produce molecules that interfere with QS in various Gram-negative bacterial species (Bjarnsholt et al. 2005; Choo et al. 2006; Gao et al. 2003; Girennavar et al. 2008; Khan et al. 2009; Mathesius et al. 2003; Singh et al. 2009; Teplitski et al. 2000).

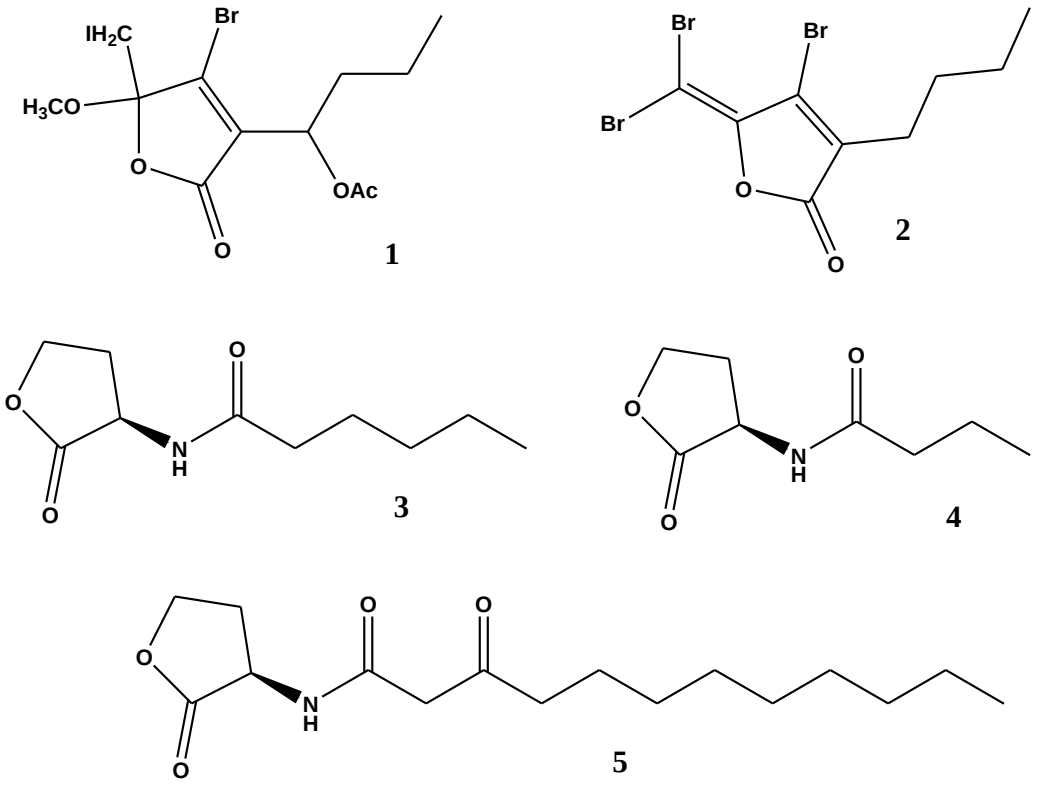

Figure 1.3 Structures of some halogenated furanones produced by marine alga *Delisea pulchra* (**1** and **2**) and *N*–acyl–homoserine lactones (AHLs) used in quorum sensing by *Chromobacterium violaceum* (**3** and **4**) and *Pseudomonas aeruginosa* (**4** and **5**).

The presence of QS modulators in plants is not surprising since they depend on the soil for nutrients and water. Like many other natural environments, the soil contains microbial populations. Plants select for only specific bacteria species (rhizobacteria) to associate with their roots in a symbiosis (Shiner et al. 2005). These plants must have evolved chemicals to prevent

colonization of other unwanted species. Plants that live in constantly wet environments such as tropical forests and cloud forests will have to face similar biofilm problems. The wet conditions are ideal for biofilm formation and we would expect these plants to have compounds interrupting QS (QQs or quorum quenchers) and/or inhibiting biofilm formation. Extracts of *Lonicera japonica* Thumb., *Boesenbergia rotunda* (L.) Mansf., *Eleutherine bulbosa* (Mill.) Urb., *Rhodomyrtus tomentosa* (Aiton) Hassk., *Reynoutria japonica* Houtt., *Leopoldia comosa* (L.) Parl., *Ballota nigra* L., and *Rubus ulmifolius* Schott were reported to inhibit *Pseudomonas aeruginosa*, staphylococcal and streptococcal biofilms (Chen et al. 2004; Limsuwan and Voravuthikunchai 2008; Quave et al. 2008; Song et al. 2007). Furthermore, garlic and Neem (*Azadirachta indica* L.) water extracts, as well as eucalyptus, peppermint, ginger grass and clove oils were shown to inhibit biofilm formation in *Candida albicans* (Agarwal et al. 2008; Polaquini et al. 2006; Shuford et al. 2005).

Recently, triterpenoids such as ursolic acid and derivatives were shown to be inhibitors of both Gram-positive and Gram-negative biofilms (Mallavadhani et al. 2004; Ren et al. 2005). Sesquiterpene lactones from *Acanthospermum hispidum* DC. and *Enydra anagallis* Gardn. showed potent inhibitory activities against *Pseudomonas aeruginosa* biofilm formation (Cartagena et al. 2007). Furthermore, diterpenoids from the roots of *Salvia sclarea* L. showed bactericidal activity against biofilms of antibiotic resistant *Staphylococcus aureus* and *Staphylococcus epidermidis* (Kuźma et al. 2007). We have also isolated antimicrobial terpenes from *Pleodendron costaricensis* N. Zamora, Hammel & Aguilar in the cloud forest of Costa Rica (Amiguet et al. 2006). These studies clearly show terpenes as one target biosynthetic group in the search for biofilm inhibitors.

Antimicrobials in traditional medicine

Ethnobotanical studies of traditional medicine have led to the discovery of many natural products and contributed to our understanding of health and diseases. Mayapple, gingko, and cinchona are a few examples of traditional plants that have had highly significant impacts on modern medicine (Gurib-Fakim 2006). Since there are many different types of infections and their occurrence is common, especially in the tropics, plants used as anti-infectives in traditional medicine are both numerous and from diverse families. Recently, Adonizio et al. (2006) showed anti-QS activity against *Chromobacterium violaceum* by extracts of six traditional anti-infective plants of southern Florida. In a later study, Adonizio et al. (2008) reported that these same extracts were able to inhibit virulence factors production in *Pseudomonas aeruginosa* PAO1. Similarly, Quave et al. (2008) showed that extracts of Italian medicinal plants interfered with QS in *Staphylococcus aureus.* Furthermore, an ethnobotanical study of the Q'eqchi' Maya healers of Belize by our research group identified 96 plant species to treat various conditions of probable bacterial and fungal origins (Amiguet et al. 2005). Further investigation of these plants could yield leads to novel biofilm inhibitors.

Neotropical forests and their botanical diversity

Tropical rainforests encompass only 6% of the world total land mass and yet these are the most biodiverse terrestrial ecosystems both in terms of flora and fauna (Corlett and Primack 2011). The world flora is estimated to have between 223,000 and 420,000 vascular plant species (Govaerts 2003; Scotland and Wortley 2003), 175,000 of which are found in tropical rainforests (Corlett and Primack 2011). These forests, described as tall, dense evergreen forests that are always hot with an absent or short dry season, are distributed throughout the neotropics, Southeast Asia, the Pacific islands, Australia and New Guinea, and parts of India and Africa

(Corlett and Primack 2011). The neotropics or tropical America is defined as the regions in the Western Hemisphere between the Tropic of Cancer (23º 27' N) and the Tropic of Capricorn (23º 27’ S), extending from southern Florida and Mexico to southern Brazil (Smith et al. 2004). Neotropical forests contain approximately 30% of the global 260,000 known angiosperms or flowering plants (Smith et al. 2004). This high biodiversity is due to distinct habitats defined by unique geological features and variation in elevation and annual precipitation (Corlett and Primack 2011).

Depending on the altitude and amount of rainfall, neotropical forests can be classified into different types: seasonal dry forests, semi-evergreen subtropical and tropical forests, evergreen tropical forests, lowland forests, montane forests, and cloud forests. Seasonally dry forests have a distinct dry season and the canopy consists only of one storey of deciduous trees less than 10 m in height. Semi-evergreen forests contain a large proportion of deciduous trees in the canopy (two to three storeys) while evergreen forests do not (Corlett and Primack 2011). The canopies in evergreen forests are three storeys and consist of very large trees that can be up to 50 m in height. Lowland forests are found below 900 to 1200 m above sea level while montane forests occur at higher elevation (Corlett and Primack 2011). Cloud forests occupy altitudes higher than 1500 m and are frequently covered in fog or mist (Montagnini and Jordan 2005). Tropical rainforests are characterized by an abundant and diverse flora of lianas and epiphytes (Corlett and Primack 2011). The epiphytic communities are especially well developed in the Neotropics (Corlett and Primack 2011). Seedlings and saplings of trees, shrubs, or vines usually cover the forest floor as herbaceous species can only grow in canopy gaps or steep slopes of montane forests due to low light levels in the canopy understorey (Corlett and Primack 2011). In contrast to the Asian dipterocarp forests, neotropical canopy understoreys have many treelets and

shrubs with flowers and fruits (Corlett and Primack 2011). Most American rainforests receive approximately 2000 to 3000 mm of rain annually; in the Caribbean coast of Central America, rainfall can be more than 3000 mm (Montagnini and Jordan 2005). The annual dry season of the neotropics lasts from 1 to 4 months (Corlett and Primack 2011).

Plant families of interest: characteristics and economic uses

In this research, major and rare neotropical plant families were examined for their biological properties and phytochemical composition. These include the Lepidobotryaceae, Marcgraviaceae, Melastomataceae, Meliaceae, and Piperaceae. Plants were collected from various types of neotropical forests in Costa Rica and Belize: lowland evergreen, seasonally dry semi-evergreen, montane, and cloud forests. Worldwide, there are only two genera in the Lepidobotryaceae (*Lepidobotrys* and *Ruptiliocarpon*), each with only one species. Only *Ruptiliocarpon* is found in the neotropics while *Lepidobotrys* is present in Africa. In Costa Rica, *Ruptiliocarpon caracolito* L. (a timber species) grows in well-drained soil in lowland tropical rainforests from sea level to 400 m (Hammel and Smith 2004).

The Marcgraviaceae is endemic to the neotropics with approximately 130 species of terrestrial and hemi-epiphytic lianas and shrubs (Dressler 2004). Most Marcgraviaceae species grow in humid evergreen tropical lowland forest, montane rainforests, and cloud forests (Dressler 2004). With the exception of *Ruyschia*, most species also prefer, but are not limited to, higher altitudes. Some Marcgraviaceae species are cultivated while others from the genera *Marcgravia*, *Norantea*, *Sarcopera*, and *Souroubea* are used for the treatment of headaches, toothaches, insect bites, diarrhoea, and syphilis by some indigenous groups (Dressler 2004).

The Melastomataceae (black mouth family) is one of the largest neotropical families with 3000 species and 167 genera found in many habitats with varying moisture and elevation (Renner 2004). These species are predominantly understorey shrubs, treelets, herbs, lianas, and epiphytes that grow in tropical montane forests; a few occur in lowland forests and seasonally dry grassland (Renner 2004). *Miconia* is the largest genus with 1000 species. Melastomataceae species have few economic uses; *Tibochina* species are cultivated as ornamental for their flowers (Renner 2004). Traditionally, the leaves, bark, and fruits of some species are also used as a blue dye (Renner 2004).

The mahogany family or Meliaceae consists of 52 genera of trees and rarely shrubs; species are well known for their timber value (Pennington 2004). The largest genera, *Guarea* (35 species) and *Trichilia* (70 species), are found in lowland forests; some *Guarea* species can grow at higher elevation in montane and cloud forests up to 2500 m (Pennington 2004). All *Ruagea* species can be found at 1500 to 3200 m while a few *Trichilia* species prefer dry forests (Pennington 2004). *Swietenia* and *Cedrela* species can grow in both wet evergreen forests and deciduous forests with well-defined dry seasons (Pennington 2004). In addition to the timber value of many species, essential oil of *Carapa guianensis* Aubl. seeds are used as soaps and insect repellent (Pennington 2004). Furthermore, Neem oil (*Azadirachta indica* L.) is a well known insecticide (Omar et al. 2003).

The pepper family (Piperaceae) is pantropical with approximately 2000 species (Nee 2004). These plants prefer to grow in moist conditions at mid to low elevation (Nee 2004). *Piper* species are gap species and often found in disturbed habitats (Nee 2004). *Peperomia* species are mostly epiphytic with a few terrestrial species adapted to long and severe dry seasons (Nee 2004). Very few neotropical Piperaceae have commercial value; some *Peperomia* are grown as

houseplants. In contrast, many neotropical Piperaceae are used in traditional medicine for treatment of various conditions (Bourbonnais-Spear 2005; Schultes and Raffauf 1990).

1.3 Objectives

The overall objective of this thesis was to identify neotropical species and their bioactive phytochemicals that can interfere with fungal growth and bacterial biofilm formation. In particular, a few prominent plant families from different regions of Costa Rica were assessed for their abilities to inhibit bacterial quorum sensing and biofilm formation (Chapter 2). Traditional Q'eqchi' Maya medicines from Belize were examined for antifungal, quorum sensing and biofilm inhibitory activities (Chapter 3). Similarly, Costa Rican and Brazilian Marcgraviaceae species were assessed for the same biological activities (Chapter 4). Bioassay-guided isolation from active plant species led to the identification of two antifungals from *Marcgravia nervosa* Triana & Planch. (Chapter 4) and *Cestrum schlechtendahlii* G.Don (Chapter 5). Chapter 6 describes the mechanistic study of antifungal action for the active principle of *C. schlechtendahlii*.

Chapter 2 – Quorum sensing and biofilm modulatory activities of neotropical rainforest plants

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Statement of author contribution

JTA and CAT conceived and designed this study. TM and ALA provided technical support for the bioassays. Plant collection was done by CAT, MO, MG, TD, KD, and JTA. Plant identification was done by MO, MG, PS, and LP. Plant extraction and bioassays were performed by CAT with assistance from MF. JAM and BJB provided alga extract for positive control. TM, ALA, and JTA contributed in manuscript preparation.

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2.1 Introduction

Bacterial biofilms develop when an exopolysaccharide matrix protecting a population of bacterial cells growing on a surface occurs through the coordinated gene expression between individual cells (Davey and O'Toole 2000; Davies et al. 1998; Donlan and Costerton 2002; Hall-Stoodley et al. 2004). In many bacterial species, this process is initiated by quorum sensing (QS), an intercellular communication system which is regulated by signalling molecules called autoinducers (Davey and O'Toole 2000; Davies et al. 1998; Donlan and Costerton 2002; Hall-Stoodley et al. 2004). These autoinducers are self-produced and species-specific; they include *N*–acyl–homoserine lactones (AHLs) in Gram-negative bacteria and autoinducing peptides (AIPs) in Gram-positive bacteria (Davey and O'Toole 2000; Davies et al. 1998; Donlan and Costerton 2002; Hall-Stoodley et al. 2004). Biofilm growth provides many advantages over the planktonic state such as protection from desiccation and antimicrobials, metabolic cooperation, and increased genetic diversity via horizontal gene transfer (Davey and O'Toole 2000; Davies et al. 1998; Donlan and Costerton 2002).

Bacterial biofilms develop on many living organisms, such as marine algae, humans and terrestrial plants. They are both beneficial (resident bacteria on plant leaves and roots to prevent unwanted colonization from potential pathogens) and harmful (chronic infections in cystic fibrosis and periodontitis) (Davey and O'Toole 2000; Donlan and Costerton 2002). Many pathogens are also biofilm formers; these include *Pseudomonas aeruginosa* and *Burkholderia cenocepacia* in cystic fibrosis (Brackman et al. 2009; Singh et al. 2000) *Campylobacter jejeuni* in food poisoning (Kalmokoff et al. 2006), and staphylococci in device-related infections (Donlan and Costerton 2002; Mukherjee and Chandra 2004). Biofilms exacerbate the current

antibiotics resistance situation since bacteria in biofilms have been reported to be up to 1000 times less susceptible to antimicrobials than their planktonic equivalents (Gilbert et al. 1997).

It is not surprising that natural product inhibitors of biofilm formation and QS have been identified since biofilms may not always be advantageous to the host organism. One of the first group of QS inhibitors was discovered in the marine alga *Delisea pulchra* (Greville) Montagne; this species produces halogenated furanones that act as natural antifouling agents against unwanted bacteria (Givskov et al. 1996; Raffa et al. 2005; Shiner et al. 2005). Among terrestrial plants, pea, tomato, vanilla, grapefruit, and garlic also produce molecules that interfere with QS in various bacteria species (Bjarnsholt et al. 2005; Choo et al. 2006; Girennavar et al. 2008; Teplitski et al. 2000). In a recent study of a medicinal plant, *Rubus ulmifolius* Schott, used for dermatological conditions, ellagic acid derivatives were shown to inhibit *Staphylococcus aureus* biofilm formation with a minimum inhibitory concentration (MIC_{90}) in the range of 50-200 μg/mL (Quave et al. 2012). Ellagitannins from *Conocarpus erectus* L. were previously shown by Adonizio (2008) to inhibit QS and biofilm formation in *P. aeruginosa*. Protolichesterinic acid, produced by lichens, also has been reported to inhibit quorum sensing in *Burkholderia cenocepacia* and *P. aeruginosa* (Riedel et al. 2008).

Plants select for specific bacteria species (rhizobacteria) to associate with their roots in a symbiosis (Shiner and Williams 2005) and these plants must have evolved chemicals to prevent colonization of other unwanted species. Plants that live in constantly wet environments such as tropical rainforests and cloud forests will have to face the similar biofilm problems. The wet conditions are ideal for biofilm formation, and the fouling of leaves provides an opportunity for detrimental growth of epiphylls on leaves and epiphytes on stems and bark. It is expected that these plants will have compounds interrupting QS (QQs or quorum quenchers) and inhibiting

biofilm formation. Work by Ren et al. (2005) has demonstrated that ursolic acid (prominent in trees of the humid tropics) inhibits *E. coli*, *P. aeruginosa*, and *Vibrio harveyi* biofilm formation. Sesquiterpene lactones from *Acanthospermum hispidum* DC. and *Enhydra anagallis* Gardn. showed potent inhibitory activities against *P. aeruginosa* biofilm formation (Cartagena et al. 2007). Furthermore, diterpenoids from the roots of *Salvia sclarea* L. showed bactericidal activity against biofilms of antibiotic resistant *S. aureus* and *S. epidermidis* (Kuźma et al. 2007). Adonizio et al. (2006, 2008) showed that medicinal plants of Southern Florida had anti-quorum sensing activity against *Chromobacterium violaceum*, *Agrobacterium tumefaciens*, and *P. aeruginosa*. Similarly, Quave et al. (2011) reported QS inhibition of *S. aureus* by Italian medicinal plants. Cech et al. (2012) also reported quorum quenching activity of goldenseal extract (*Hydrastis canadensis* L.) against methicillin-resistant *S. aureus.*

As yet, little is known about the taxonomic and chemical ecology determinants of biofilm inhibition. In the present study, we examined several prominent neotropical families for their potential to inhibit biofilms and quorum sensing. The Meliaceae are a family of emergent and mid-canopy trees; the Melastomataceae are mainly understory trees and shrubs and the Piperaceae are shrubs of forest gaps, and edges. In addition we investigated several families where the leaves were remarkably free of fouling (Lepidobotryaceae, Sapindaceae and Simaroubaceae). In total, we examined 126 plant extracts from 71 species and six families.

2.2 Materials and Methods

2.2.1 Plant collection

All plant materials were collected from Costa Rica and Fairchild Tropical Botanic Garden (Florida, USA). Plant specimens were preserved in 70% ethanol immediately after collection. Vouchers were deposited at the University of Ottawa Herbarium (OTT), Herbario Juvenal Valerio Rodriguez (JVR), and Fairchild Tropical Botanic Garden Herbarium (FTG). Plant species identification was confirmed by M. Garcia, M. Otárola-Rojas, P. Sanchez-Vindas, and L. Poveda. Algal collection of *Delisea pulchra* was made at Palmer Station, Antarctica and a voucher was deposited at this research station. Taxonomic identification was performed by Charles Armsler (University of Alabama at Birmingham). Voucher numbers are listed in Table 2.1.

2.2.2 Plant extraction

Plant materials were ground with a blender (Waring commercial LR 8992) and extracted with 80% EtOH using a 1:10 biomass to solvent (w/v) ratio. This solvent system has been determined previously to extract most types of phytochemicals with varying polarities. The plant material/ethanol mixture was shaken overnight at 200 rpm (shaker: New Brunswick Scientific, Edison, NJ, USA) and then filtered using vacuum filtration. A second extraction was performed with the residue using a 1:5 w/v ratio and the mixture was again shaken and filtered. The two extracts were combined, dried *in vacuo* using a rotary evaporator (Yamato RE 500) at 45°C, lyophilized to remove any residual water using a freeze-dryer (EC Super Modulyo, ~ -55 °C, 10^{-2} mbar), and stored at -20°C in the dark until needed.

2.2.3 Bacteria strains and culture

Chromobacterium violaceum ATCC 12472 was used in the quorum sensing bioassay and *Pseudomonas aeruginosa* PA14 in the biofilm bioassay. *Chromobacterium violaceum* was purchased from American Type Culture Collection (ATCC) and *Pseudomonas aeruginosa* PA14 was obtained from T. Mah (University of Ottawa). These strains were maintained as liquid culture with shaking (200 rpm) at 30^{o}C (incubator: New Brunswick Scientific Series 25, Edison, NJ, USA) in nutrient broth (BD, Sparks, MD, USA) and LB broth (Fisher Scientific, Fairlawn, NJ, USA), respectively.

2.2.4 Quorum sensing (QS) bioassay

A modified disc diffusion assay described by Adonizio et al. (2006) was used to determine whether the plant extracts can interfere with the QS of *C. violaceum. C. violaceum* produces a purple pigment, violacein, which is under QS control. The inhibition of violacein production will indicate the disruption of QS. Briefly, sterile paper disks (Oxoid, Basingstoke, Hants, UK) loaded each with 1 mg of extract were placed onto TGY agar plates (BD, Sparks, MD, USA) inoculated with 100 µL of overnight cultures then incubated without agitation for 24 hours at 30^{o}C (incubator: Precision Automatic CO_2 Incubator). QS inhibition was indicated by a colourless opaque halo around the disc and growth inhibition by a clear halo. Plates were examined under a dissecting microscope to confirm whether the extract has anti-QS and/or antibacterial activity. The controls used were: 70% ethanol as vehicle control and *N*–decanoyl–L–homoserine lactone (C10–HSL) (400 µg/disc, ≥96%, Cayman Chemical, Ann Arbor, MI, USA) as positive control. Extract of *Delisea pulchra* (Greville) Montagne (1 mg/disc) was also included for comparative purposes as this alga contains known QS inhibitors, the halogenated

furanones (Givskov et al. 1996; Raffa et al. 2005; Shiner and Williams 2005). Each sample was tested in triplicate.

2.2.5 Biofilm bioassay

A spectrophotometric assay was adapted from Ren et al. (2005) to assess the biofilm inhibitory activity of the plant extracts. Extracts were prepared in 50% methanol and tested at a final concentration of 400 μg/mL. Overnight culture of *P. aeruginosa* was diluted 1:50 in M63 medium broth (Amresco, Solon, OH) supplemented with 0.4% arginine and 1mM $MgSO_4$. This inoculum was grown in a 24-well flat bottom microtiter plate (Costar 3526) without agitation for 24 hours at 37ºC to allow biofilm formation (incubator: Precision Automatic CO_2 Incubator). The plate was then decanted and washed with distilled water three times to eliminate non-adherent cells. The remaining biofilm was stained with 0.1% crystal violet (Fisher Scientific, Fairlawn, NJ) for 15 minutes. The excess dye was decanted, the plate was washed three times with distilled water, and then air dried overnight. The biofilm was quantified by dissolving the dye in ethanol and reading the absorbance at 570 nm (spectrophotometer: SpectraMax M5, Molecular Devices, Sunnyvale, CA, USA). The controls used were: vehicle control with 50% methanol, negative control with M63 medium broth, and positive control with allicin (1 μL/mL or 1.08 mg/mL, 97%, AK Scientific Inc., Mountain View, CA, USA). Treatments were added before incubation. Biofilm formation was expressed relative to the vehicle control. Samples were tested in triplicate.

2.2.6 Statistical analysis

Statistical analyses were done using SigmaPlot 11 software for Windows (Systat Software, San Jose, CA, USA). Results are presented as means ± S.E.M. (standard error of the mean) for at least 3 independent experiments. One-way ANOVA was performed with posthoc Tukey's test and $p < 0.01$ considered as significant.

2.3 Results

Plant extracts were tested for their ability to interfere with bacterial quorum sensing (QS) and biofilm formation (Table 2.1). Extract yields ranged from 7% to 10% of raw plant materials. Preliminary assessments showed that there was a broad range of QS and biofilm inhibitory activities with a third of the plant species showing significant inhibition. These species belong to the Lepidobotryaceae, Melastomataceae, Meliaceae, Sapindaceae, and Simaroubaceae families. Piperaceae species showed little or no activity. A summary of inhibitory activities (Figure 2.1) for the three main plant families examined (Meliaceae, Melastomataceae, and Piperaceae)

showed there was no statistical difference in average QS inhibition between the families. However, Melastomataceae and Meliaceae bark and wood showed significantly more inhibitory activities against biofilm formation than Piperaceae. For many Meliaceae species, bark and wood showed more inhibitory activities than leaves except for *C. fissilis*, *C. salvadorensis*, *T. martiana*, and *T. pleeana* where leaves displayed more inhibition than other plant parts. C10–HSL, a positive control (McClean et al. 1997), showed only modest anti-QS activity by comparison (10.2 ± 0.1 mm). Extract of *Delisea pulchra* (Greville) Montagne containing halogenated furanones (known QS inhibitors) showed an inhibition of 12.8 ± 0.7 mm. Of the 19 Melastomataceae species tested, nine displayed more QS inhibition than *D. pulchra*; these include *A. rubricaulis*, *C. dentata*, *C. rhodopetala*, *L. melanodesma*, *M. longifolia*, *M. floribundum*, *M. vulcanicum*, *O. brenesii*, and *T. maurofernandeziana*. For the 30 Meliaceae species, a third showed stronger anti-QS activities than *D. pulchra* (*C. fissilis* leaves, *C. salvadorensis* leaves, *C. tabularis* leaves/bark/wood, *G. guidonia* leaves, *G.pterorhachis* leaves and bark, *G. pyriformis* bark, *S. macrophylla* bark, *S. mahogani* wood, *T. pleeana* leaves, and *T. septentrionalis* leaves and wood). In other families, all parts of *Ruptiliocarpon caracolito* L.

(Lepidobotryaceae) except bark and *Simarouba glauca* DC. (Simaroubaceae) leaves and bark also displayed more QS inhibition than the positive control *D. pulchra.* Many plant extracts also had growth inhibition of 1 to 5 mm beyond the disc while *R. caracolito* and *C. tabularis* showed inhibitory zones of 10 to 12 mm.

For biofilm growth, 40% of extracts showed either no inhibition or actually enhanced the formation of the biofilm mass when compared to the vehicle control (50% methanol). Thirteen Meliaceae and Melastomataceae species showed significantly more biofilm inhibition than the positive control allicin (74.4 ± 8.1% growth). These species include *A. rubricaulis*, *C. dentata, C. pittieri, L. melanodesma, M. floribundum, T. maurofernadeziana*, *C. guianensis* bark and wood, *C. odorata* bark, *C. tabularis* all parts, *G. pterorhachis* leaves, *S. macrophylla* bark, *T. martiana* leaves, and *T. septentrionalis* wood. In other families, all parts of *Ruptiliocarpon caracolito* L. (Lepidobotryaceae) and the leaves and bark of *Cupania glabra* Sw. (Sapindaceae) also showed significant inhibition when compared to allicin.

QS and biofilm inhibition are not always in accordance in these extracts with some displaying only QS inhibitory activities, some only biofilm inhibitory activities, and others with both types of inhibition. Species with both types of inhibition include: *Ruptiliocarpon caracolito* L. (leaves, fruits, and wood), *Aciotis rubricaulis* (Mart. ex DC.) Triana, *Clidemia dentata* D. Don, *Monochaetum floribundum* Naudin, *Topobea maurofernadeziana* Cogn., *Chukrasia tabularis* A. Juss. (bark, wood, and leaves), *Guarea pterorhachis* Harms (bark), and *Trichilia septentrionalis* C. DC. (wood). IC_{50}s for biofilm inhibition some of the most active species ranged from 45 to 266 μg/mL (Table 2.2).

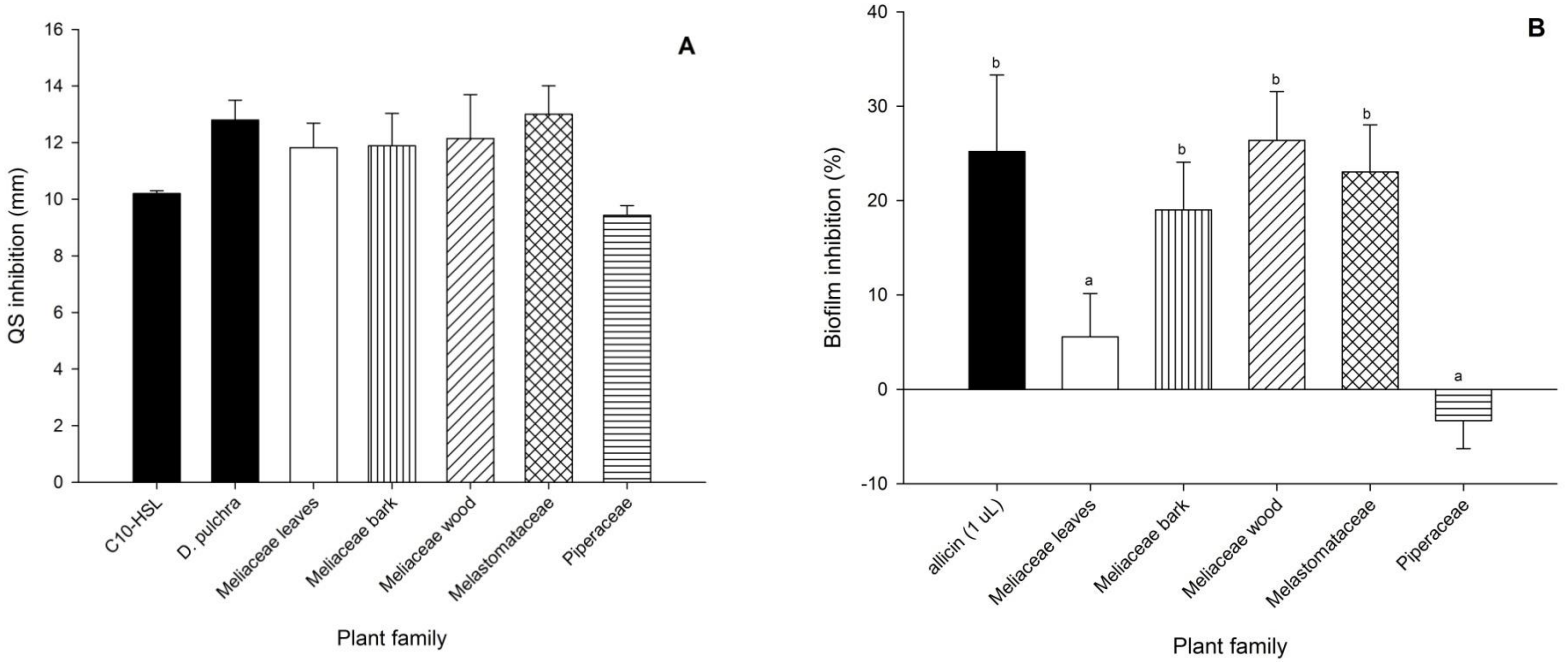

Figure 2.1 Average inhibition of quorum sensing in *Chromobacterium violaceum* (A) and biofilm formation in *Pseudomonas aeruginosa* PA14 (B) by three main plant families (n = 15–28) compared to the positive controls C10–HSL (400 µg) and allicin (1 µL or 1.08 mg); Groups with different letters are statistically different ($p < 0.01$).

Table 2.1 Effects of plant extracts on violacein production in *Chromobacterium violaceum* ATCC 12472 (1 mg/disc, N = 3–7, disc diameter = 7 mm, vehicle control: 70% EtOH) and biofilm formation in *Pseudomonas aeruginosa* PA14 (400 µg/mL, N = 3–7, vehicle control: 50% MeOH).

Voucher	Family	Species	Part(s)[a]	Location	Elevation	Violacein inhibition ± SEM (mm)	% Biofilm growth ± SEM
OTT19109	Lepidobotryaceae	*Ruptiliocarpon caracolito* L.	B	Golfito	100 m	11.1 ± 1.6	54.1 ± 4.6[b]
			L			28.8 ± 0.3[b,e]	23.2 ± 2.0[b]
			F			28.3 ± 0.2[b,e]	53.5 ± 5.9[b]
			Wo			21.6 ± 0.3[b,e]	40.5 ± 2.9[b]
JVR12816	Melastomataceae	*Aciotis rubricaulis* (Mart. ex DC.) Triana	A	Horquetas, Sarapiqui	155 m	16.5 ± 0.4[b,c]	44.1 ± 1.7[b]
JVR12807	Melastomataceae	*Blakea gracilis* Hemsl.	L	San Geraldo de Rivas	1300 m	9.3 ± 0.1	69.9 ± 6.3
JVR12802	Melastomataceae	*Centradenia grandifolia* (Schltdl.) Endl.	A	Reserva Cloud Bridge	1500 m	12.4 ± 0.2[c]	81.9 ± 5.6
JVR12815	Melastomataceae	*Clidemia dentata* D. Don	A	Horquetas, Sarapiqui	164 m	17.9 ± 0.3[b,d]	54.7 ± 4.4[b]
JVR12809	Melastomataceae	*Conostegia monteleagreana* Cogn. en A. DC.	L	Parque Nacional Braulio Carillo	1200 m	8.8 ± 0.1	82.8 ± 4.7
JVR7634	Melastomataceae	*Conostegia pittieri* Cogn. ex T.Durand	L	Monte de la Cruz	2081 m	8.9 ± 0.2	53.7 ± 5.1[b]
JVR12805	Melastomataceae	*Conostegia rhodopetala* Donn. Sm.	L	Reserva Cloud Bridge	1500 m	17.5 ± 0.3[b,c]	81.8 ± 4.8
JVR12811	Melastomataceae	*Conostegia subcrustilata* (Beurl.) Triana	A	Horquetas, Sarapiqui	48 m	8.7 ± 0.1	112.1 ± 4.9
JVR12808	Melastomataceae	*Conostegia xalapensis* (Bonpl.) D. Don ex DC.	A	Juan Viñas	600 m	8.6 ± 0.1	104.1 ± 13.7
JVR12804	Melastomataceae	*Leandra melanodesma* (Naudin) Cogn.	L, S	Reserva Cloud Bridge	1558 m	14.3 ± 0.9	54.7 ± 4.0[b]
JVR12812	Melastomataceae	*Miconia affinis* DC.	L	Horquetas, Sarapiqui	155 m	8.6 ± 0.1	102.8 ± 5.1

Table 2.1 Continued

Voucher	Family	Species	Part(s)[a]	Location	Elevation	Violacein inhibition ± SEM (mm)	% Biofilm growth ± SEM
JVR12818	Melastomataceae	*Miconia barbinervis* (Benth.) Triana	L	Rio Costa Rica	203 m	8.8 ± 0.1	115.9 ± 9.6
JVR12801	Melastomataceae	*Miconia caudata* (Bonpl.) DC.	A	Reserva Cloud Bridge	1558 m	9.9 ± 0.3	86.4 ± 2.6
JVR12813	Melastomataceae	*Miconia longifolia* (Aubl.) DC.	L	Horquetas, Sarapiqui	155 m	19.3 ± 0.3 [b,d]	82.2 ± 4.4
JVR13216	Melastomataceae	*Miconia pittieri* Cogn.	L, S	Monte de la Cruz	1600 m	8.0 ± 0.1	77.9 ± 12.1
JVR12806	Melastomataceae	*Monochaetum floribundum* Naudin	A	Monte de la Cruz	1919 m	16.7 ± 0.2 [b,c]	46.0 ± 4.4 [b]
JVR12820	Melastomataceae	*Monochaetum vulcanicum* Cogn.	A	Monte de la Cruz	2094 m	16.6 ± 0.2 [b,c]	71.8 ± 4.2
JVR12810	Melastomataceae	*Ossaea brenesii* Standl.	A	Parque Nacional Braulio Carillo	1200 m	16.4 ± 0.1 [b,c]	83.1 ± 4.9
JVR12817	Melastomataceae	*Topobea maurofernandeziana* Cogn.	A	Rio Costa Rica	220 m	19.9 ± 0.3 [b,d]	56.2 ± 9.6 [b]
JVR12252	Meliaceae	*Azadirachta indica* A.Juss.	B	Cañas	100 m	14.0 ± 0.7	62.6 ± 2.2
			L			8.8 ± 0.4	100.3 ± 2.2
			Wo			8.0 ± 0.1	85.0 ± 4.1
FTG65568	Meliaceae	*Aphanamixis polystachya* (Wall.) R. Parker	L	Fairchild Tropical Garden	3 m	8.9 ± 0.2	90.5 ± 3.6
			B			12.4 ± 0.2	57.4 ± 4.9
OTT19049	Meliaceae	*Cabralea canjerana* (Vell.) Mart.	Wo	Turrialba	600 m	7.8 ± 0.1	107.2 ± 10.2
			B			8.3 ± 0.1	90.5 ± 6.2
JVR10721	Meliaceae	*Carapa guianensis* Aubl.	B	Puerto Viejo	40–60 m	9.0 ± 0.1	54.8 ± 5.4 [b]
			L			8.2 ± 0.2	79.6 ± 4.6
			Wo			8.8 ± 0.1	50.7 ± 4.9 [b]
JVR11573	Meliaceae	*Cedrela fissilis* Vell.	B	Finca La Selva	220 m	13.0 ± 0.2	66.7 ± 1.8
			L			18.4 ± 0.8 [b]	98.7 ± 2.0

Table 2.1 Continued

Voucher	Family	Species	Part(s)[a]	Location	Elevation	Violacein inhibition ± SEM (mm)	% Biofilm growth ± SEM
OTT19009	Meliaceae	*Cedrela odorata* L.	Wo	Monteverde	1300 m	10.0 ± 0.3	85.2 ± 10.8
			B			8.0 ± 0.3	41.4 ± 3.5[b]
			L			11.2 ± 0.2	100.0 ± 5.1
			F			8.4 ± 0.2[c]	109.3 ± 4.0
OTT19015	Meliaceae	*Cedrela salvadorensis* Standl.	L	Ciudad Colon	800 m	21.1 ± 0.4	108.6 ± 4.2
			B			7.6 ± 0.1	70.7 ± 5.6
OTT19008	Meliaceae	*Cedrela tonduzii* C. DC.	F	Cerro del	2300 m	8.2 ± 0.1	NT[f]
			Wo	Chompipe		9.0 ± 0.2	89.2 ± 0.1
			L			8.9 ± 0.2	87.1 ± 4.9
			B			8.8 ± 0.1	74.2 ± 4.9
FTG24968	Meliaceae	*Chukrasia tabularis* A.Juss.	B	Fairchild Tropical	3 m	27.8 ± 0.1[b,e]	57.2 ± 6.2[b]
			Wo	Garden		23.6 ± 0.3[b,e]	45.1 ± 4.5[b]
			L			26.0 ± 1.3[b,e]	55.6 ± 6.0[b]
JVR12114	Meliaceae	*Guarea bullata* Radlk.	L, S	Reserva Biologica Tirimbina	189 m	9.4 ± 0.2	70.3 ± 5.6
JVR11804	Meliaceae	*Guarea glabra* Vahl.	L	San Juan de	900 m	7.3 ± 0.1	64.2 ± 1.8
			B	Puriscal		7.7 ± 0.2	98.3 ± 1.9
JVR12108	Meliaceae	*Guarea grandifolia* DC.	L, S	Parque Nacional	1400 m	10.8 ± 0.2	97.8 ± 4.1
			B	Tapanti		10.3 ± 0.2	90.9 ± 5.5
			F			7.9 ± 0.1	103.1 ± 2.4
JVR3412	Meliaceae	*Guarea guidonia* (L.) Sleumer	B	Palmar Norte	25 m	12.8 ± 1.4	90.9 ± 5.5
			L			17.2 ± 0.6[b]	84.0 ± 6.8
JVR8390	Meliaceae	*Guarea pterorhachis* Harms	B	Golfito	500 m	20.3 ± 0.2[b]	54.6 ± 6.5[b]
			L			15.6 ± 0.1	57.5 ± 5.2
JVR13220	Meliaceae	*Guarea pyriformis* T.D. Penn.	B	Palmar Norte	25 m	24.9 ± 0.2[b]	80.8 ± 8.7
			L			8.0 ± 0.1	99.9 ± 0.1
JVR12252	Meliaceae	*Ruagea insignis* (C. DC.) T.D. Penn.	B	Parque Nacional	1000 m	8.5 ± 0.2	108.0 ± 8.5
			L	Braulio Carillo		8.7 ± 0.2	100.5 ± 7.6

Table 2.1 Continued

Voucher	Family	Species	Part(s)[a]	Location	Elevation	Violacein inhibition ± SEM (mm)	% Biofilm growth ± SEM
OTT19019	Meliaceae	*Swietenia macrophylla* King	B	La Pacifica,	250 m	14.4 ± 0.3	48.5 ± 5.3 [b]
			L	Guanacaste		9.9 ± 0.1	93.9 ± 2.4
			Wo			12.9 ± 0.1	61.4 ± 6.7
FTG63640	Meliaceae	*Swietenia mahogani* (L.)	B	Fairchild Tropical	3 m	12.3 ± 0.2	58.0 ± 4.7
		Jacq.	Wo	Garden		19.6 ± 0.5 [b]	63.3 ± 2.6
			L			8.6 ± 0.1	85.7 ± 7.2
JVR4354	Meliaceae	*Toona ciliata* M.Roem.	L	Cercanias del	1150 m	9.0 ± 0.2	152.3 ± 12.7
			Wo	Pirro		8.9 ± 0.2	72.1 ± 4.4
			B			7.3 ± 0.2	102.6 ± 2.8
JVR12697	Meliaceae	*Trichilia americana* (Sesse	B	Aguas Zarcas	500 m	10.8 ± 0.1[c]	139.6 ± 15.3
		& Moc.) T.D. Penn.	L			10.6 ± 0.2[c]	112.7 ± 9.7
			S			10.4 ± 0.1[c]	112.7 ± 9.7
			F			8.1 ± 0.0	134.6 ± 17.5
JVR4168	Meliaceae	*Trichilia glabra* L.	F	Parque Nacional	200 m	10.3 ± 0.1	127.5 ± 7.6
			L	Palo Verde		8.1 ± 0.1	104.7 ± 3.0
			Wo			10.4 ± 0.3	85.9 ± 6.2
JVR7072	Meliaceae	*Trichilia havanensis* Jacq.	B	Cerro del	2300 m	8.6 ± 0.2	119.5 ± 4.8
			L	Chompipe		10.2 ± 0.1	144.6 ± 5.6
			Wo			8.3 ± 0.2	111.7 ± 13.3
JVR647	Meliaceae	*Trichilia hirta* L.	Wo	Naranjo Parque	10 m	12.2 ± 0.6	74.2 ± 6.4
			B	Santa Rosa		10.2 ± 0.2	104.2 ± 7.1
			L			8.3 ± 0.1	104.8 ± 8.5
JVR3000	Meliaceae	*Trichilia martiana* C. DC.	F	Caño Negro	300 m	8.0 ± 0.1	87.3 ± 7.3
			B			12.3 ± 0.3	93.6 ± 2.1
			L			11.9 ± 0.2	42.0 ± 4.6 [b]
JVR6832	Meliaceae	*Trichilia pittieri* C. DC.	S	Reserva Biologica	200 m	8.1 ± 0.1	90.4 ± 7.3
			L	Carara		11.3 ± 0.2	102.1 ± 5.9

Table 2.1 Continued

Voucher	Family	Species	Part(s)[a]	Location	Elevation	Violacein inhibition ± SEM (mm)	% Biofilm growth ± SEM
JVR360	Meliaceae	*Trichilia pleeana* (A. Juss.) C. DC.	B	Reserva Biologica Carara	200 m	9.8 ± 0.1	78.6 ± 4.9
			Wo			9.6 ± 0.2	59.3 ± 2.5
			L			17.8 ± 0.3 b	79.1 ± 4.9
JVR12118	Meliaceae	*Trichilia quadrijuga* Kunth.	L, S	Parque Nacional Tapanti	1419 m	10.1 ± 0.2	105.5 ± 5.4
			B			7.0 ± 0.1	100.0 ± 0.6
JVR7069	Meliaceae	*Trichilia quadrijuga* ssp. *cinerascens* Kunth, (C. DC.) T.D. Penn.	L	Reserva Biologica Carara	200 m	11.4 ± 0.3	128.9 ± 9.7
JVR6031	Meliaceae	*Trichilia septentrionalis* C. DC.	L	Estacion Biologica Marenco	100 m	15.3 ± 0.3c	93.2 ± 2.8
			Wo			26.1 ± 0.2 b,c	56.1 ± 3.1[b]
JVR7311	Meliaceae	*Trichilia trifolia* L.	Wo	Parque Nacional Santa Rosa	250 m	7.6 ± 0.1	57.9 ± 2.4
JVR12106	Piperaceae	*Peperomia angularis* C. DC	W	Volcan Poas	2023 m	7.8 ± 0.2	100.4 ± 0.3
JVR12117	Piperaceae	*Peperomia hernandifolia* (Vahl) A. Dietr.	W	Parque Nacional Tapanti	1217 m	10.0 ± 0.2	97.5 ± 6.8
JVR12105	Piperaceae	*Peperomia palmana* C .DC.	W	Volcan Poas	2023 m	10.0 ± 0.2	138.7 ± 10.8
JVR12121	Piperaceae	*Peperomia vinasiana* C. DC.	W	Parque Nacional Tapanti	1400 m	7.5 ± 0.1	97.4 ± 4.3
JVR12119	Piperaceae	*Piper arboreum* Aubl.	L, S	Parque Nacional Braulio Carillo	1000 m	9.1 ± 0.1	105.3 ± 8.2
JVR12122	Piperaceae	*Piper bredemeyeri* Jacq.	A	Parque Nacional Braulio Carillo	1000 m	10.1 ± 0.2	103.1 ± 4.0

Table 2.1 Continued

Voucher	Family	Species	Part(s)[a]	Location	Elevation	Violacein inhibition ± SEM (mm)	% Biofilm growth ± SEM
JVR1197	Piperaceae	*Piper bisasperatum* Trel.	A	San Jose	1100 m	8.7 ± 0.1	87.9 ± 4.9
JVR13217	Piperaceae	*Piper carpinteranum* C. DC. ex Pittier	A	Monte de la Cruz	1600 m	8.8 ± 0.1	103.1 ± 4.0
OTT19060	Piperaceae	*Piper decurrens* C. DC.	L	Ciudad Colon	800 m	8.7 ± 0.2	76.0 ± 3.1
JVR2098	Piperaceae	*Piper guanacastense* C. DC.	L	Palmar Norte	25 m	9.8 ± 0.3[c]	109.5 ± 5.4
JVR12113	Piperaceae	*Piper imperiale* L.	L	Volcan Poas	1927 m	7.4 ± 0.1	107.4 ± 12.6
			F			7.0 ± 0.1	131.5 ± 7.3
			Wo			9.1 ± 0.3	111.5 ± 12.6
			B			9.0 ± 0.2	109.5 ± 5.4
JVR12112	Piperaceae	*Piper lanceifolium* Kunth.	L	Volcan Poas	2019 m	11.3 ± 0.2[c]	107.0 ± 9.4
JVR12124	Piperaceae	*Piper papantlense* C. DC.	L	Ciudad Colon	1000 m	13.1 ± 0.8[c]	104.0 ± 3.2
OTT19063	Piperaceae	*Piper pseudolindenii* C. DC.	L	Parque Nacional Braulio Carillo	800 m	10.2 ± 0.2	99.5 ± 1.1
JVR2134	Piperaceae	*Piper reticulatum* L.	L	Cuidad Quesada	700 m	8.3 ± 0.1	96.8 ± 5.9
			S			10.3 ± 0.1	101.0 ± 1.6
JVR12116	Piperaceae	*Piper subsessilifolium* C.DC.	A	Parque Nacional Tapanti	1250 m	8.2 ± 0.1	117.1 ± 4.6
JVR12126	Piperaceae	*Piper urostachyum* Hemsley	L, S	Parque Nacional Braulio Carillo	1000 m	10.4 ± 0.3	99.2 ± 5.7
JVR12110	Piperaceae	*Sarcorhachis naranjoana* (C.DC.) Trel.	L	Parque Nacional Tapanti	1217 m	10.4 ± 0.2	109.7 ± 6.0
JVR12107	Sapindaceae	*Cupania glabra* Sw.	L	Volcan Poas	1418 m	10.3 ± 0.4	53.6 ± 4.0[b]
			B			8.6 ± 0.3	44.5 ± 4.3[b]
			Wo			7.2 ± 0.1	65.3 ± 2.8
JVR13218	Simaroubaceae	*Simarouba amara* Aubl.	L	Finca La Selva	100 m	12.8 ± 0.1	77.0 ± 7.3
JVR13219	Simaroubaceae	*Simarouba glauca* DC.	L	Puntarenas	100 m	20.6 ± 0.7[b]	69.6 ± 4.0
			B			15.6 ± 0.7	79.8 ± 3.0

Table 2.1 Continued

Voucher	Family	Species	Part(s)[a]	Location	Elevation	Violacein inhibition ± SEM (mm)	% Biofilm growth ± SEM
PSC08-85	Bonnemaisoniaceae	*Delisea pulchra* (Greville) Montagne	T	Palmer Station, Antarctica	0 m	12.8 ± 0.7[c]	NT
positive control (QS)		C10–HSL (400 µg)				10.2 ± 0.1	NT
positive control (biofilm)		allicin (1 µl or 1.08 mg)				NT	74.8 ± 8.1

[a] plant parts: A – aerial, B – bark, L – leaves, S – stems, T – thallus, W – whole plant, Wo – wood
[b] statistically significant ($p < 0.01$)
[c] growth inhibition zones of 1–3 mm beyond disc
[d] growth inhibition zones of 3–5 mm beyond disc
[e] growth inhibition zones of 10–12 mm beyond disc
[f] NT - not tested

Table 2.2 IC_{50} values for some plant species inhibiting biofilm formation of *Pseudomonas aeruginosa* PA14.

Family	Species	Part(s)	IC_{50} (μg/mL)
Lepidobotryaceae	*Ruptiliocarpon caracolito* L.	leaves	126
Melastomataceae	*Monochaetum floribundum* Naudin	whole plant	266
Meliaceae	*Carapa guianensis* Aubl.	bark	208
Meliaceae	*Chukrasia tabularis* A. Juss.	leaves	45
Meliaceae	*Swietenia mahogani* L.	bark	202
Meliaceae	*Trichilia martiana* C. DC.	leaves	127
Sapindaceae	*Cupania glabra* Sw.	leaves	70

2.4 Discussion

To our knowledge, this is the first report of anti-QS and anti-biofilm activities in the Lepidobotryaceae, Sapindaceae, and Simaroubaceae. Adonizio et al. (2006) showed QS inhibition by *Tetrazygia bicolor* (Mill.) Cogn. leaves, a Melastomataceae. Interestingly, all of the active plant species from the Melastomataceae reported here have little or no previous phytochemical information and biological activities reported. Meliaceae species have been shown to have many biological activities such as antifeedant (Omar et al. 2003), insecticidal (Zhang et al. 2013), anti-malarial (Kvist et al. 2006; Miranda Júnior et al. 2012), antimicrobial (Chowdhury et al. 2003; Govindachari et al. 1993; Kanwal et al. 2011; Malairajan et al. 2012; Nagalakshmi et al. 2001; Sahgal 2011) and antiviral activities (Cella et al. 2004). *Swietenia macrophylla* King leaves, *Toona ciliata* M. Roem leaves, bark and wood, *Cabralea canjerana* (Vell.) Mart. leaves, *Azadirachta indica* A. Juss. leaves, *Chukrasia tabularis* A. Juss. leaves and bark, *Aphanamixis polystachya* (Wall.) Parker fruits, and *Swietenia mahogani* (L.) Jacq. seeds have been shown to have antimicrobial activities against various fungal and bacterial pathogens (Chowdhury et al. 2003; Govindachari et al. 1993; Kanwal et al. 2011; Malairajan et al. 2012; Nagalakshmi et al. 2001; Sahgal 2011; Zhang et al. 2013).

The various biological activities in these Meliaceae extracts have been attributed to the presence of limonoids and flavonoids. Limonoids such as cedrelone (found in *Toona ciliata* M. Roem wood), 6–acetylswietenine, 6–acetyl–3–tigloyl–swietenolide (from *Swietenia mahogani* (L.) Jacq. seeds) have been shown to inhibit the growth of *Candida albicans* and groundnut rust *Puccinia arachidis* (Govindachari et al. 1993; Malairajan et al. 2012). The leaves and bark of *Chukrasia tabularis* A. Juss are rich in tannic acid (Cella et al. 2004) which was reported by

Chung et al. (1993) to have antibacterial activity. Tannic acid also inhibited the growth of *Pseudomonas aeruginosa* PA14 biofilm in our assay (72% inhibition at 200 μg/mL). *Azadirachta indica* A. Juss leaves extract at 25 mg/g has been shown to prevent the formation of dental plaque in a 6-week clinical trial (Pai et al. 2004). Dental plaques are formed by mixed community bacterial biofilms. Genistein–7–*O*–glucoside and (–)-epicatechin are responsible for the antibacterial activity in *Azadirachta indica* A. Juss leaves (Kanwal et al. 2011). Our results, however, do not agree with these studies as the leaf extract has little anti-QS or anti-biofilm activities. This difference could be due to our lower tested concentrations (400 – 1000 μg/mL).

Although the phytochemistry of the active Meliaceae species such as *Chukrasia tabularis* A. Juss, *Carapa guianensis* Aubl., and *Swietenia mahogani* (L.) Jacq. has been studied extensively, no active compounds have been reported for the QS and/or biofilm inhibition. The majority of the Meliaceae extracts tested here have little or no phytochemical information available. At this point, we have not done any fractionation of the active species; however, the active compounds could be polyphenols similar to tannic acid or other polar compounds.

From all the Melastomataceae species tested, only one has been previously studied: *Monochaetum vulcanicum* Cogniaux. Ursolic acid and other derivatives have been isolated from *Monochaetum vulcanicum* Cogn. by Chaturvedula et al. (2004). Although, ursolic acid has been shown to inhibit *E. coli*, *P. aeruginosa* PAO1, and *V. harveyi* biofilms by Ren et al. (2005), this compound and betulinic acid did not have the same effect against *P. aeruginosa* PA14 biofilm in our assay at the tested concentration of 5 μg/mL (data not shown). These two compounds also did not exhibit any QS inhibition. Thus, the active principles in these Melastomataceae are unlikely to be triterpenoids.

All plant parts of *Cupania glabra* Sw. showed high biofilm inhibition. This activity could be due to cupanioside, the antibacterial compound isolated by Setzer et al. (2005) from bark of this species. Both *Simarouba amara* Aubl. and *Simarouba glauca* DC. have been well characterized phytochemically. These two species contain anti-plasmodial quassinoids (Franssen et al. 1997; O'Neill et al. 1988). *Simaruba glauca* DC. extract has been reported to inhibit the growth of enterobacteria (Carceres et al. 1990). The most active plant in our bioassays was *Ruptiliocarpon caracolito* L. All parts tested of *R. caracolito* except the bark showed significant QS and biofilm inhibition. Omar et al. (2007) reported the antifeedant properties of the spirocaracolitones, unique to this species. However, spirocaracolitones-rich fractions did not possess any QS or biofilm inhibitory activity (data not shown), suggesting that these are not the active principles in our bioassays.

These results showed that tropical plants contain phytochemicals capable of interfering with bacterial quorum sensing and biofilm formation. This provides support for the use of plant-based therapies as treatment for infections by antibiotic resistant biofilms. The bioassays used are appropriate for the rapid screening of large number of samples; however, there are a number of limitations that should be stated. In the QS bioassay, growth inhibition and QS inhibition can be distinguished visually; antibacterial activity is indicated by a clear lawn whereas QS inhibition is shown by a white pigmentless zone. As with other disc diffusion assays, the detection of activity can be affected by the limited mobility in agar and by the presence of active compounds in low concentrations in these extracts. QS modulatory activities can be verified by quantifying the amount of violacein produced in the presence and absence of specific extracts using spectrophotometry or HPLC. Similarly, biofilm formation can also be affected by growth inhibition; thus, the activity should be tested using different concentrations of extracts as well as

at different time points. Finally, plant extracts typically contain several hundreds to over a thousand different compounds. It is possible that compounds with opposite modes of action are present in the same extract and that the active compounds are not present in high enough concentrations to illicit a response. The opposite scenario is also possible where the different compounds act in synergy to amplify the observed activity. All of these factors result in false positives and/or false negatives and thus secondary bioassays should be performed to confirm the biological activities of these extracts.

Chapter 3 – Antimicrobial and biofilm inhibitory activities of Q'eqchi' Maya medicinal plants

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Statement of author contribution

JTA and CAT conceived and designed this study. Plant collection was done by CAT, BWR, FC, FC, and JTA. Plant identification was done by MO, PS, and LP. Plant extraction and bioassays were performed by CAT with assistance from ER. Ethnobotanical data were provided by BWR and VTA. VC coordinated project in Belize. BWR, MLS, and JTA contributed in manuscript preparation.

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3.1 Introduction

The living Maya are descendants of indigenous people who created one of the great Mesoamerican civilizations that began over 3000 years ago. The height of the Maya civilization was during the Classic period (250 to 900 A.D) (Coe 2002), but many of the central Maya area ceremonial sites were abandoned after this period. In recent years, Maya culture has undergone a substantial revival with renewed interest in traditional ceremonies and medicine. In Mesoamerica today, there are over 15 spoken Maya languages. The Q'eqchi' language is spoken in Guatemala and Belize by indigenous farming families who live a largely traditional lifestyle in small villages. Much of the primary health care depends on traditional healers and the rainforest plants they select.

Over the years, there have been numerous ethnobotanical studies conducted with Maya communities in Guatemala (Comerford 1996; Michel et al. 2007), Mexico (Ankli et al. 1999; Alcorn 1984; Stepp 2002), and Belize (Arnason et al. 1980; Bourbonnais-Spear et al. 2005; Amiguet et al. 2005; Walshe-Roussel 2014) documenting traditional knowledge of medicinal plants. Treatment of infections and dermatological conditions are often cited in the top use categories; Amiguet et al. (2005) reported 96 species used to treat various infections and associated symptoms and Ankli et al. (1999) reported 150 species for dermatological conditions. Further studies of these plants often provide a pharmacological basis for their use in traditional medicine. For example, leaf extract of *Helianthemum glomeratum* (Lag.) Lag. (Cistaceae), a plant used to treat diarrhoea by the Maya, inhibited the growth of enteric pathogens such as *Vibrio cholera*, *Escherichia coli*, as well as *Salmonella* and *Shigella* isolates (Meckes et al. 1997). Furthermore, the fruits of various *Capsicum* species are widely used in Maya medicine to treat ailments of probable bacterial origins (Alcorn 1984). Extracts of five *Capsicum* species

fruits have been shown to have antimicrobial activities against *Bacillus*, *Clostridium*, and *Streptococcus* species that are often implicated in gastrointestinal discomforts, food poisonings, and cutaneous infections (Cichewicz and Thorpe 1996).

As part of our ongoing studies of Maya ethnobotany in collaboration with Q'eqchi' Maya healers, we collected 61 plants in the Toledo District of Belize that were used by them as medicines for treatment of various conditions including infections. Their potential for interference with bacterial quorum sensing (QS) and biofilm formation as well as fungal growth were examined using model organisms.

3.2 Materials and Methods

3.2.1 Plant collection

Ethnobotanical studies with the Q'eqchi' Maya were conducted by Amiguet et al. (2005) and Walshe-Roussel (2014). Secondary usage of ethnobotanical data for this project received ethical approval from the University of Ottawa Research Ethics Board (permit #H11–11–09). All plant materials were collected from three locations of the Toledo District in southern Belize: Punta Gorda, Jalacte, and Itzamma Botanical Garden. Plant collection was done with ethical approval (permit #H03–070–01). Plant specimens were preserved in 70% ethanol immediately after collection. Vouchers were deposited at the University of Ottawa Herbarium (OTT), the Belize Forestry Department, and Herbario Juvenal Valerio Rodriguez (JVR) in Costa Rica. Plant species identification was confirmed by M. Otárola-Rojas, P. Sanchez-Vindas, and L. Poveda. Algal collection of *Delisea pulchra* was made at Palmer Station, Antarctica and a voucher was deposited at this research station. Taxonomic identification was performed by Charles Armsler (University of Alabama at Birmingham). Voucher numbers are listed in Table 3.1.

3.2.2 Plant extraction

Plant materials were ground with a blender (Waring commercial LR 8992) and extracted with 80% EtOH using a 1:10 biomass to solvent (w/v) ratio. The plant material/ethanol mixture was shaken overnight at 200 rpm (shaker: New Brunswick Scientific, Edison, NJ, USA) and then filtered using vacuum filtration. A second extraction was performed with the residue using a 1:5 w/v ratio and the mixture was again shaken and filtered. The two extracts were combined, dried *in vacuo* using a rotary evaporator (Yamato RE 500) at 45°C, lyophilized to remove any residual water using a freeze-dryer (EC Super Modulyo, ~ -55 °C, 10^{-2} mbar), and stored at -20°C in the dark until needed.

3.2.3 Microbial strains and culture

Bacteria species *Chromobacterium violaceum* ATCC 12472 was used in the quorum sensing bioassay and *Pseudomonas aeruginosa* PA14 in the biofilm bioassay. *Chromobacterium violaceum* was purchased from ATCC and *Pseudomonas aeruginosa* PA14 was obtained from T. Mah (University of Ottawa). These strains were maintained as liquid culture with shaking (200 rpm) at 30°C (incubator: New Brunswick Scientific Series 25, Edison, NJ, USA) in nutrient broth (BD, Sparks, MD, USA) and LB broth (Fisher Scientific, Fairlawn, NJ, USA), respectively. Fungal species *Saccharomyces cervisiae* S288C, *Cryptococcus neoformans*, and *Candida albicans* D10 provided by M. L. Smith (Carleton University) were maintained in liquid culture with shaking (200 rpm) at 30°C (incubator: New Brunswick Scientific Innova 40, Edison, NJ, USA) in Sabouraud's dextrose medium (BD, Sparks, MD, USA).

3.2.4 Quorum sensing (QS) bioassay

A modified disc diffusion assay described by Adonizio et al. (2006) was used to determine whether the plant extracts can interfere with the quorum sensing (QS) of *Chromobacterium violaceum*. *Chromobacterium violaceum* produces a purple pigment, violacein, which is under QS control. The inhibition of violacein production will indicate the disruption of QS. Briefly, sterile paper disks (Oxoid, Basingstoke, Hants, UK) loaded each with 1 mg of extract were placed onto TGY agar plates (BD, Sparks, MD, USA) inoculated with 100 µL of overnight cultures then incubated without agitation for 24 hours at 30ºC (incubator: Precision Automatic CO_2 Incubator). QS inhibition was indicated by a colourless opaque halo around the disc and growth inhibition by a clear halo. Plates were examined under a dissecting microscope to confirm whether the extract has anti-QS and/or antibacterial activity. The controls used were: 70% ethanol as vehicle control and *N*–decanoyl–L–homoserine lactone (C10–HSL) (400 µg/disc, ≥96%, Cayman Chemical, Ann Arbor, MI, USA) as positive control. Extract of *Delisea pulchra* (Greville) Montagne (1 mg/disc) was also included for comparative purposes as this alga contains known QS inhibitors, the halogenated furanones (Givskov et al. 1996; Raffa et al. 2005; Shiner et al. 2005). Each sample was tested in triplicate.

3.2.5 Biofilm bioassay

A spectrophotometric assay was adapted from Ren et al. (2005) to assess the biofilm inhibitory activity of the plant extracts. Extracts were prepared in 50% methanol and tested at a final concentration of 400 µg/mL. Overnight culture of *P. aeruginosa* was diluted 1:50 in M63 medium broth (Amresco, Solon, OH) supplemented with 0.4% arginine and 1mM $MgSO_4$. This inoculum was grown in a 24-well microtiter plate (Costar 3526) without agitation for 24 hours at 37ºC to allow biofilm formation (incubator: Precision Automatic CO_2 Incubator). The plate was

then decanted and washed with distilled water three times to eliminate non-adherent cells. The remaining biofilm was stained with 0.1% crystal violet (Fisher Scientific, Fairlawn, NJ) for 15 minutes. The excess dye was decanted, the plate was washed three times with distilled water, and then air dried overnight. The biofilm was quantified by dissolving the dye in ethanol and reading the absorbance at 570 nm (spectrophotometer: SpectraMax M5, Molecular Devices, Sunnyvale, CA, USA). The controls used were: vehicle control with 50% methanol, negative control with M63 medium broth, and positive control with allicin (1 μL/mL or 1.08 mg/mL, 97%, AK Scientific Inc., Mountain View, CA, USA). Biofilm formation was expressed relative to the vehicle control. Samples were tested in triplicate.

3.2.6 Antifungal disc diffusion assay

Saccharomyces cerevisiae S288C was used for the initial evaluation of plant extracts for antifungal activity, performed in triplicate. Plant extracts were prepared to a final concentration of 40mg/mL, using HPLC grade methanol (Fisher Scientific, Fairlawn, NJ, USA) as a solvent. Berberine (95%, Sigma-Aldrich, Mississauga, ON, Canada) was used as an antifungal positive control and HPLC grade methanol was used as a negative control. *Saccharomyces cerevisiae* was inoculated into Sabouraud's broth medium and grown to an optical density of 600 nm (OD_{600}) of ~1.0 and diluted 1:100. Aliquots (100 μL) of the diluted broth culture were spread over the surface of Sabouraud's agar plates. Paper discs (7.0 mm diameter) were amended with crude extract (2 mg/disc), berberine (1mg/disc) or HPLC methanol and allowed to air-dry. All treatments were subsequently incubated in the dark for 48 h at 30 ℃. Inhibition zones from active extracts were then measured. For active plants, growth inhibition was also tested with *Candida albicans* D10 and *Cryptococcus neoformans* using the same assay.

3.2.7 Statistical analysis

Statistical analyses were done using SigmaPlot 11 software for Windows (Systat Software, San Jose, CA, USA). Results are presented as means ± S.E.M. (standard error of the mean) for at least 3 independent experiments. One-way ANOVA was performed with posthoc Tukey's test and $p < 0.01$ considered as significant.

3.3 Results

Extracts of 61 Q'eqchi' Maya medicinal plant species, representing 35 families and 22 orders, collected over the last 5 years for general and focused ethnobotanicals purposes were tested for their ability to interfere with bacterial quorum sensing (QS) and biofilm formation as well as fungal growth inhibition. These species are used by the healers to treat many conditions including infections. When the ethnobotanical use reports were classified according to the Cook (1995) use categories, the top four categories with the largest number of species were infections/infestations (INF), digestive system disorders (DIG), pain (PAI), and nervous system disorders (NER) with 40 species, 35 species, 31 species, and 30 species cited, respectively (Figure 3.1). Muscular skeletal system disorders (MUS), circulatory systems disorders (CIR), poisonings (POI), and skin/subcutaneous cellular tissue disorders (SKI) are the next highest categories with 23 species, 20 species, 17 species, and 17 species used, respectively. Many plants are used by the Q'eqchi' Maya healers for multiple treatments and thus appear in more than one category. The complete list of specific uses for each species can be found in handbooks by Amiguet et al. (2004) and Walshe-Roussel et al. (2014). When the top eight use categories were considered, SKI was the category with the highest percentage of active species (53%); INF and MUS were the next highest with 48%, followed by PAI, POI, and DIG with 45%, 41%, and 40%, respectively (Figure 3.2).

Considering the taxonomic affiliation of the tested plants, the orders with the highest activity were Malpighiales, Myrtales, and Cucurbitales with 100%, 83%, and 75%, respectively (Figure 3.3). The Malpighiales includes families such as Euphorbiaceae and Passifloraceae; the Myrtales includes the Melastomataceae and Combretaceae while the Cucurbitales includes the Begoniaceae and Cucurbitaceae.

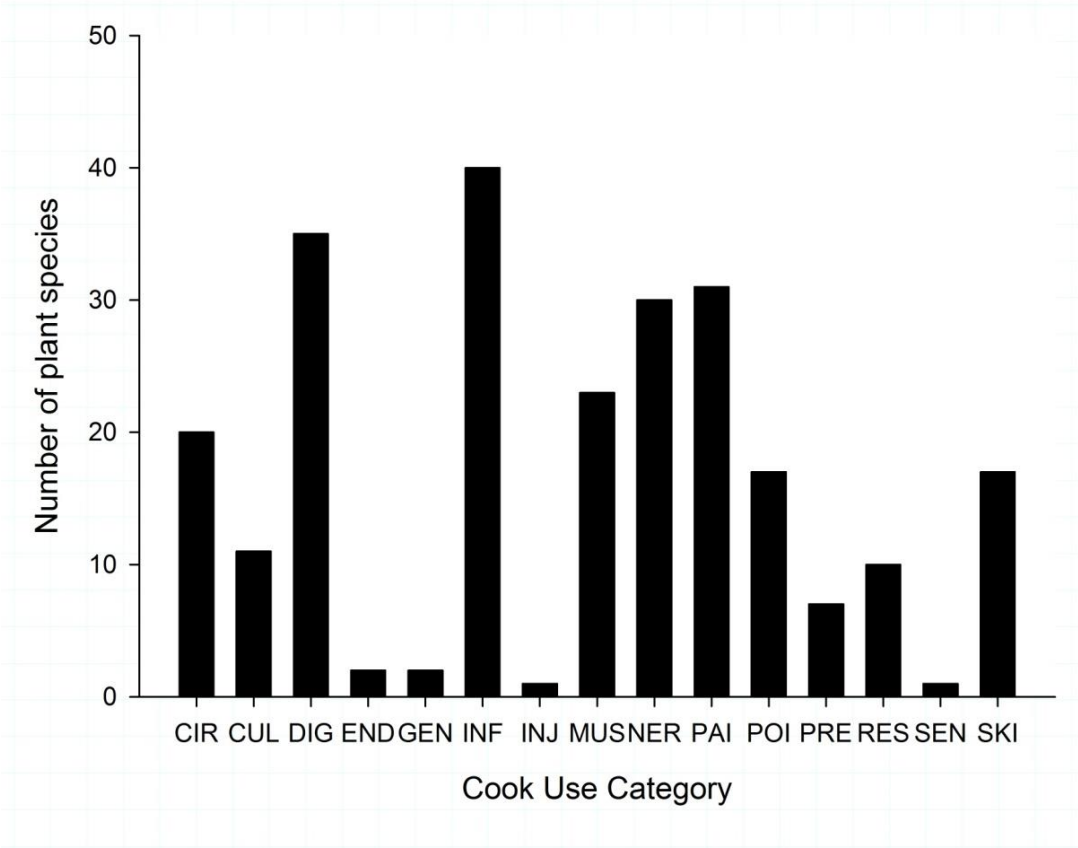

Figure 3.1 Distribution of Q'eqchi' Maya medicinal plants in relation to Cook (1995) use categories. Plant species can be used for more than one use category. Total species = 61. Use categories: CIR = circulatory system disorders, CUL = culture-bound syndromes, DIG = digestive system distorders, END = endocrine system disorders, GEN =genitourinary system disorders, INF = infections/infestations, INJ = injuries, MUS = muscular-skeletal system disorders, NER = nervous system disorders, PAI = pain, POI = poisonings, PRE = pregnancy/birth/puerperal disorders, RES = respiratory system disorders, SEN = sensory system disorders, SKI = skin/subcutaneous cellular tissue disorder.

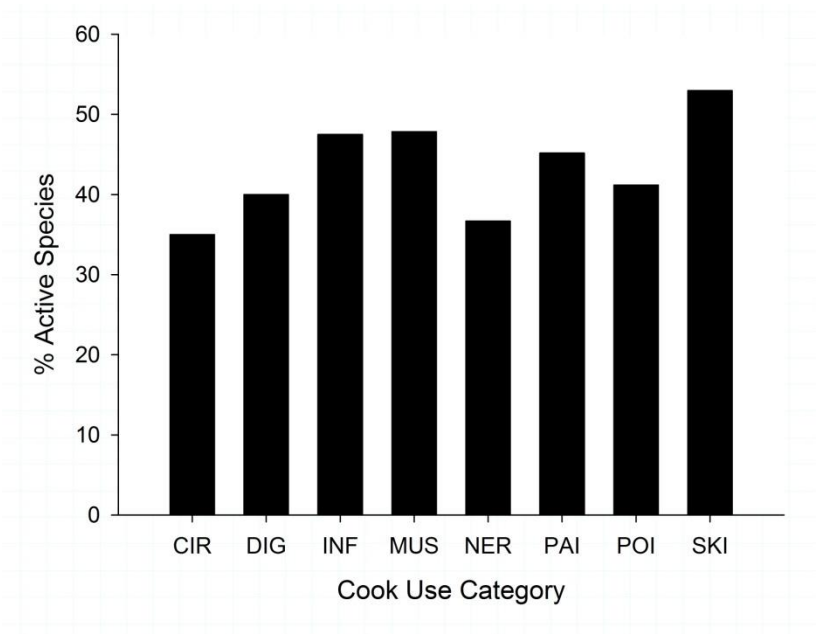

Figure 3.2 Percent active species for the top eight Cook (1995) use categories. A species is considered active if there is inhibition in at least one of the following: bacterial quorum sensing, bacterial biofilm formation, and fungal growth. Number of active species ranged from 7 to 19.

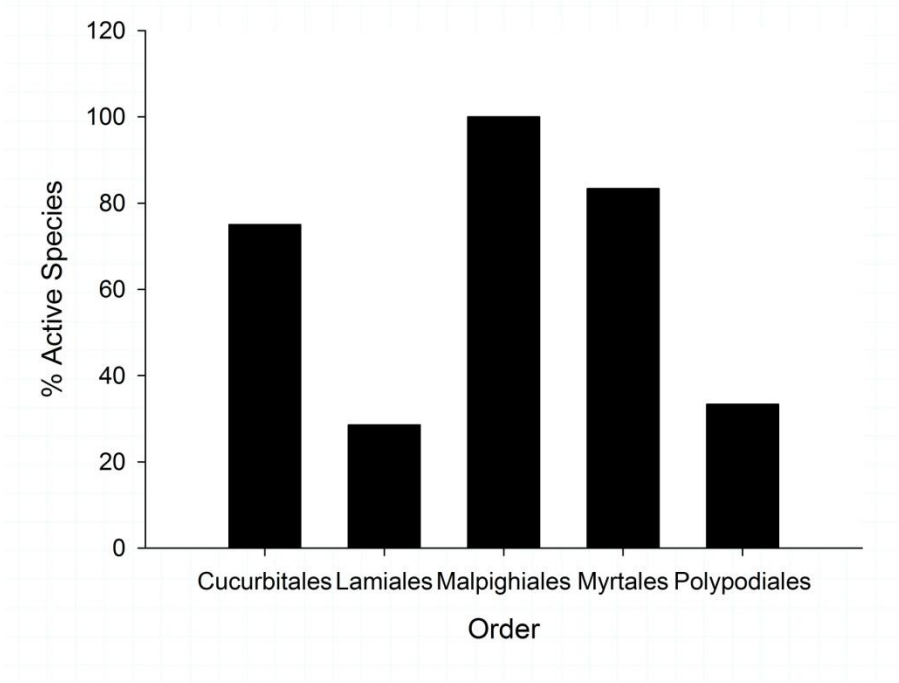

Figure 3.3 Percent active species for the top five plant orders. A species is considered active if there is inhibition in at least one of the following: bacterial quorum sensing, bacterial biofilm formation, and fungal growth. Number of active species ranged from 3 to 7.

Table 3.1 summarized the biological activities of the tested extracts. Preliminary assessments showed that QS inhibitory activities in *C. violaceum* ATCC 12472 ranged from 7.0 ± 0.1 mm to 26.1 ± 0.3 mm. In particular, four species displayed significant QS inhibition when compared to the positive control *D. pulchra* extract (12.8 ± 0.7 mm): *Combretum fruticosum* (Loefl.) Stuntz. leaves (Combretaceae), *Chamaesyce hyssopifolia* (L.) Small. leaves (Euphorbiaceae), *Euphorbia lancifolia* L. leaves (Euphorbiaceae), and *Blakea cuneata* Standl. leaves (Melastomataceae). The leaves of *Hyptis capitata* Jacq. (Lamiaceae), stems of *Blakea cuneata*, and leaves of *Petiveria alliacea* L. (Phytolaccaceae) showed similar QS inhibitory activities of that of *D. pulchra*. Furthermore, a third of the extracts (21) had similar QS inhibition to that of the other positive control C10–HSL (10.2 ± 0.1 mm).

For biofilm growth, most species showed either little to no inhibition or actually enhanced the formation of the biofilm mass when compared to the vehicle control (50% methanol). However, the leaves of *Croton schiedeanus* Schtldl. (Euphorbiaceae) and *Blakea cuneata* Standl. (Melastomataceae) significantly inhibited the growth of *P. aeruginosa* PA14 biofilm more than the positive control allicin (74.4 ± 8.1% control) with growths of 49.1 ± 2.9 % and 27.1 ± 3.1 %, respectively. Five species showed similar inhibitory activities to that of allicin: *Begonia heracleifolia* Schltdl. & Cham. (Begoniaceae), *Momordica charantia* L. (Cucurbitaceae), *Hyptis capitata* Jacq. (Lamiaceae), *Blakea cuneata* stems (Melastomataceae), and *Miconia gracilis* Triana (Melastomataceae).

Many tested extracts showed little to no growth inhibition against *Saccharomyces cerevisiae* S288C except *Passiflora oerstedii* Mast. var. *choconiana* S. Watson (Passifloraceae), *Campyloneurum brevifolium* (Lodd. ex Link) Link (Polypodiaceae), and *Cestrum schlechtendhalii* G.Don. *Campyloneurum brevifolium* and *Cestrum schlechtendahlii*, which

showed significant activities against S288C similar to that of berberine (19.3 ± 0.7 mm) with inhibitory zones of 17.7 ± 0.3 mm and 15.1 ± 0.6 mm, respectively. These two species also inhibited the growth of *Candida albicans* D10 and *Cryptococcus neoformans* but to a lesser extent (Figure 3.4).

Table 3.1 Effects of Q'eqchi' Maya plant extracts on violacein production in *Chromobacterium violaceum* ATCC 12472 (1 mg/disc, N = 3–7, disc diameter = 7 mm, vehicle control: 70% EtOH), biofilm formation in *Pseudomonas aeruginosa* PA14 (400 μg/mL, N = 3–7, vehicle control: 50% MeOH), and growth of *Saccharomyces cerevisiae* S288C (2 mg/disc, N=3–7, disc diameter = 7 mm, vehicle control: 70% EtOH).

Voucher	Family	Species	Q'eqchi' name	Part(s)[a]	Violacein inhibition ± SEM (mm)	% Biofilm growth ± SEM	Growth inhibition ± SEM (mm)
JVR7915	Acanthaceae	*Aphelandra scabra* (Vahl.) Sm.	Jolom chacmut	L	9.6 ± 0.3	100.0 ± 0.1	7.0 ± 0.1
UOH19745	Acanthaceae	*Justicia albobracteata* Leonard.	Xna kejen	L	9.6 ± 0.2	97.5 ± 2.4	7.0 ± 0.1
UOH19764	Acanthaceae	*Justicia aurea* Schltdl.	Saxjolom chacmut	L	7.1 ± 0.1	99.0 ± 1.0	7.0 ± 0.1
JVR8003	Acanthaceae	*Justicia pectoralis* Jacq.	Xucoy' i'kok	L	10.2 ± 0.2	103.0 ± 0.8	7.0 ± 0.1
UOH19731	Adiantaceae	*Adiantum latifolium* Lam.	Roq chit cuan	L	10.5 ± 0.4	101.0 ± 1.0	7.0 ± 0.1
UOH19705	Adiantaceae	*Adiantum princeps* T. Moore.	Roq chit cuan	L	7.0 ± 0.1	99.6 ± 2.6	7.0 ± 0.1
UOH19750	Adiantaceae	*Adiantum tetraphyllum* Humb. & Bonpl. ex Willd.	Roq chit cuan	L	10.4 ± 0.2	101.0 ± 1.0	7.0 ± 0.1
JVR7920	Adiantaceae	*Adiatum wilsonii* Hook.	Ruj'i'rak'aj'tza	L	10.2 ± 0.2	87.0 ± 3.8	7.0 ± 0.1
JVR7983	Araliaceae	*Dendropanax arboreus* (L.) Decne.& Planch.	Cojl	L	8.0 ± 0.1	88.1 ± 8.1	NT
UOH19736	Aristolochiaceae	*Aristolochia grandiflora* Sw.	Patz pim	L	10.5 ± 0.4	99.0 ± 1.7	7.0 ± 0.1
JVR8054	Asteraceae	*Baccharis trinervis* Pers.	Cherek sak	L	9.9 ± 0.3	104.3 ± 19.2	7.0 ± 0.1
JVR7916	Asteraceae	*Mikania micrantha* Kunth.	Chak'onoob'	L	7.0 ± 0.1	99.5 ± 0.6	7.0 ± 0.1
JVR8074	Asteraceae	*Neurolaena lobata* (L.) R. Br. ex Cass.	K'a mank	L	9.7 ± 0.1	109.0 ± 2.3	7.0 ± 0.1
UOH19711	Asteraceae	*Porophyllum ruderale* (Jacq.) Cass.	So'sol pim	L	9.0 ± 0.1	100.1 ± 0.1	7.0 ± 0.1
JVR7945	Begoniaceae	*Begonia heracleifolia* Schltdl. & Cham.	Rutzaj k'opopo'	L	9.2 ± 0.1	71.5 ± 7.5	7.0 ± 0.1
JVR7984	Begoniaceae	*Begonia sericoneura* Liebm.	Xak pek	L	9.1 ± 0.1	100.1 ± 0.1	7.0 ± 0.1
JVR8079	Bursaraceae	*Bursera simaruba* (L.) Sarg.	Kak kajl	B	7.0 ± 0.1	99.4 ± 0.4	7.0 ± 0.1
JVR7999	Combretaceae	*Combretum fruticosum* (Loefl.) Stuntz.	K'an shan k'aham	B	10.4 ± 0.1	100.5 ± 9.9	7.0 ± 0.1
				L	26.1 ± 0.3[b]	82.9 ± 8.1	7.0 ± 0.1
JVR8005	Cucurbitaceae	*Gurania makoyana* (Lem.) Cogn.	Kum pim	L	10.3 ± 0.	128.4 ± 20.5	7.0 ± 0.1

Table 3.1 Continued

Voucher	Family	Species	Q'eqchi' name	Part(s)[a]	Violacein inhibition ± SEM (mm)	% Biofilm growth ± SEM	Growth inhibition ± SEM (mm)
JVR8065	Cucurbitaceae	*Momordica charantia* L.	Ya'mor	L	9.7 ± 0.2	71.1 ± 6.3	7.0 ± 0.1
JVR8071	Davalliaceae	*Nephrolepis biserrata* (Sw.) Schott.	Ixqu'oq mo'coch	L	9.5 ± 0.1	103.4 ± 0.6	7.7 ± 0.3
JVR8023	Dryopteridaceae	*Bolbitis permagataceae* (Maxon) Ching.	Re'quaxiru	L	9.8 ± 0.6	99.3 ± 2.4	7.0 ± 0.1
JVR8008	Euphorbiaceae	*Chamaesyce hyssopifolia* (L.) Small.	None	L	19.2 ± 2.2[b]	82.6 ± 1.2	7.0 ± 0.1
JVR7952	Euphorbiaceae	*Croton schiedeanus* Schltdl.	Copal chi’	B	7.8 ± 0.1	99.8 ± 0.3	7.0 ± 0.1
				L	7.2 ± 0.2	49.2 ± 2.9[b]	7.0 ± 0.1
JVR8043	Euphorbiaceae	*Euphorbia lancifolia* Schltdl.	Ixbut	L	16.5 ± 1.0[b]	102.1 ± 1.1	NT
JVR7963	Fabaceae	*Acosmium panamense* (Benth.) Yakovlev	K'a che	B	7.0 ± 0.1	93.1 ± 3.5	7.0 ± 0.1
UOH19748	Fabaceae	*Mimosa pudica* L.	Quare kix	L	9.3 ± 0.1	103.3 ± 0.6	NT[c]
JVR7987	Fabaceae	*Desmodium adcendens (*Sw.) DC.	Chint pim	L	7.1 ± 0.1	93.2 ± 7.5	7.0 ± 0.1
JVR8057	Haemodoraceae	*Xiphidium caeruleum* Aubl.	Ixcua'i'kuch	L	10.1 ± 0.2	99.0 ± 8.0	7.0 ± 0.1
JVR7996	Lamiaceae	*Hyptis capitata* Jacq.	Se ruj kaway	L	13.1 ± 0.2	70.7 ± 5.2	7.0 ± 0.1
JVR8002	Malvaceae	*Sida acuta* Burm. f.	Mes b'eel	L	10.3 ± 0.1	106.6 ± 4.8	7.1 ± 0.1
JVR8044	Melastomataceae	*Abdelobotrys adscendens* (Sw.) Triana.	Chunac kejen	L	10.2 ± 0.1	103.4 ± 0.6	7.8 ± 0.3
JVR131	Melastomataceae	*Arthrostemma ciliatum* Pav. ex D. Don	Selek sak	L	8.8 ± 0.4	NT	8.0 ± 0.1
JVR8012	Melastomataceae	*Blakea cuneata* Standl.	Oxlaho chajom	L	25.9 ± 0.6[b]	27.1 ± 3.1[b]	7.0 ± 0.1
				S	13.9 ± 0.2	73.8 ± 4.1	7.0 ± 0.1
JVR8046	Melastomataceae	*Miconia oinochrophylla* Donn. Sm.	Purple Maya	L	11.0 ± 0.1	100.3 ± 14.6	NT
JVR111	Melastomataceae	*Miconia gracilis* Triana	Roq muqui	L	11.4 ± 0.2	71.8 ± 8.1	7.8 ± 0.2
JVR8030	Menispermaceae	*Cissampelos pareira* L.	Ch'up i al (male)	L	7.1 ± 0.1	97.9 ± 2.9	7.0 ± 0.1

Table 3.1 Continued

Voucher	Family	Species	Q'eqchi' name	Part(s)[a]	Violacein inhibition ± SEM (mm)	% Biofilm growth ± SEM	Growth inhibition ± SEM (mm)
JVR7950	Menispermaceae	*Cissampelos tropelifolia* DC.	Ch'up i al female)	L	8.2 ± 0.3	93.4 ± 3.6	7.0 ± 0.1
JVR8032	Monimiaceae	*Mollinedia guatamalensis* Perkins	Saki kejen	L	10.0 ± 0.1	124.9 ± 30.9	7.1 ± 0.1
JVR8028	Moraceae	*Dorstenia contrajerva* L.	Ch'up i al	L	9.0 ± 0.1	108.8 ± 0.1	7.2 ± 0.2
JVR8025	Passifloraceae	*Passiflora oerstedii* Mast. var *choconiana* S. Watson	Tu kej kejen	L	10.8 ± 0.3	108.1 ± 2.1	11.0 ± 0.2
UOH19738	Phytolaccaceae	*Petiveria alliacea* L.	Par'i' pim	L	11.8 ± 0.2	100.7 ± 0.1	7.0 ± 0.1
JVR7917	Piperaceae	*Piper amalago* L.	Tziritok kejen	L	10.3 ± 0.1	87.9 ± 4.9	7.0 ± 0.1
JVR7909	Piperaceae	*Piper tuerckheimii* C. DC. ex Donn. Sm.	Cux sawi kejen	L	9.9 ± 0.3	126.5 ± 10.1	7.0 ± 0.1
UOH19706	Plantaginaceae	*Scoparia dulcis* L.	Colantra pim	L	8.0 ± 1.0	100.4 ± 2.9	7.0 ± 0.1
JVR7901	Polygalaceae	*Securidaca diversifolia* (L.) S.F. Blake	Se'ru' k'an tyaj	W	7.0 ± 0.1	99.9 ± 0.2	7.1 ± 0.1
JVR8083	Polypodiaceae	*Campyloneurum brevifolium* (Lodd. ex Link) Link	Rix i xul	L	7.7 ± 0.7	100.1 ± 0.1	17.7 ± 0.3[b]
UOH19751	Pteridaceae	*Pityrogramma calomelanos* (L.) Link.	Roq chit cuan	L	8.9 ± 0.2	102.9 ± 1.7	7.0 ± 0.1
JVR7998	Rubiaceae	*Gonzalagunia panamensis* (Cav.) K. Schum.	Tzu'ul che	L	8.3 ± 0.3	99.9 ± 0.5	7.0 ± 0.1
JVR8009	Rubiaceae	*Morinda citrifolia* L.	K'an i che	B	8.8 ± 0.3	97.8 ± 3.2	7.0 ± 0.1
UOH19727	Rubiaceae	*Psychotria poeppigiana* Mull. Arg.	Saxjolom chilan	L	8.6 ± 0.3	100.8 ± 7.5	7.0 ± 0.1
UOH19741	Rubiaceae	*Psychotria tenuifolia* Sw.	Xa'ab maus	L	9.5 ± 0.1	102.2 ± 1.9	7.0 ± 0.1
JVR030	Sapindaceae	*Paullinia costata* Schltdl. & Cham.	Korona kix	L	8.7 ± 0.1	113.7 ± 11.7	7.0 ± 0.1
JVR8068	Schizaeaceae	*Lygodium heterodoxum* Kunze.	Ruxb'i'kaak	L	10.1 ± 0.1	81.6 ± 10.5	7.1 ± 0.1
JVR8069	Schizaeaceae	*Lygodium venustum* Sw.	Ruxb'i'kaak	L	9.6 ± 0.2	109.3 ± 10.6	7.1 ± 0.1
JVR7979	Selaginellaceae	*Selaginella aff. stellata Spring.*	none	L	11.0 ± 0.3	105.3 ± 13.2	7.0 ± 0.1

Table 3.1 Continued

Voucher	Family	Species	Q'eqchi' name	Part(s)[a]	Violacein inhibition ± SEM (mm)	% Biofilm growth ± SEM	Growth inhibition ± SEM (mm)
JVR8010	Selaginellaceae	*Selaginella umbrosa* Lem. Ex Hieron	Choql pim	L	7.7 ± 0.4	103.1 ± 2.3	7.0 ± 0.1
UOH19776	Solanaceae	*Cestrum schlechtendahlii* G.Don	Ik che	L	7.3 ± 0.2	92.9 ± 4.6	15.1 ± 0.6[b]
UOH19726	Verbenaceae	*Stachytarpheta frantzii* Pol.	Xtye aj pak	L	7.0 ± 0.1	100.0 ± 0.1	7.0 ± 0.1
JVR8029	Vitaceae	*Cissus microcarpa* Vahl.	Roq ab	L	11.1 ± 1.2	89.7 ± 4.5	7.0 ± 0.1
PSC08-85	Bonnemaisoniaceae	*Delisea pulchra* (Greville) Montagne		T	12.8 ± 0.7	NT	NT
CON (QS)		C10–HSL (400 μg)			10.2 ± 0.1	NT	NT
CON (biofilm)		allicin (1 μL or 1.08 mg)			NT	74.5 ± 8.1	NT
CON (fungal)		berberine (1 mg)			NT	NT	19.3 ± 0.7[b]

[a] plant parts: B – bark, L – leaves, S – stems, T – thallus, W – whole plant
[b] statistically significant ($p < 0.01$)
[c] NT - not tested

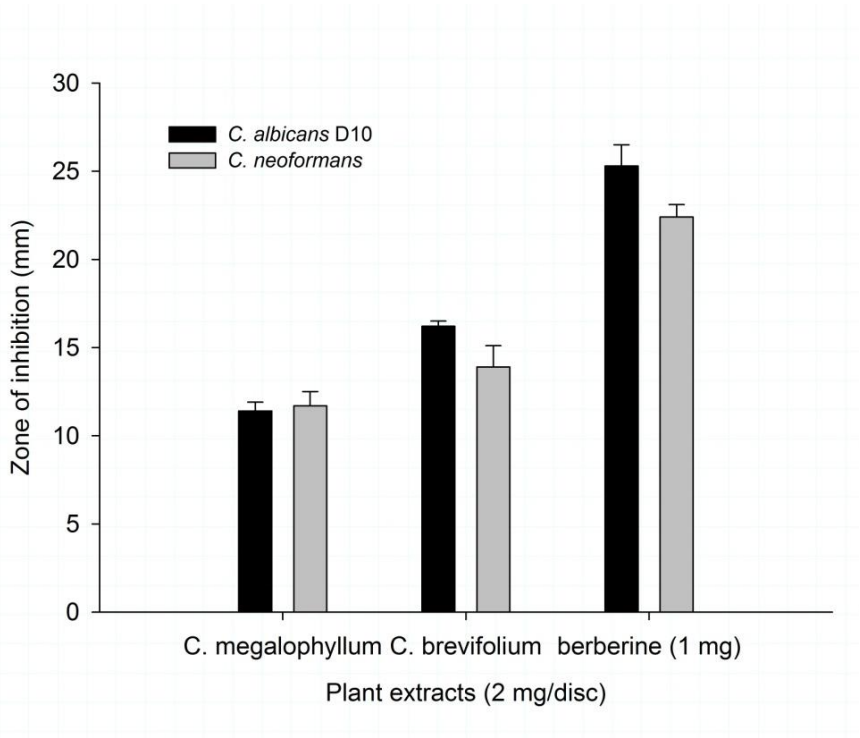

Figure 3.4 Average growth inhibition (± SEM) against *Candida albicans* D10 and *Cryptococcus neoformans* by extracts of *Cestrum schlechtendahlii* G.Don and *Campyloneurum brevifolium* (Lodd. ex Link) Link (N = 3).

3.4 Discussion

Although there was not a strong differentiation in the percentage of active species in the traditional Q'eqchi' Maya use categories, the uses skin/subcutaneous cellular tissue disorders, pain, infections/infestations, and muscular-skeletal system disoders have the highest percentage of active plants. These uses could clearly have bacterial conditions associated with them such as erysipelas, itchy sores, toothache, furuncles/carbuncles, and swelling. On the other hand, the next highest use categories circulatory system disorders, digestive system disorders, nervous system disorders, and poisonings are less likely to involve a microbial origin with the exception digestive system disorders (ulcers, diarrhoea). Since the plant collection was done based on the basis of general ethnobotanical study, it is possible that more use reports for more specific conditions related to infections were not documented. If another ethnobotanical study were to be conducted solely on infections, the number of active plants identified might be enlarged. The lack of a clear trend for the use categories may be explained by the observation that most plants have some inherent antimicrobial phytochemicals to defend against potential attacks. Thus, it is important to also look at the biological activities for these plants on a case by case basis and not just use categories.

To our knowledge, this is the first report of biological activities for *Blakea cuneata* Standl (Melastomatacaea), *Miconia gracilis* Triana (Melastomataceae), *Campyloneurum brevifolium* (Lodd. ex Link) Link (Polypodiaceae), and *Cestrum schlechtendahlii* G.Don (Solanaceae). Interestingly, there also have been no phytochemical studies for any of these species. *Blakea cuneata* (Oxlaho chajom) is used by the Q'eqchi' Maya healers for fever, swelling, insect bites and treatment of digestive system troubles such as stomach cramps and ulcers. Both the leaves and stems of this species showed inhibitory activities against bacterial QS

and biofilm growth with the leaves having stronger inhibition. This suggests that the active phytochemicals are present in both the stems and leaves. *Miconia gracilis,* another Melastomataceae, also showed anti-QS and biofilm inhibition. QS inhibition by a Melastomataceae extract was previously reported by Adonizio et al. (2006) in *Tetrazygia bicolor* (Mill.) Cogn. leaves. Preliminary results for the identification of active principles responsible for the inhibiting bacterial QS and biofilm formation in *B. cuneata* are included in Appendix I.

Another family found to have strong QS inhibitory activities is the Euphorbiaceae. In this study, two of the three of the tested Euphorbiaceae species, *Chamaesyce hyssopifolia* (L.) Small. and *Euphorbia lancifolia* Schltdl., showed greater QS inhibition than the positive control *D. pulchra*, which contains QS inhibiting halogenated furanones (Givskov et al. 1996; Raffa et al. 2005; Shiner et al. 2005). *Chamaesyce hyssopifolia* leaves are used by the healers to treat athlete's foot; however, this species did not show any growth inhibition against *Saccharomyces cerevisiae* S288C. The aqueous extracts of this plant have been reported to have anti-viral activities against HIV-1 (Matsuse et al. 1999) and corilagin, quercetin 3–*O*–β–D–glucopyranoside, and 1,3,4,6–tetra–*O*–galloyl–β–D–glucopyranose have been identified as the active principles (Lim et al. 1997). *Euphorbia lancifolia* (Ixbut in Q'eqchi') is used by Q'eqchi' Maya healers for treatment of athlete's foot and dry skin. Ixbut is also used in traditional medicine throughout Mesoamerica as a galactogogue (Rosengarten 1982). Phytochemical analyses of *E. lancifolia* leaves by Jewell (2009) showed the presence of triterpenoids such as lupeol acetate, germanicol pentanoate, and β–amyrin. Betulinic acid and ursolic acid (related triterpenes) did not inhibit QS in *C. violaceum* at concentration of 0.25 mg/disc (data not shown). Therefore, it is unlikely that triterpenoids are the active principles in *E. lancifolia.*

Croton schiedeanus Schltdl. (Copal chi') leaves are used to treat diarrhoea by the healers. *Croton schiedeanus* leaves inhibited *P. aeruginosa* PA14 biofilm formation by 50% (25% more than the positive control allicin). Aerial parts of *C. schiedeanus* have been shown to have vasorelaxant activities due to the presence of quercetin 3,7–dimethyl ether (Guerrero et al. 2002) and neoclerodane diterpenoids (12*R*)–12–hydroxycascarillone, *cis*–dehydrocrotonin, 5β–hydroxy–*cis*–dehydrocrotonin, *trans*–dehydrocrotonin (Guerrero et al. 2004). Clerodane diterpenoids isolated from a related species *Croton macrostachyus* Hochst. ex Delile have been reported to have antimicrobial activities against *Staphylococcus aureus, Candida albicans*, and *Candida krusei* (Tene et al. 2009). It is possible that these neoclerodane diterpenoids isolated by Guerrero et al. (2004) are responsible for the biofilm growth inhibition observed.

Combretum fruticosum (Loefl.) Stuntz. (K'an shan k'aham) leaves are used by Q'eqchi' Maya healers to treat diarrhoea. *Combretum fruticosum* (Combretaceae) leaves showed strong QS inhibitory activities and minor interference to biofilm formation (~17% inhibition). Extracts of the leaves and stems of this species have been reported by Braga et al. (2007) to show potent inhibition of angiotensin-converting enzyme (ACE). Leaves extract also showed antimicrobial activity against both Gram-positive and Gram-negative bacteria such as *Staphylococcus aureus*, *Escherichia coli*, *Proteus mirabilis*, *Pseudomonas aeruginosa* (Smith et al. 2000). Furthermore, *C. fruticosum* leaves extract was also effective against different strains of methicillin-resistant *S. aureus* (MRSA) with minimum inhibitory concentrations ranging from 250 to 500 μg/mL (Barneche et al. 2011). However, no phytochemical investigation has been reported for this species.

Hyptis capitata Jacq. (Se ruj kaway) also showed good inhibition in both the QS and biofilm (~30% inhibition) bioassays. This plant is used for treatment of diarrhoea, bay sore,

fever, sores, insect bites, swelling, and rash. Leaf extract of *H. capitata* was documented to have potent inhibitory activities against methicillin-resistant *S. aureus* strains (Nogodula et al. 2012) and moderate inhibition against *Bacillis subtilis* and *Mycobacterium intracellulare* (Lentz et al. 1998). Other reported biological activities for this species include antiviral by oleanic acid (Kashidawa et al. 2000) and *in vitro* cytotoxicity in human colon HCT-8 cancer cells (Yamagishi et al. 1988).

Petiveria alliacea L. (par'i'pim) is used by the Q'eqchi' Maya for epilepsy, seizures, madness, and headache. This plant is also used to treat diarrhoea, skin infections, erysipelas, and urinary tract and respiratory tract infections in Guatemala and Argentina (Cáceres et al. 1987; Logan 1973; Perez and Anesini 1994). *Petiveria alliacea* showed inhibition of QS similarly to that of *D. pulchra*. Moderate antimicrobial activities against bacteria and fungi were reported by Lentz et al. 1998). Phytochemical isolation of the roots resulted in 7 polysulfides (Benevides et al. 2001); thiosulfates, trisulfides and benzylsufinic acid were identified to be responsible for the antimicrobial activities (Kim et al. 2006).

Begonia heracleifolia Schltdl. & Cham. (Begoniaceae) and *Momordica charantia* L. (Cucurbitaceae) both showed ~30% inhibition of biofilm growth. *Begonia heracleifolia* leaves (pa'u'lul) is used for athlete's foot, bay sore, fever, rashes, and swelling while *Momordica charantia* leaves (ya'mor) to treat diabetes, fever, and malaria. The phytochemical investigations of these two species have been well documented. Frei et al. (1998) isolated six cucurbitacins from the rhizomes of *B. heracleifolia*, all of which showed anti-proliferative activities. Leaf extracts of *M. charantia* have been reported to show antimicrobial activities against *Escherichia coli*, *Salmonella paratyphi*, *Shigella dysenteriae*, *Streptomyces griseus*, and *Mycobacterium tuberculosis* (Frame et al. 1998; Khan and Omoloso 1998; Ogata et al. 1991; Omoregbe et al.

1996). *Momordica charantia* fruits also have antimicrobial (Mahmood et al. 2012) as well as antiviral (Lee-Huang et al. 1990) and antidiabetic activities (Ali et al. 1993). Phytochemical analyses identified cucurbitacins (Murakami et al. 2001) to be responsible for cytotoxic activities (Frei et al. 1998), charantins (Raman and Lau 1996) for antidiabetic activities (Ali et al. 1993), and specific proteins (α–momorcharin and β–momorcharin, MAP 30) for antiviral and antimicrobial activities (Lee-Huang et al. 1990; Au et al. 2000; Zheng et al. 1999).

Of the tested extracts, only three showed growth inhibition against *Saccharomyces cerevisiae* S288C: *Passiflora oerstedii* Mast. var. *choconiana* S. Watson (Passifloraceae), *Campyloneurum brevifolium* (Lodd. ex Link) Link (Polypodiaceae), and *Cestrum schlechtendahlii* G.Don (Solanaceae). *Passiflora oerstedii* (Tu kej kejen) is used by the Q'eqchi' Maya for ulcer/heart burn, pink eyes, fever, headache, allergies, coughs, and the common cold. The only phytochemical report for this plant showed the presence of 2"–xylosyl vitexin in the leaves (Ulubelen et al. 1981). Vitexin and other related flavonoids such as apigenin and isovitexin did not show any growth inhibition of S288C at a test concentration of 0.5 mg/disc (data not shown). Thus, it is unlikely that flavonoids are responsible for the observed antifungal activity in *P. oerstedii.* Both *Campylonerum brevifolium* (Rix i xul) and *Cestrum schlechtendahlii* (Ik che) showed strong antifungal activities against S288C similar to that of the positive control berberine. *Campyloneurum brevifolium* is used to treat snake bites and *Cestrum schlechtendahlii* athlete's foot and thrush. As mentioned, neither of these two species has been investigated phytochemically. Due to limited yield, only *C. schlechtendahlii* will be further studied in order to identify the active principles responsible for the antifungal activities. This is presented in Chapter 5 and mechanistic studies are the focus of Chapter 6.

In Chapter 2, five families (Lepidobotryaceae, Melastomataceae, Meliaceae, Sapindaceae, and Simaroubaceae) were identified to have quorum quenching (QQ) and/or biofilm inhibitory activities. In this chapter, six more families (Begoniaceae, Combretaceae, Cucurbitaceae, Euphorbiaceae, Lamiaceae, and Phytolaccaceae) showed good inhibitory activities against bacterial quorum sensing and/or biofilm formation. Furthermore, the Solanaceae and Polypodiaceae were identified to have promising antifungal activity. It is clear that the healers have a wide diversity of plant usage. From these results, some pharmacological validation for the use of traditional Q'eqchi' Maya medicinal plants for the treatment of infections and related symptoms is provided. Further investigation such as bioassay-guided fraction of active species such as *Blakea cuneata* could lead to the development of botanical therapies for treatment of biofilm-associated infections. This is presented in Appendix I. Moreover, pharmacological validation of traditional knowledge could increase interest in and conservation of Maya traditional medicine for future generations.

Chapter 4 – Antimicrobial activities of Marcgraviaceae species and isolation of a naphthoquinone from *Marcgravia nervosa* Triana & Planch. (Marcgraviaceae)

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Statement of author contribution

JTA, CAT, and AFC conceived and designed this study. Plant collection was made by MER, MO, MG, TD, and JTA. Plant identification was performed by MER, MO, MG, PS, and LP. Plant extraction was undertaken by AFC and MER. Bioassays were performed by CAT with assistance from CDM. Column chromatography and NMR interpretation were done by AFC with assistance from IH. Phytochemical analyses were performed by AFC and RL. RS, MLS, and JTA contributed in manuscript preparation.

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4.1 Introduction

The Marcgraviaceae is a neotropical plant family of lianas and shrubs consisting of 7 genera and 130 species distributed throughout Central and South America mainly in evergreen and semi-evergreen tropical forests (Dressler 2004; Woodson et al. 1970). The largest genus *Marcgravia* L. has 60 species; 12 of which are found in Central America (Dressler 2004) and 22 in Brazil (Teixeira et al. 2013). The other genera include *Marcgraviastrum* (Wittm. Ex Szyszyl.) de Roon & S. Dressler (15 species), *Norantea* Aubl. (2 species), *Ruyschia* Jacq. (7 species), *Sarcopera* Bedell (10 species), *Schwartzia* Vell. (14 species), and *Souroubea* Aubl. (19 species) (Dressler 2004). Although phylogenetic studies have been published in the literature (Balthazar and Schönenberger 2013; Misa Ward and Price 2002), few phytochemical studies and biological activities have been documented for the Marcgraviaceae family. Mullally et al. (2011) reported the presence of betulinic acid in the leaves of *Souroubea sympetala* Gilg and its anxiolytic activity in rats. The presence of other triterpenoids including amyrins, and lupeol as well as phenolics was reported by Puniani et al. (2014). This paper also reported anxiolytic activity in extracts of *Souroubea gilgii* V.A. Ritcht. demonstrated in elevated plus maze experiments with rats. *Souroubea* species have been traditionally used in Amazonian and Maya traditional medicines to treat "*susto*" (fear or witchcraft), a folk illness which may be associated with anxiety (Bourbonnais-Spear et al. 2007; Schultes and Raffauf 1990, Puniani et al. 2014). The leaves of *Souroubea gilgii* V.A. Richt. are also used by Q'eqchi' Maya healers of Belize to treat infections and as a weight control tonic (Amiguet 2004).

Because of the hyper humid tropical environment in which Marcgraviaceae species grow, we suspected it might have interesting antimicrobial activities. The growth of bacteria in these humid environments depends on the secretion of extracellular factors. The presence of these

factors allows colonies to perform vital functions such as nutrient acquisition, protection from the environment, and helps them establish a competitor-free space (Annous et al. 2009; Davey and O'Toole 2000; Davies et al. 1998; Donlan and Costerton 2002). Recently, there has been a growing awareness about the social behaviour of microorganisms. Microorganisms communicate with one another in order to exhibit multicellular behaviour such as dispersal, foraging, biofilm formation, "chemical warfare", and quorum sensing (Davey and O'Toole 2000; Davies et al. 1998; Donlan and Costerton 2002; Popat et al. 2012). Furthermore, this behaviour plays a key role in bacterial virulence (Popat et al. 2012).

Biofilms are communities of microorganisms that form on surfaces of natural and artificial environments. This community is embedded in a matrix of polysaccharides, proteins, and nucleic acids (Davey and O'Toole 2000; Davies et al. 1998; Donlan and Costerton 2002; West et al. 2006). Within these biofilms, bacteria and fungi are protected from antibacterial chemicals (including natural antibiotics), environmental bacteriophages, and phagocytic amoebae. In medicine, chronic biofilm infections resist antibiotic therapy and become resistant to clearance mechanisms such as antibodies and phagocytes (Donlan and Costerton, 2002, Popat et al. 2012). This is especially prevalent in opportunistic pathogens like *Pseudomonas aeruginosa*, which is involved in many nosocomial infections such as urinary tract and respiratory system infections, dermatitis, chronic wounds, as well as soft-tissue and various systemic infections, particularly in patients with severe burns or those who are immunocompromised (Singh et al. 2001;Yang et al. 2009). Chronic biofilm infections are also linked to the development of chronic lung infections of patients who suffer cystic fibrosis (Singh et al. 2001; Yang et al. 2009).

In Gram-negative bacteria, biofilm formation is initiated by quorum sensing (QS), a cell to cell signalling communication system (Davey and O'Toole 2000; Davies et al. 1998; Donlan

and Costerton 2002; Hall-Stoodley et al. 2004). Small diffusible signalling molecules such as *N*–acyl–homoserine lactones (AHLs) and autoinducing peptides (AIPs) are self-produced and released continuously into the environment (Cady et al. 2012; Davey and O'Toole 2000; Davies et al. 1998). Once a threshold concentration is reached, certain genes can trigger the expression of virulence factors, adhesion proteins, and reduced metabolism (Davey and O'Toole 2000; Popat et al. 2012; Williams et al. 2007). Targeting QS is an attractive route for drug discovery since QS disruption can diminish virulence without providing a selective pressure for resistance.

Because of the potential for rapid microbial growth in the warm and humid habitat of tropical species, we hypothesized that these plants may produce antifungal and biofilm inhibiting compounds to prevent colonization of unwanted species. In particular, biofilm formation on leaves can lead to fouling with soil and other organisms like mosses which greatly reduce light reaching photosynthetic tissue. In a previous study (Ta et al. 2014) we showed that several tropical families of plants have this activity. The Marcgraviaceae was not investigated in the previous study and we present data here for this family that has a range covering the entire neotropics. In the current study, we investigated the antimicrobial activities of 12 Marcgraviaceae species (10 from Costa Rica and 2 from Brazil) using model fungal and bacterial organisms as well as a selection of pathogens. Bioassay-guided isolation of the leaves of *Marcgravia nervosa* Triana & Planch. resulted in the identification of an active principle and characterization of its secondary metabolite profile was completed by HPLC–MS.

4.2 Materials and Methods

4.2.1 General experimental procedures

NMR spectra were recorded on a Bruker Avance 400 spectrometer in $CDCl_3$ at 400 MHz. Open column chromatography was carried out on silica gel 60 (70-230 mesh, Merck). TLC analyses were performed on silica gel 60 F254 plates (Merck), and visualization of the plates was carried out using a Hanessian stain**.**

4.2.2 Materials

Extraction and HPLC grade solvents were purchased from Fisher Scientific (Ottawa, ON. Canada). Betulinic acid, ursolic acid, lupeol, α–amyrin and β–amyrin (95%) were purchased from Extrasynthase (Lyon, France) or were purified by preparative HPLC (Agilent Technologies, Mississauga, Canada). Berberine (>95%) was purchased from Sigma-Aldrich (Mississauga, Canada). Extract of *Delisea pulchra* was provided by B. J. Baker (University of South Florida). *N*–decanoyl–L–homoserine lactone (C10–HSL) (≥96%) was purchased from Cayman Chemical (Ann Arbor, MI, USA) and allicin (97%) from AK Scientific Inc. (Mountain View, CA, USA).

4.2.3 Plant collection

All plant materials were collected from Costa Rica and Brazil. Vouchers were deposited at the Herbario Juvenal Valerio Rodriguez (JVR), Museu Nacional/Universidade federal do Rio de Janeiro (MN/UFRJ) and Rio de Janeiro Botanical Garden (RB). Plant species identification was confirmed by M. E. N. Rocha (UFRJ), M. Nadruz (RB), M. Garcia, M. Otárola-Rojas, P. Sanchez-Vindas, and L. Poveda (JVR). Voucher numbers are listed in Table 4.1.

4.2.4 Plant extraction

Plant materials were dried at 35ºC with a plant drier for 72 hours and then ground with a Wiley mill (mesh size 0.2 cm) and extracted with 80% EtOH using a 1:10 biomass to solvent (w/v) ratio. The plant material/ethanol mixture was shaken overnight at 200 rpm (shaker: New Brunswick Scientific, Edison, NJ, USA) and then filtered using vacuum filtration. A second extraction was performed with the residue using a 1:5 w/v ratio and the mixture was again shaken and filtered. The two extracts were combined, dried *in vacuo* using a rotary evaporator (Yamato RE 500) at 45ºC, lyophilized to remove any residual water using a freeze-dryer (EC Super Modulyo, ~ -55 ℃, 10^{-2} mbar), and stored at -20ºC in the dark until needed.

4.2.5 Microbial strains and culture

Bacteria species *Chromobacterium violaceum* ATCC 12472 was used in the quorum sensing bioassay and *Pseudomonas aeruginosa* PA14 in the biofilm bioassay. *Chromobacterium violaceum* was purchased from ATCC and *Pseudomonas aeruginosa* PA14 was obtained from T. Mah (University of Ottawa). These strains were maintained as liquid culture with shaking (200 rpm) at 30ºC (incubator: New Brunswick Scientific Series 25, Edison, NJ, USA) in nutrient broth (BD, Sparks, MD, USA) and LB broth (Fisher Scientific, Fairlawn, NJ, USA), respectively. Fungal species *Saccharomyces cerevisiae* S288C and *Candida albicans* D10 were provided by M. L. Smith (Carleton University); *S. cerevisiae* strains BY4741 and BY4743 were provided by R. Subramaniam (Agriculture and Agri-Food Canada). These strains were maintained in liquid culture with shaking (200 rpm) at 30ºC (incubator: New Brunswick Scientific Series 25, Edison, NJ, USA) in YPD broth (1% yeast extract, 2% peptone, 2% dextrose w/v) (VWR International, Mississauga, ON, Canada) or Sabouraud's medium (Fisher Scientific, Fairlawn, NJ, USA).

4.2.6 Quorum sensing (QS) bioassay

A modified disc diffusion assay described by Adonizio et al. (2006) was used to determine whether the plant extracts can interfere with the QS of *C. violaceum. C. violaceum* produces a purple pigment, violacein, which is under QS control. The inhibition of violacein production will indicate the disruption of QS. Briefly, sterile paper disks (Oxoid, Basingstoke, Hants, UK) loaded each with 1 mg of extract were placed onto TGY agar plates (BD, Sparks, MD, USA) inoculated with 100 µL of overnight cultures then incubated without agitation for 24 hours at 30^{o}C (incubator: Precision Automatic CO_2 Incubator). QS inhibition was indicated by a colourless opaque halo around the disc and growth inhibition by a clear halo. Plates were examined under a dissecting microscope to confirm whether the extract has anti-QS and/or antibacterial activity. The controls used were: 70% ethanol as vehicle control and *N*–decanoyl–L–homoserine lactone (C10–HSL) (400 µg/disc, $\geq$96%, Cayman Chemical, Ann Arbor, MI, USA) as positive control. Extract of *Delisea pulchra* (Greville) Montagne (1 mg/disc) was also included for comparative purposes as this alga contains known QS inhibitors, the halogenated furanones (Givskov et al. 1996; Raffa et al. 2005; Shiner et al. 2005). Each sample was tested in triplicate.

4.2.7 Biofilm bioassay

A spectrophotometric assay was adapted from Ren et al. (2005) to assess the biofilm inhibitory activity of the plant extracts. Extracts were prepared in 50% methanol and tested at a final concentration of 400 µg/mL. Overnight culture of *P. aeruginosa* PA14 was diluted 1:50 in M63 medium broth (Amresco, Solon, OH, USA) supplemented with 0.4% arginine (Fisher Scientific, Fairlawn, NJ, USA) and 1mM $MgSO_4$ (Fisher Scientific, Fairlawn, NJ, USA). This inoculum was grown in a 24-well flat bottom microtiter plate (Costar 3526, Fisher Scientific,

Fairlawn, NJ, USA) without agitation for 24 hours at 37°C to allow biofilm formation (incubator: Precision Automatic CO_2 Incubator). The plate was then decanted and washed with distilled water three times to eliminate non-adherent cells. The remaining biofilm was stained with 0.1% crystal violet (Fisher Scientific, Fairlawn, NJ, USA) for 15 minutes. The excess dye was decanted, the plate was washed three times with distilled water, and then air dried overnight. The biofilm was quantified by dissolving the dye in ethanol and reading the absorbance at 570 nm (spectrophotometer: SpectraMax M5, Molecular Devices, Sunnyvale, CA, USA). The controls used were: vehicle control with 50% methanol, negative control with M63 medium broth, and positive control with allicin (1 μL/mL or 1.08 mg/mL, 97%, AK Scientific Inc., Mountain View, CA, USA). Biofilm formation was expressed relative to the vehicle control. Samples were tested in triplicate.

4.2.8 Antifungal disc diffusion assay

Saccharomyces cerevisiae S288C was used for the initial evaluation of plant extracts for antifungal activity, performed in triplicate. Plant extracts were prepared to a final concentration of 40 mg/mL, using HPLC grade methanol as a solvent. Berberine was used as an antifungal positive control and HPLC grade methanol was used as a negative control. *Saccharomyces cerevisiae* was inoculated into Sabouraud's medium (Fisher Scientific, Fairlawn, NJ, USA) and grown to an optical density of 600 nm (OD_{600}) of ~1.0 and diluted 1:100 (1.5×10^5 CFU/mL). Aliquots (100 μL) of the diluted broth culture were spread over the surface of Sabouraud's agar plates. Sterile paper discs (7.0 mm diameter, Oxoid, Basingstoke, Hants, UK) were amended with crude extract (2 mg/disc), berberine (1 mg/disc) or HPLC methanol and allowed to air-dry. All treatments were subsequently incubated in the dark for 48 h at 30 ℃ without agitation.

Inhibition zones from active extracts were then measured. *C. albicans* D10 was also used to assess the antifungal activity of pure compounds.

4.2.9 Minimum inhibitory Concentration (MIC) determination

Saccharomyces cerevisiae BY4741 and BY4743 were cultured in YPD broth (1% yeast extract, 2% peptone, 2% dextrose w/v) at 30ºC. Overnight cultures were adjusted to an OD_{600} of 0.1 (1.5×10^6 CFU/mL). In a 96-well flat bottom plate (Costar 3596), 50 µL of this inoculum ($\sim 7.5 \times 10^4$ CFU/well) was mixed with 50 µL YPD in which the test compounds were dissolved at varying concentrations. Wells containing berberine or appropriate quantities of the MeOH carrier solvent were also included to act as positive and negative antifungal controls, respectively. Yeast growth was monitored using a Biotek Powerwave XS2 microplate reader (Biotek, Winooski, Vermont, USA), running the Gen5® Microplate Data Collection & Analysis software ver. 2.00. The plate was incubated at 30ºC shaking at 600 rpm, with absorbance readings taken at 600 nm every 10 minutes for 24 hours. All experiments were repeated three times, with three technical repetitions per biological repetition.

4.2.10 Isolation of naphthoquine from Marcgravia nervosa

Plant leaves material was dried at 35 ℃ in a plant drier for 2 hours and ground to 0.2 cm mesh in a Willey mill. Ground materials (42.87 g) were extracted by dynamic maceration overnight at room temperature using three different solvents (ethanol, ethyl acetate, and hexane). The extracts obtained were concentrated under reduced pressure using a Yamato Rotary Evaporator RE50 (Yamato Scientific, Japan) at 40 ℃ and then lyophilized (Super Modulo, Thermo Electron, USA) to give three dried fractions. The fractions were stored at 4ºC in amber vials. The ethanolic (EtOH) fraction (2.56 g) was placed in 160 g of silica gel and the column

was eluted with solvent volumes of 300 mL going from 100% hexanes to 100% ethyl acetate (EtOAc), with increments of 5% EtOAc until 60% hexanes and thereafter increments of 10% until 100% EtOAc. Finally the column was flushed using a mixture of ethyl acetate: methanol (80:20). A total of 299 eluates were collected and then combined into 18 fractions based upon their analytical TLCs. Compound **1** was collected in fractions corresponding to 85%-90% hexane; NMR analysis of which showed the presence of betulinic acid.

This fraction (2.6 g) was placed in 100g of silica and the column was eluted with solvent volumes of 200 mL going from 100% hexane to 20% EtOAc, with increments of 5% EtOAc. A total of 20 eluates were collected with compound **1** present in fractions corresponding to 85% hexane. These fractions were combined and dried to yield a total of 1.4 g of compound **1** or 3.4% of the dry plant weight.

A second sample was used to confirm the presence of the isolated metabolite. For this isolation, 2.6 g of the ethanolic extract was chromatographed on 160 g silica gel column and separated into five fractions (MNE–C1 to MNE–C5), using a solvent system from 100% hexane to 100% ethyl acetate with increments of 25 %, the column was flushed with 10% methanol. The results obtained from this column were as followed: fraction one MNE–C1 collected at 100% hexane (0.0 g), fraction MNE–C2, 75% hexane (0.9 g), fraction MNE–C3, 50% hexane (4.3 g), fraction MNE–C4 100 ethyl acetate (2.2 g), and final fraction MNE–C5, 10% methanol (2.1 g).

The highest concentration and activity was detected in fraction MNE–C3. This fraction was rechromatographed on a silica gel column to yield compound **1** as a pure compound a 35 mg/g dry plant, for a total yield of 3.5 %.

4.2.11 HPLC–APCI–MS analysis

HPLC–APCI–MS analyses were carried out on a 3200 QTRAP (ABSciex, Concord, ON, Canada) connected to a 1200 series HPLC system (Agilent Technologies, Santa Clara, CA, USA). The HPLC system consisted of a high performance autosampler, a binary pump and, a column thermostat, and an online degasser. The MS system consisted of a turbo V source and a mass analyser. The separations were performed at 1 mL/min on a Kinetex C18 column, particle size 2.6 μm, particle diameter 100Å, 100 mm × 2.1 mm I.D. (Phenomenex, Torrance, CA, USA). Mobile phases were H_2O (A) and acetonitrile (B). The column thermostat was maintained at 50 ℃. The gradient elution method was initiated with 30% B and then increased to 100% B in 10 min. The column was then flushed with 100% B for 8 min and changed back to the initial conditions for 7 min. The flow rate was set at 0.4 mL/min. The MS was operated in the Q1M1 negative ionization mode for compounds **2** and **3** and in the Q1M1 positive ionization mode for compounds **4**, **5** and **6** with a dwell time set at 100 msec. Optimal negative mode Q1M1 conditions were: declustering and entrance potentials -60 V, -10 V, nebulizer current -1.0 V, source temperature 500 ℃, nebulizer gases 1 and 2 set at 50 psig and 30 pisg respectively. Optimal positive mode Q1M1 conditions were: declustering potential, entrance potential and nebulizer current 60 V, 10V and 3V respectively, source temperature 500 ℃ with ion source gases 1and 2 50 and 55 pisg respectively. Curtain gas was set at 20 L/min in both modes. Positive identification was determined by comparison with the retention times and masses of authentic standards.

4.2.12 UPLC–PDA–MS analysis

Plant extract was analyzed on a Shimadzu UPLC–PDA–MS system (Mandel Scientific Company Inc, Guelph, Ontario) which consisted of LC30AD pumps, a CTO20A column oven, a SIL-30AC auto sampler and a LCMS-2020 mass spectrometer with an electrospray-ionization source. Briefly, 1 μL of each fraction was injected through the autosampler to an Acquity CSH C18 column (100 × 2.1 mm, 1.7 μm particle size, Waters, Mississauga, Ontario) with an Acquity CSH C18 VanGuard Pre-column (5 × 2.1 mm). The mobile phases are H_2O (A) and acetonitrile (B) with 0.1% formic acid in both. The gradient elution method initiated with 30% B then increased to 95% B in 3 min. The column was then washed with 95% B for 3 min and changed back to the initial condition in 1 min. The flow rate was set at 0.8 mL/min with the column temperature set at 50˚C. The photodiode array detector was set to monitoring wavelengths from 190 to 400 nm. The mass spectrometer with electrospray ionization (ESI) interface was operating in positive and negative scan modes ; the nebulizing gas flow was set at 1.5 L/min and drying gas flow was at 10 L/min. The desolvation line temperature and heat block temperature was set at 300 ℃ and 450 ℃, respectively. The m/z range of both positive and negative scan is from 150 to 600 with 938 u/sec scan speed.

4.2.13 Statistical analysis

Statistical analyses were done using SigmaPlot 11 software for Windows (Systat Software, San Jose, CA, USA). Results are presented as means ± S.E.M. (standard error of the mean) for at least 3 independent experiments. One-way ANOVA was performed with posthoc Tukey's test and $p < 0.01$ considered as significant.

4.3 Results

Extracts of the 12 Marcgraviaceae species were assessed for antimicrobial activities using model bacterial and fungal organisms. Table 4.1 summarized the biological activities of these Marcgraviaceae extracts. Quorum sensing (QS) inhibitory activities ranged from 7.7 ± 0.3 to 21.9 ± 0.8 mm. In particular, three extracts showed significantly more QS inhibition than the control *D. pulchra* extract (12.8 ± 0.7 mm): *Marcgravia nervosa* Triana & Planchs leaves and stems and *Marcgravia schippii* Standl. leaves. The roots of *Schwartzia brasiliensis* (Choisy) Bedell ex Gir-Cañas and stems of *Schwartzia costaricensis* (Gilg.) Bedell displayed similar anti-QS activities to that of *D. pulchra*. *Sarcopera* and *Souroubea* species showed little to no QS inhibition.

For biofilm formation, a large range of inhibition was observed. Both the stems and roots of *Marcgravia polyantha* Delp. significantly inhibited biofilm growth of *P. aerguinosa* PA14 at 45.4 ± 3.1% and 30.3 ± 3.2 % of growth in vehicle control, respectively, when compared to the positive control allicin (1 µL or 1.08 mg) with 74.8 ± 8.1 % growth. However, *M. polyantha* leaves only showed moderately inhibition at 78.5 ± 3.0 % growth. Furthermore, five extracts displayed from 30% to 40% biofilm inhibition: *Marcgraviastrium subsessile* (Benth.) Bedell stems, *Schwartzia brasiliensis* (Choisy) Bedell ex Gir-Cañas leaves and root, and *Schwartzia costaricensis* (Gilg.) Bedell leaves and stems. *Marcgravia nervosa*, *Sarcopera*, and *Souroubea* species had little or no activity in this bioassay.

Table 4.1 Effects of Marcgraviaceae extracts on violacein production in *Chromobacterium violaceum* ATCC 12472 (1 mg/disc, N = 3–7, disc diameter = 7 mm, vehicle control: 70% EtOH), biofilm formation in *Pseudomonas aeruginosa* PA14 (400 µg/mL, N = 3–7, vehicle control: 50% MeOH), and growth of *Saccharomyces cerevisiae* S288C (2 mg/disc, N=3–7, disc diameter = 7 mm, vehicle control: 70% EtOH).

Voucher	Species	Part[a]	Location	Violacein inhibition ± SM (mm)	% Biofilm growth ± SEM	Growth inhibition ± SEM (mm)
JVR13150	*Marcgravia mexicana* Gilg	L	Tortugero, Limón	8.4 ± 0.4	100.3 ± 0.3	8.6 ± 0.2
JVR13145	*Marcgravia nepenthoides* Seem.	L	Tortugero, Limón	9.0 ± 0.4	83.4 ± 3.1	8.1 ± 0.2
JVR13157	*Marcgravia nervosa* Triana & Planch.	L	Rio Pacuare, Limón	21.8 ± 0.3[b]	100.4 ± 0.2	23.9 ± 0.2[b]
		S		21.9 ± 0.8[b]	97.1 ± 0.9	23.6 ± 0.2[b]
RB495172	*Marcgravia polyantha* Delp.	L	Rio de Janeiro	9.4 ± 0 .4	78.5 ± 3.0	9.0 ± 0.2
		R		8.2 ± 0.1	45.4 ± 3.1[b]	8.1 ± 0.3
		S		10.4 ± 0.2	30.3 ± 3.2[b]	7.8 ± 0.3
JVR14272	*Marcgravia schippii* Standl.	L	Mastatal de Puriscal	16.7 ± 0.4[b]	100.0 ± 0.1	9.0 ± 0.6
JVR13130	*Marcgraviastrum subsessile* (Benth.)	L	Rio Costa Rica,	10.1 ± 0.4	87.9 ± 3.6	8.0 ± 0.1
	Bedell	S	Cartago	10.2 ± 0.2	70.4 ± 3.1	8.4 ± 0.2
JVR13142	*Sarcopera rosulata* de Roon & Bedell.	L	Tapanti, Cartago	9.0 ± 0.1	100.5 ± 0.2	7.3 ± 0.3
JVR13154	*Sarcopera sessiliflora* (Triana & Planch.)	L	Lago Arenal,	10.9 ± 0.8	100.3 ± 0.3	8.6 ± 0.4
	Bedell		Alajuela			
MN/UFRJ	*Schwartzia brasiliensis* (Choisy) Bedell	L	Rio de Janeiro	7.7 ± 0.3	71.1 ± 4.1	8.4 ± 0.3
178118	ex Gir.-Cañas	R		12.7 ± 0.5	64.7 ± 7.3	8.6 ± 0.3
JVR13298	*Schwartzia costaricensis* (Gilg) Bedell	L	Tapanti, Cartago	11.1 ± 0.3	59.5 ± 1.7	9.0 ± 0.4
		S		12.2 ± 0.3	60.8 ± 3.7	8.7 ± 0.2
JVR13321	*Souroubea gilgii* V.A. Richt.	L	Tortugero, Limón	8.0 ± 0.1	99.0 ± 0.8	7.9 ± 0.3
JVR13300	*Souroubea sympetala* Gilg	L	Tortugero, Limón	7.8 ± 0.2	86.9 ± 1.7	8.2 ± 0.2
PSC08-85	*Delisea pulchra* (Greville) Montagne	T	Palmer Station, Antartica	12.8 ± 0.7	NT	NT
CON (QS)	C10–HSL (400 µg)			10.2 ± 0.1	NT	NT
CON (biofilm)	allicin (1 µL or 1.08 mg)			NT[c]	74.8 ± 8.1	NT
CON (fungal)	berberine (1 mg)			NT	NT	19.3 ± 0.7

[a] plant parts: L – leaves, R – roots, S – stems, T – thallus; [b] statistically significant ($p < 0.01$); [c]NT - not tested

All tested Marcgraviacae extracts showed little to no inhibition against the growth of *Saccharomyces cerevisiae* S288C except *M. nervosa.* When compared to the positive control berberine at 1 mg/disc (19.3 ± 0.7 mm), both leaves and stems extracts showed inhibitory zones of 23.9 ± 0.2 and 23.6 ± 0.2 mm, respectively.

Using the analytical method developed for other Marcgraviaceae (Mullally et al. 2011), HPLC–APCI–MS analysis of the crude extract of *M. nervosa* leaves identified the presence of ursolic acid, betulinic acid, α–amyrin, β–amyrin, and lupeol (Figures 4.1, 4.2, and 4.3). However, all of these compounds were inactive in the QS and antifungal bioassays at 250 μg/disc (data not shown). The leaves extract was then fractionated using liquid-liquid extraction with hexane, ethyl acetate (EtOAc), and ethanol (EtOH) to give three fractions. These fractions were tested using the same bioassays, which identified the EtOH fraction as the most active (Figure 4.4). Both EtOAc and EtOH fractions showed antifungal activities comparable to the positive control berberine. For QS inhibition, the hexane fraction showed little or no activity while both EtOH and EtOAc fractions were more active than the positive control *Delisea pulchra* extract, which contains known QS inhibitors, the halogenated furanones (Giskov et al. 1996; Manefield et al. 1999; Raffa et al. 2005; Shiner et al. 2005).

The ^{1}H NMR spectra of the three fractions showed the presence of the same metabolite which featured a prominent O-CH_3 group in the 3.9 ppm range and two low field aromatic multiplets (Appendix IIa). The key difference among the extracts was the concentration of this compound with the highest concentration residing in the EtOH fraction. This fraction was further fractionated using silica gel open glass column chromatography (CC) to give 18 secondary fractions. Fraction 4 (85–90% hexane) showed the same activity as the EtOH fraction; however, NMR analysis showed that the bioactive component has co-eluted with betulinic acid. A second

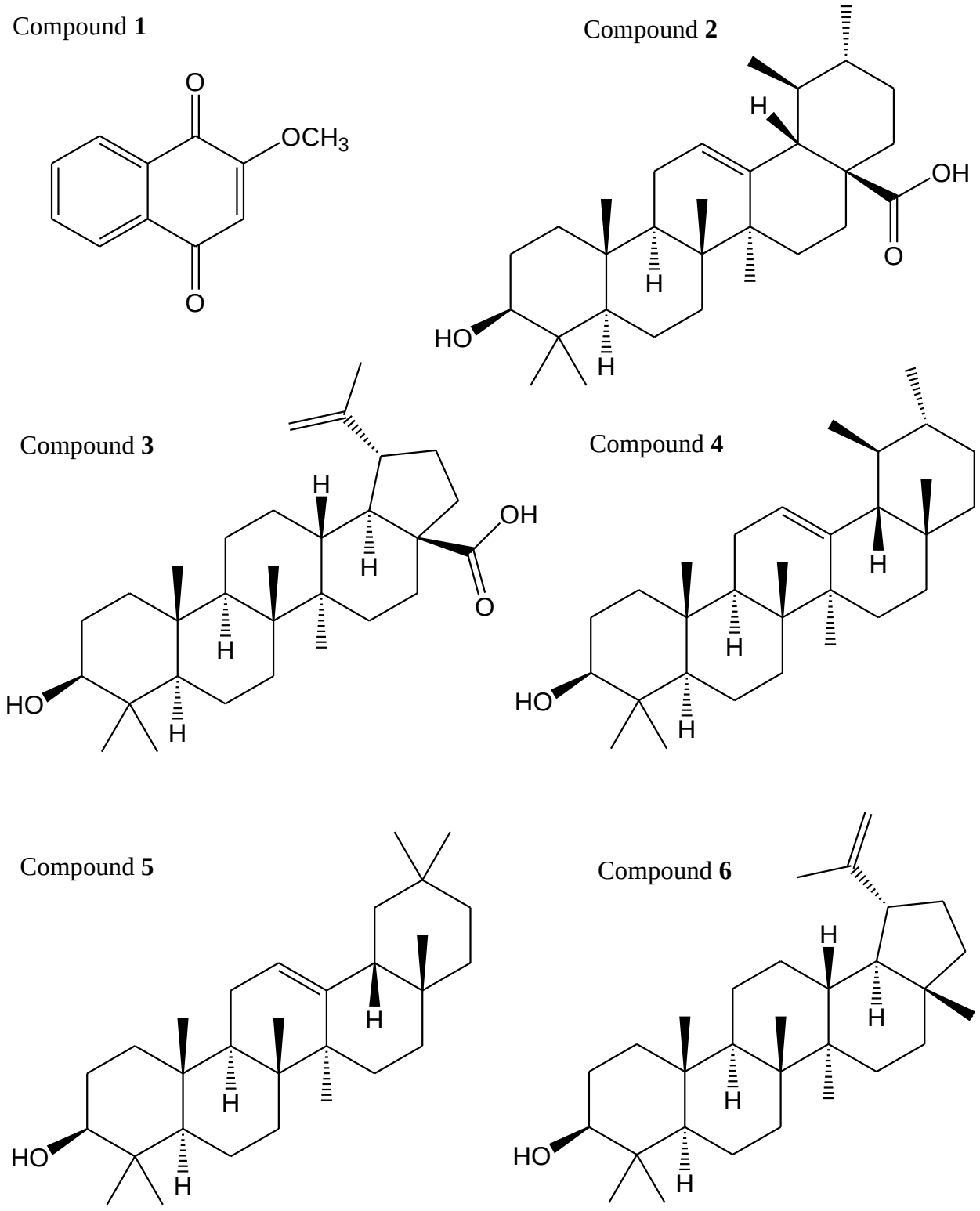

Figure 4.1 Chemical structures of compounds **1–6** isolated from the leaves of *Marcgravia nervosa* Triana & Planch. Compound **1** is a naphthoquinone: 2–methoxy–1,4–naphthoquinone; compounds **2–6** are triterpenes: ursolic acid (**2**), betulinic acid (**3**), α–amyrin (**4**), β–amyrin (**5**), and lupeol (**6**).

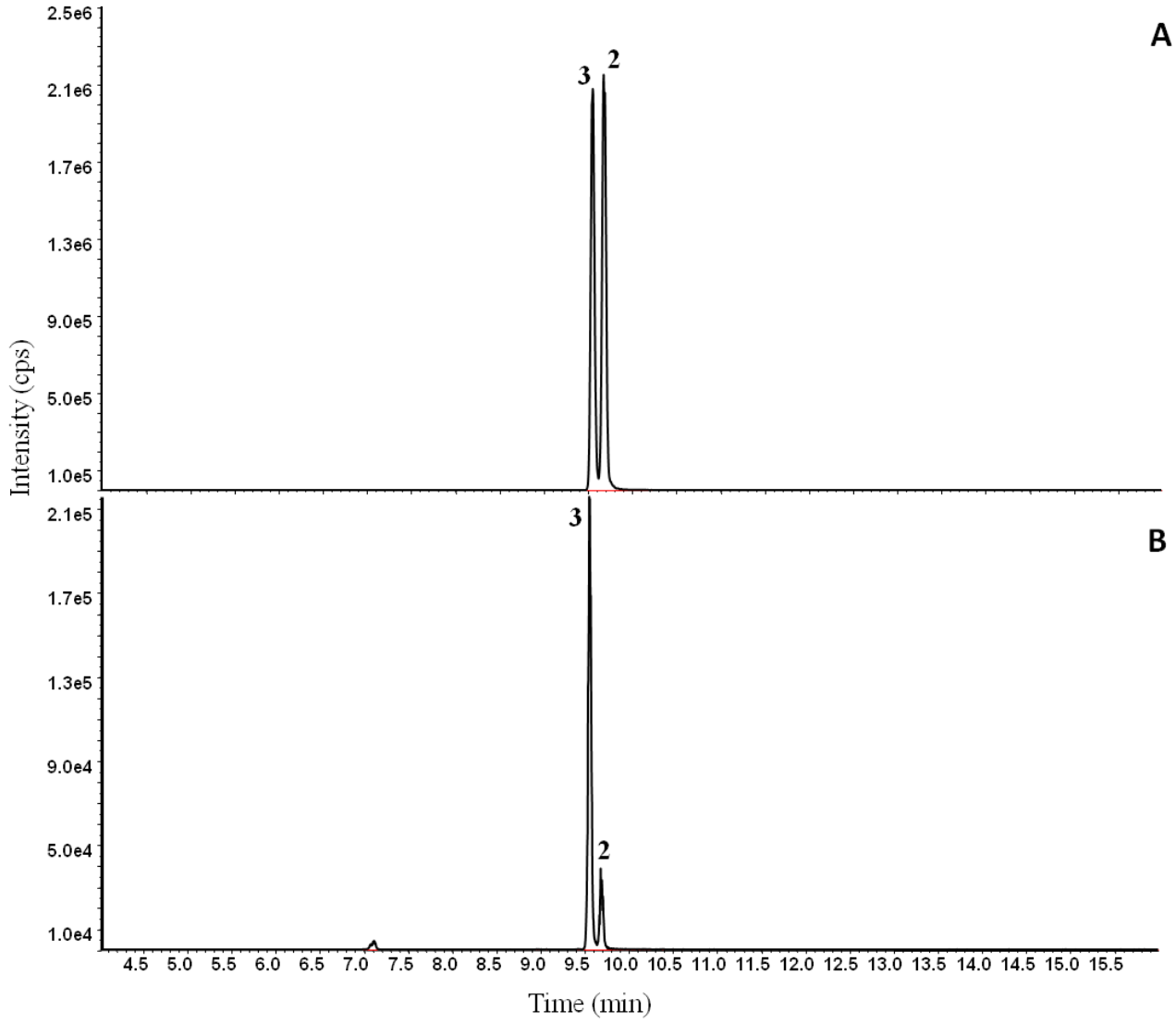

Figure 4.2 HPLC–APCI–MS analysis for the identification of ursolic acid **(2**) and betulinic acid (**3**) in the crude extract of *Marcgravia nervosa* leaves (B) compared with the standard mix (A). Extracted ion currents shown for 455.3 *m/z*.

column was performed on this fraction to obtain a pure sample of the active metabolite. ^{1}H and ^{13}C spectra of the isolated metabolite were obtained (Appendix IIb). The simplicity of the spectra enabled us to identify this compound as 2–methoxy–1,4–naphthoquinone (compound **1**). The identification and mass were verified via UPLC–PDA–MS analysis (Figures 4.1 and 4.5).

Compound **1** was obtained as yellow crystals with a molecular formula of $C_{11}H_8O_3$ (MS (positive mode) 189.20). The spectroscopic values of compound **1** matched those reported in the literature (Ding et al. 2008). The yield of 2–methoxy–1,4–naphthoquinone was 3.5% based on dry leaf material. This represents a remarkably high concentration for a secondary metabolite. Extraction of the leaves of the same plant collected in a different season and a second sample collected at a very different collection site also showed somewhat lower, but still very substantial levels of compound **1** in a qualitative analysis performed using UPLC-DAD system at 280 nm. Compound **1** showed potent QS inhibition at the tested concentration of 20 µg/disc (Figure 4.4). The minimum inhibitory concentration (MIC) for compound **1** was determined to be 85–100 µM against *Saccharomyces cerevisiae* BY4741 (haploid) and BY4743 (diploid). These MICs are at least fivefold lower than that of berberine (positive control) against the same strains (600 µM for both). This compound was also effective against *Candida albicans* D10 with an average zone of inhibition of 26.0 ± 0.6 mm at 20 µg/disc (106 nmol/disc). Berberine at 600 µg/disc (1.64 µmol) had an inhibitory zone of 21.9 ± 1.8 mm. Compound **1** was not detected in any other Marcgraviacae species tested (at 189.2 *m/z*).

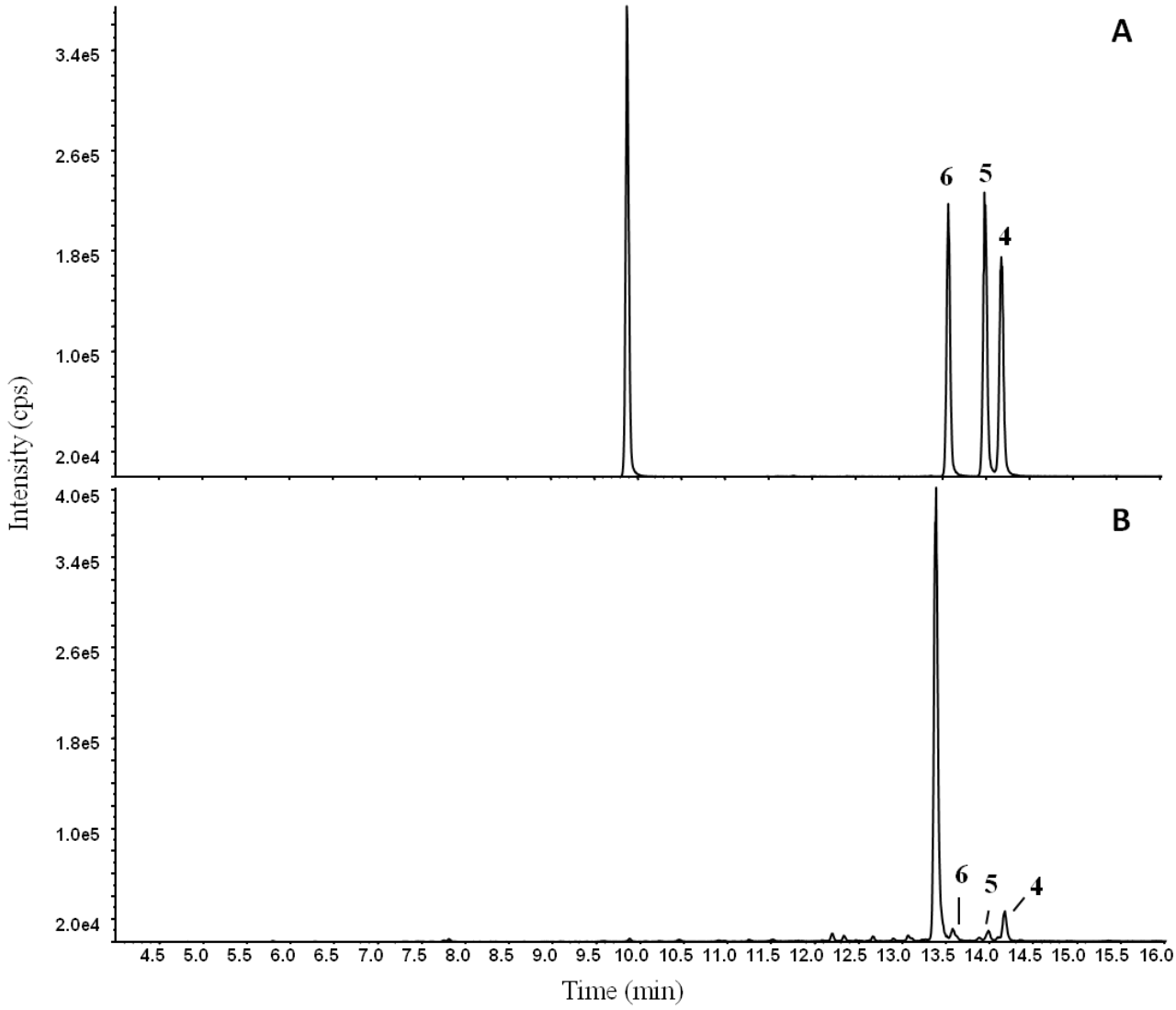

Figure 4.3 HPLC–APCI–MS analysis for the identification of α–amyrin (**4**), β–amyrin (**5**), and lupeol (**6**) in the crude extract of *Marcgravia nervosa* leaves (B) compared with the standard mix (A). Extracted ion currents for 425.4 *m/z*.

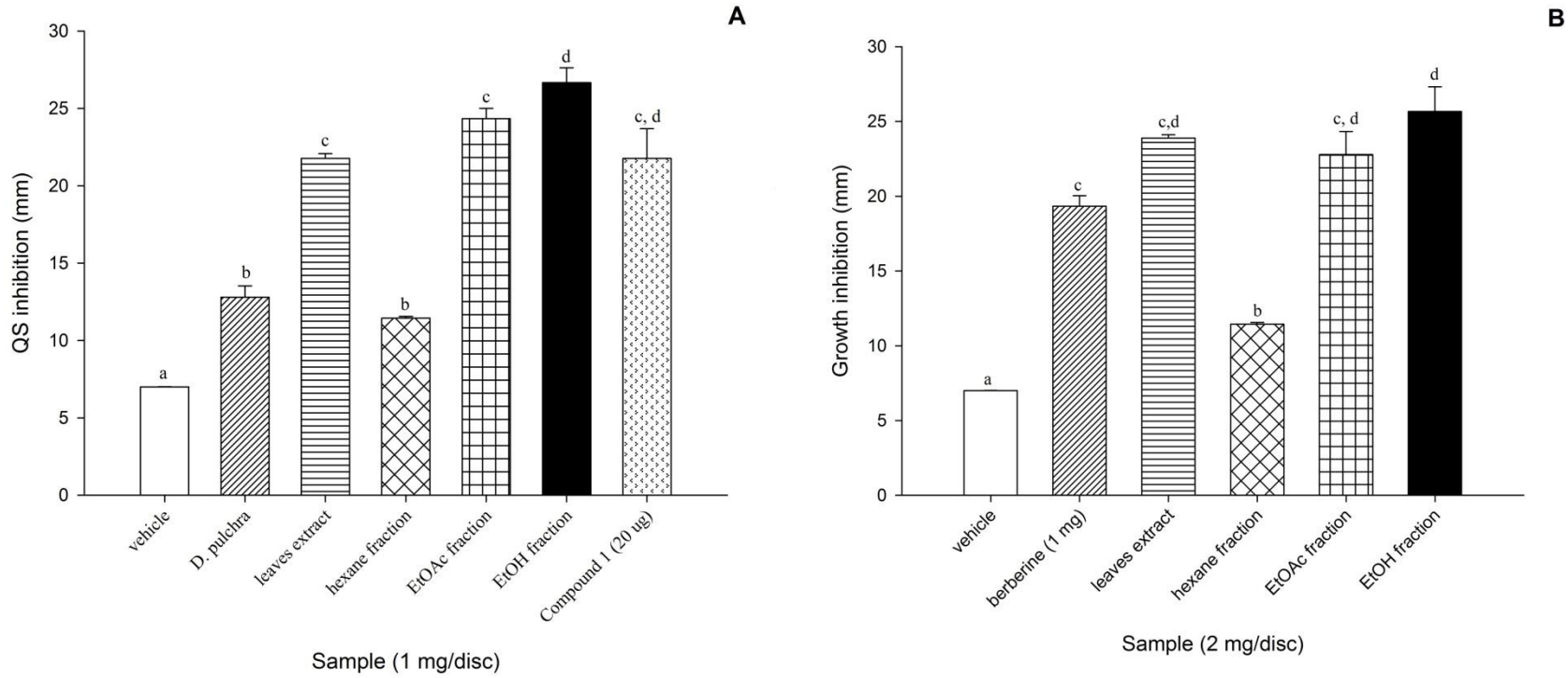

Figure 4.4 (A) Inhibition of quorum sensing (QS) in *Chromobacterium violaceum* by *Marcgravia nervosa* leaves crude extract, fractions, and compound **1** (20 µg/disc) compared to positive control *D. pulchra* (disc diameter = 7 mm; N = 3). (B) Growth inhibition of *Saccharomyces cerevisiae* S288C by *Marcgravia nervosa* leaves crude extract and fractions compared to positive control berberine (1 mg/disc) (disc diameter = 7 mm; N = 3). Treatments with different letters are statistically significant ($p < 0.01$).

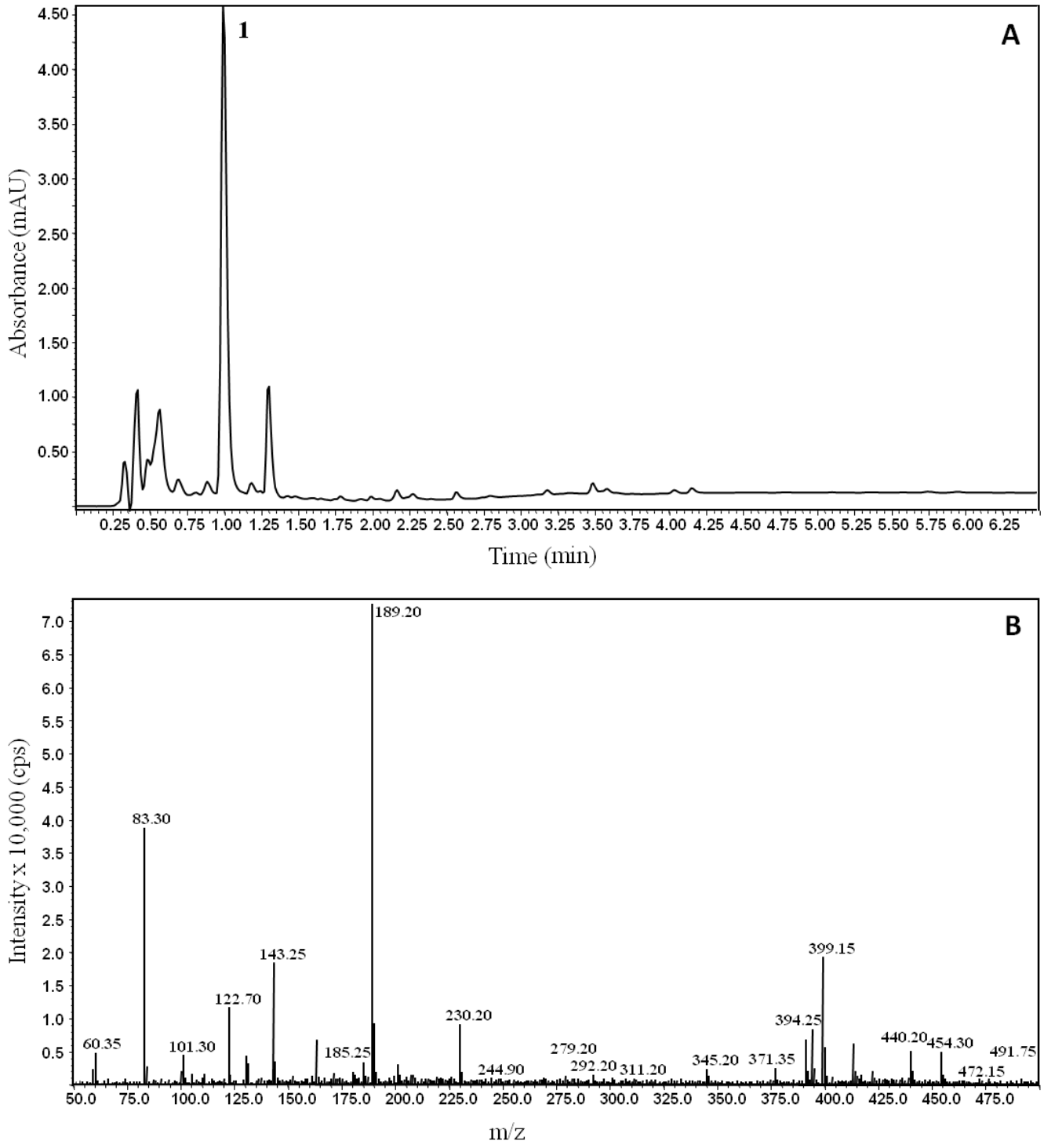

Figure 4.5 UPLC–PDA analysis (A) (254 nm) and ESI–MS spectrum (B) for the identification of 2–methoxy–1,4–naphthoquinone (**1**) from *Marcgravia nervosa*.

4.4 Discussion

To our knowledge, this is the first study of the bacterial biofilm and quorum sensing inhibition by Marcgraviaceae species and only the second type of biological activity reported for these plants. The anxiolytic activity of *Souroubea* species has been documented in rats (Mullally et al. 2011; Puniani et al. 2014) and dogs (Villalobos et al. 2014). Interestingly, none of the active species (*Marcgravia nervosa* Triana & Planch., *Marcgravia polyantha* Delp., *Marcgravia schippii* Standl., *Marcgraviastrum subsessile* (Benth.) Bedell, *Schwartzia brasiliensis* Choisy) Bedell ex Gir.-Cañas, and *Schwartzia costaricensis* (Gilg.) Bedell) has been previously studied in terms of biological activities and phytochemistry. The presence of triterpenoids such as ursolic acid and betulinic acid is characteristic for this plant family. However, these compounds do not appear to be responsible for the observed anti-QS, antifungal, and biofilm inhibitory activities as they were inactive at tested concentrations of 5 µg/mL for biofilm and 250 µg/disc for QS and fungal bioassays. Preliminary fractionation of *M. polyantha* stems showed biofilm inhibition in the polar region, suggesting that the active phytochemicals could be polyphenols or other polar compounds.

The isolation of 2–methoxy–1,4–naphthoquinone (compound **1**) from the leaves of *M. nervosa* is the first report of a naphthoquinone in the Marcgraviaceae family. Quinones are known for their antibacterial and antifungal activities (Savoia 2012). Ding *et al.*. (2011) found that the anthroquinone emodin inhibited biofilm formation of *P. aeruginosa* and *S. maltophilia* at 20 µM. Compound **1** was first isolated from *Impatiens balsamina* L. (Panichayupakaranant et al. 1995; Panichayupakaranant 2001; Yang et al. 2001), a widely used plant in Traditional Chinese Medicine (Ding et al. 2008). The antimicrobial activity of this naphthoquinone (compound **1**) against several bacterial strains such as *Staphylococcus aureus*, *Bacillus cereus*, *Aeromonas*

salmonicida and fungi such as *Fusarium oxysporum* has been reported (Fostel and Lartey 2000). More recently, compound **1** showed promising activity in the treatment of multiple antibiotic resistant strains of a bacterium responsible for stomach ulcers, *Helicobacter pylori* (Wang et al. 2011), and the mechanism of action for this compound was proposed to occur via oxidative stress. A comparison of the antifungal activity of chimaphilin, another naphthoquinone previously isolated in our research group, shows that compound **1** is more active by a factor of 30 (Galván et al. 2008).

Since this report provides new data on occurrence of phytochemicals in the family, chemotaxonomic comparisons at the level of order is appropriate. The occurrence of quinones in the order Ericales, to which the Marcgraviaceae family belongs, has been estimated to be approximately 10% (Rocha et al.. 2014). In particular, naphthoquinones such as plumbagin and derivatives have been isolated from the ironwood family, Ebenaceae, a related plant family in the Ericales (Higa et al. 2002; Kuo, Chang, and Kuo 1996; Lee and Lee 2008). The occurrence of naphthoquinone secondary metabolite in *Marcgravia nervosa,* and some Ericales species and its absence in the other Marcgraviaceae and Ericales species evaluated could be the result of specific microbial evolutionary pressure for the production of napthoquinone in these plants. Secondary metabolites can evolve in a specific species or genus as they originate as a strategy for survival and to overcome threats in a specific environment (Kennedy and Wightman 2011). The environment from which *M. nervosa* has been collected is a high humidity and temperature ecosystem (humid evergreen lowland forest), making this the perfect climate for the development of microorganisms, including plant and animal pathogens. The evolution of a biosynthetic pathway of a compound like the naphthoquinone with a broad spectrum of antimicrobial activity provides a selective advantage for these plants to survive in this climate.

We suggest that napthoquinone evolution has occurred independently in plants that are not closely related, but could possibly start with a common shikimate derivative common to Ericales. The identification of novel biologically active species such as *Marcgravia polyantha*, *Marcgravia nervosa*, and *Schwartzia brasiliensis* shows support for using plants as botanical therapies for the treatment of infections.

Chapter 5 – Antifungal saponins from the Maya medicinal plant *Cestrum schlechtendahlii* G.Don

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Statement of author contribution

JTA and CAT conceived and designed this study. Plant collection was done by FC, FC, and JTA. Plant identification was done by MOR, PS, and LP. Plant extraction and bioassays were performed by CAT with assistance from ER and CDM. Column chromatography was done by CAT and JAG with assistance from ER. NMR interpretation was done by JAG. Phytochemical analyses were performed by CAT, RL, and AS. VC coordinated project in Belize. JAG, CDM, RS, MLS, and JTA contributed in manuscript preparation.

Will be submitted to *Phytotherapy Research*

5.1 Introduction

The Mesoamerican region of Central America is a world biodiversity hotspot of mainly semi-evergreen tropical forest (Myers et al. 2000). Indigenous Maya cultures in this region have strong traditions of healer apprenticeship and continue to use traditional medicines derived from local plants for primary health care. Ethnobotanical studies show the Maya have an extensive knowledge of useful flora, and quantitative methodologies show that there is a high degree of consensus for many usage categories (Ankli et al. 1999; Bourbonnais-Spear et al. 2005; Amiguet et al. 2005).

The use of plants for infections is one of the areas that show a high degree of consensus. For dermatological infections, Heinrich (2000) reported an informant consensus factor of 0.52 among Yucatec Maya. For the Q'eqchi, our own research calculated a consensus factor of 0.68 for treating infections using 96 plant species (Amiguet et al. 2005). Laboratory studies have shown that there is often a sound pharmacological basis for the use of many of these plants. In a lab study of 38 plant species used by the Tzeltal and Tzotzil Maya communities from Chiapas (Mexico), 65% of plants showed antimicrobial activity against Gram-positive *Staphylococcus aureus*, Gram-negative *Escherichia coli* bacteria and the fungal pathogen *Candida albicans* (Meckes et al. 1995).

The Solanaceae family is of particular interest in the Maya area for treatment of mycoses. For example, the Yucatec Maya community healers from San Jose Succotz Belize use *Solanum torvum* Sw. and *Solanum mammosum* L. for treatment of athlete's foot (Arnason et al. 1980). Furthermore, leaves of *Cestrum dumetorum* Schltdl. are used by the Huastec Maya to treat warts and infected wounds (Alcorn 1984). A pilot blinded clinical study on *Tinae pedes* infections (athlete's foot) was undertaken by Lozoya et al. (1992) with *Solanum chrysotrichum* Schltdl., a

plant widely used by Maya communities in Chiapas for severe athlete's foot. A cream containing a 5% MeOH extract of the leaves provided a statistically better response rate than the maconozole control with complete remission in 45% of cases (Lozoya et al. 1992). Further information on their active constituents and mode of action of antimicrobial plants is required.

In the present study, we examined the antifungal activity of a Q'eqchi' Maya plant *Cestrum schlechtendahlii* G.Don. This plant is known as ik che (pepper tree) or ik kejen (pepper plant) and crushed leaves are used by the Q'eqchi' healers of Belize for skin problems such as athlete's foot and thrush. Calderón et al. (2006) reported weak antileishmanial activity of *C. schlechtendahlii* leaves extract; however, no phytochemical work has been presented on this species. In the present study, the objective was to determine if the plant has antifungal activity and to isolate active principles and any other characteristic phytochemicals.

5.2 Materials and Methods

5.2.1 General experimental procedures

IR spectra were recorded on a Shimadzu 8400-S FT/IR spectrometer. Optical rotations were registered on a Perkin-Elmer 241 digital polarimeter. NMR spectra were recorded on a Bruker Avance 500 spectrometer in CD_3OD at 500 MHz (^1H) and 125 MHz (^{13}C) using tetramethylsilane (TMS) as an internal standard. High Resolution Electrospray Ionization Mass Spectrometry (HRESIMS) was done using a Waters XEVO G2 UPLC–QTOF–ESI system. Electrospray Ionization Mass Spectrometry (ESI–MS) was done using a Shimadzu LCMS 2020 Series system. Open column chromatography was carried out on silica gel 60 (70–230 mesh, Merck). TLC analyses were performed on silica gel 60 F254 plates (Merck) and visualization of the plates was carried out using a ceric sulfate (10%) solution in H_2SO_4. For sugar analyses,

silica gel 0.25 mm plates (Merck) were used and visualization was done with an anisaldehyde reagent (5% *p*-anisaldehyde, 5% concentrated H_2SO_4 in EtOH).

5.2.2 Plant material

Leaves of *Cestrum schlechtendahlii* were collected in Jalacte, Belize in May 2010 under ethical approval (permits #H11–11–09, #H03–070–01). Plant material was preserved in 70% ethanol immediately after collection. Plant identification was performed by M. Otárola-Rojas, P. Sanchez-Vindas, and L. Poveda from the Universidad Nacional Autonoma of Costa Rica. Voucher specimens (UOH19776) have been deposited at the University of Ottawa Herbarium (OTT), the Herbario Juvenal Valerio Rodriguez (UNA), and the Belize Forest Department.

5.2.3 Extraction and isolation

Leaves (600 g) were ground using a blender (Waring commercial LR 8992) and extracted with 80% EtOH in a 1:10 biomass to solvent (w/v) ratio. The plant material/ethanol mixture was shaken at room temperature overnight at 200 rpm (shaker: New Brunswick Scientific) then filtered using vacuum filtration. A second extraction was performed with the plant residue, using a 1:5 w/v ratio and the mixture was again shaken and filtered. The two extracts were combined, dried *in vacuo* using a rotary evaporator (Yamato RE 500) at 45ºC, lyophilized to remove any residual water using a freeze-dryer (EC Super Modulyo, ~ -55 ℃, 10^{-2} mbar), and stored at -20ºC in the dark until needed. The crude leaf extract (79 g) was then fractionated using silica gel chromatography (column size 80 × 10 cm I.D.) with elution gradients hexane–EtOAc (1:0 → 0:1), EtOAc, and EtOAc–MeOH (1:0 → 0:1) and 26 primary fractions were collected (CME–I to CME–XXVI). Active fraction CME–XVIII (10 g) eluted with 80:20 EtOAc–MeOH was then chromatographed using another silica gel column (180 x 5 cm I.D.) using the same solvent

systems to result in 39 secondary fractions (CME–XVIII–1 to CME–XVIII–39). Compound **1** (800 mg) was eluted from fraction CME–XVIII–13 with 85:15 EtOAc–MeOH, and crystalized via spontaneous precipitation. Fraction CME–XVIII–19 was further chromatographed with a Sephadex LH-20 column (60 x 2 cm I.D.) with MeOH eluted isocratically to yield compound **2** (3.4 mg).

5.2.4 (25R)–1β,2α–dihydroxy–5α–spirostan–3–β–yl–O–α–L–rhamnopyranosyl–(1→2)–β–D–galactopyranoside (1)

Amorphous white powder (MeOH–EtOAc 80:20); $[\alpha]^{25}_{D}$ -53.5 (*c* 0.001, MeOH); IR(KBr) ν_{max} 3360, 2905, 1235 and 1050 cm^{-1}; ^{1}H NMR (CD_3OD, 500 MHz) and ^{13}C NMR (CD_3OD, 500 MHz), see Table 1; HRESIMS (negative mode) *m/z* 755.3107 $[M–H]^-$ (calculated for $C_{39}H_{63}O_{14}$: 755.4296). ESI-MS *m/z* 779 $[M + Na]^+$ (80), 757 $[M+H]^+$ (40), 595 $[M – C_6O_5H_{11}]^+$ (40), 449 $[M - C_6H_{11}O_5 – C_6H_{11}O_4]^+$ (55), 431 $[M – C_6H_{11}O_5 – C_6H_{11}O_4 - H_2O]^+$ (30), 413 $[M - C_6H_{11}O_5 – C_6H_{11}O_4 - 2H_2O]^+$ (100).

5.2.5 (25R)–1β,2α–dihydroxy–5α–spirostan–3–β–yl–O–β–D–galactopyranoside (2)

Amorphous white powder (MeOH). $[\alpha]^{25}_{D}$ -50.5; (*c* 0.001, MeOH); IR(KBr) ν_{max} 3365, 2900, 1240 and 1065 cm^{-1}; ^{1}H NMR (CD_3OD, 500 MHz) and ^{13}C NMR (CD_3OD, 500 MHz), see Table 1; HRESIMS (negative mode) *m/z* 609.2575 $[M–H]^-$ (calculated for $C_{33}H_{53}O_{10}$: 609.3768); ESI–MS *m/z* 633 $[M + Na]^+$ (100), 611 $[M+H]^+$ (40), 449 $[M – C_6O_5H_{11}]^+$ (25), 431 $[M – C_6H_{11}O_5 – H_2O]^+$ (25), 413 $[M – C_6H_{11}O_5 – 2H_2O]^+$ (70).

5.2.6 Acid Hydrolysis of compounds 1 and 2

Samples of **1 (**10 mg) and **2** (1 mg) were dissolved separately in 5 mL of 2M HCl (dioxane–H_2O 1:1). The solutions were refluxed for 4 h at 100^oC. The reaction mixture was diluted with H_2O and extracted three times with DCM (10 mL each). The organic layer from compound **1** hydrolysis was passed through anhydrous magnesium sulfate and dried *in vacuo* to give sapogenin 1A (6 mg). The structure of 1A was confirmed by ^{1}H-NMR and ESI–MS (see Appendices IIIa and IIIb). The aqueous phases from the hydrolysis of compounds **1** and **2** were neutralized by passing through an Amberlite IRA-93ZU column (Organo, Japan) and concentrated by lyophilization. The sugar identities were confirmed by TLC methods [EtOAc:MeOH:CH_3COOH:H_2O (11:2:2:2)] using authentic monosaccharide standards (Sigma-Aldrich, St. Louis, MI, USA). TLC plates were visualized with an anisaldehide solution. In support of the TLC results, optical rotational measurements confirmed the presence of L–rhamnose ([α_D] -13.6) on **1** and D–galactose on both **1** and **2** ([α_D] +18.5 and [α_D] +17.9 , respectively).

5.2.7 Phytochemical analysis

Chromatographic analyses of the crude extract and fractions were performed on an Agilent 1100 series HPLC system consisted of a quaternary pump, a degasser, an auto-sampler with 100 µL loop, a column thermostat and a diode array detector (DAD). The identification of the phenolics was corroborated by comparing the retention time and maximum UV absorption values with authentic commercial standards (Sigma-Aldrich, St. Louis, MI, USA). The analyses were performed using a Luna C18 column (250 mm × 4.6 mm, 5µm particle size) with column temperature set at 55 ℃ and a flow rate of 1.5 ml/min. The mobile phase A is acetonitrile containing 0.05% trifluoroacetic acid and B is water containing 0.05% trifluoroacetic acid.

Optimized separation was achieved with the following method: initial conditions of 5% A and 95% B with an increasing gradient to 100% A in 25 min; the column was flushed with 100% A for 5 min and then set back to the initial conditions. DAD was set to monitor wavelengths 254 nm, 280 nm and 330 nm.

5.2.8 Fungal strains

Three yeast-like fungal strains, *Saccharomyces cerevisiae* S288C, *Cryptococcus neoformans* and *Candida albicans* D10 were used for the disc diffusion assays. These strains were provided by M. L. Smith at Carleton University. The strains used to determine minimum inhibitory concentrations (MICs) in liquid culture were: *Saccharomyces cerevisiae* BY4741 (haploid), *Saccharomyces cerevisiae* BY4743 (diploid) (purchased from Thermo Scientific), and *Fusarium graminearum* [teleomorph *Gibberella zeae* (Schwein.) Petch]. The *F. graminearum* strains are maintained by the Canadian Collection of Fungal Cultures; *F. graminearum* ZTE-2A (DAOM 227650) is a generous gift from Robert Proctor (USDA Agricultural Research Service), which constitutively expresses green fluorescent protein (GFP), and is a transformant of *F. graminearum* GZ3639 (DAOM 223423, NRRL 38155) (Skadsen and Hohn 2004).

5.2.9 Antifungal disc diffusion assay

Saccharomyces cerevisiae S288C, *Cryptococcus neoformans and Candida albicans* D10 were cultured in Sabouraud dextrose broth (Difco) at 30 ℃. Berberine (95%, Sigma-Aldrich, St. Louis, MI, USA) was used as an antifungal positive control and methanol a negative control. Overnight cultures were grown to an optical density of ~1.0 or 1.0×10^7 CFU/mL at 600 nm (OD_{600}) and diluted 1:100. Aliquots (100 μL) of this inoculum were spread over the surface of Sabouraud agar plates. Paper discs (7.0 mm diameter) were loaded with crude extract (2

mg/disc), berberine (1 mg/disc), fractions (0.5 mg/disc), saponin (0.5 mg/disc), or methanol (carrier solvent) and allowed to air dry. The amended discs were placed treated side down on the prepared media, and incubated in the dark at 30 ℃ for 48 h. Inhibition zones from active extracts were then measured with a ruler. All experiments were repeated three times, with three technical repetitions per biological repetition.

5.2.10 Yeast growth assay

Saccharomyces cerevisiae BY4741 and BY4743 were cultured in YPD broth (1% yeast extract, 2% peptone, 2% dextrose w/v) at 30ºC. Overnight cultures were adjusted to an OD_{600} of 0.1 (1.5×10^6 CFU/mL). In a 96-well flat bottom plate (Costar 3596), 50 µL of this inoculum (~7.5×10^4 CFU/well) was mixed with 50 µL YPD in which the test compounds were dissolved at varying concentrations. Wells containing berberine (95%, Sigma-Aldrich, St. Louis, MI, USA), or appropriate quantities of the MeOH carrier solvent were also included to act as positive and negative controls. Yeast growth was monitored using a Biotek Powerwave XS2 microplate reader (Biotek, Winooski, Vermont, USA), running the Gen5® Microplate Data Collection & Analysis software ver. 2.00. The plate was incubated at 30ºC shaking at 600 rpm, with absorbance readings taken at 600 nm every 10 minutes for 24 hours. All experiments were repeated three times, with three technical repetitions per biological repetition.

5.2.11 Serial dot dilution assay

Overnight cultures of *S. cerevisiae* BY4741 and BY4743 grown in YPD media at 30ºC were adjusted to 1.5×10^6 CFU/mL and dilutions of 1:10, 1:100, and 1:1000 produced. In a 6-well flat bottom plate (Falcon 3046), compound **1** at chosen concentrations was dissolved in YPD agar before solidification. Once the media had set, 1 µL of each diluted inoculum was

spotted twice into each well in a defined pattern to make comparisons easier. The plate was then incubated in the dark at 30°C for up to 72 hours. Growth was inspected every 24 hours. An experiment was also performed using berberine to act as the positive control. All experiments were repeated three times, with three technical repetitions per biological repetition.

5.2.12 Fusarium growth assay

To prepare conidiospores, ~1 µL of frozen glycerol stock conidiospores were used to inoculate 100 mL of CMC broth (Capellini and Peterson 1965). Cultures were shaken at 28 ℃ for 3 to 5 days to generate conidia. Mycelial solids were separated from conidia by passing through one layer of sterile miracloth. Conidia were then washed with sterile water twice by centrifugation at 4,000 rpm for 15 min at room temperature. The lightly pelleted conidia were resuspended in sterile water and then stored at 4 ℃. Conidia were inspected and counted with a haemocytometer prior to use. Conidiospores were adjusted to 1.0×10^4 CFU/mL in GYEP broth. In a 96-well flat bottom white plate (Costar 3632), 100 µL of this inoculum (~1000 CFU/well) mixed with 100 µL GYEP in which the test compounds were dissolved at varying concentrations. Wells containing berberine or appropriate quantities of the MeOH carrier solvent were also included to act as positive and negative controls, respectively. Growth was monitored via fluorescence using a Polarstar Optima Microplate Reader (BMG Labtech, Gmbh, Offenberg, Germany) running FLUOstar OPTIMA Ver. 2.20R2. The plate was incubated at 28°C shaking at 600 rpm; readings (excitation/emission at 485 nm/520 nm) were taken every 23 minutes for 72 hours. For growth assessment with mycelia, spores were allowed to germinate (24 hours) before the compounds were added. All experiments were repeated three times, with three technical repetitions per biological repetition.

5.3 Results

The leaf extract of *Cestrum schlechtendahlii* G.Don (CSE) showed growth inhibition against three yeast-like fungi (*Saccharomyces cerevisiae* S288C, *Candida albicans* D10, and *Cryptococcus neoformans*) in the disc diffusion assay (Table 5.1). HPLC–DAD analysis of the crude extract identified the presence of caffeic acid (**3**), *p*–coumaric acid (**4**), and rosmarinic acid (**5**), all of which showed no antifungal activity in the current assay (Figure 5.1). The crude extract was then fractionated using silica gel open glass column chromatography (CC), from which 26 primary fractions were collected (CSE–I to CSE–XXVI). Fractions CSE–XVII to CSE–XXII were active in disc diffusion assays with growth inhibition comparable to that of the positive control berberine (Table 5.1). Since CSE–XVIII (13.5 g) eluted with 80:20 EtOAc–MeOH was most active, this fraction was chosen for further isolation work. CSE–XVIII was chromatographed using another silica gel column resulting in 39 secondary fractions (CSE–XVIII–1 to CSE–XVIII–39). From sub-fraction CSE–XVIII–13 eluted with 85:15 EtOAc–MeOH, compound **1** (800 mg) was obtained via spontaneous precipitation. All secondary fractions and **1** were tested in antifungal disc assays.

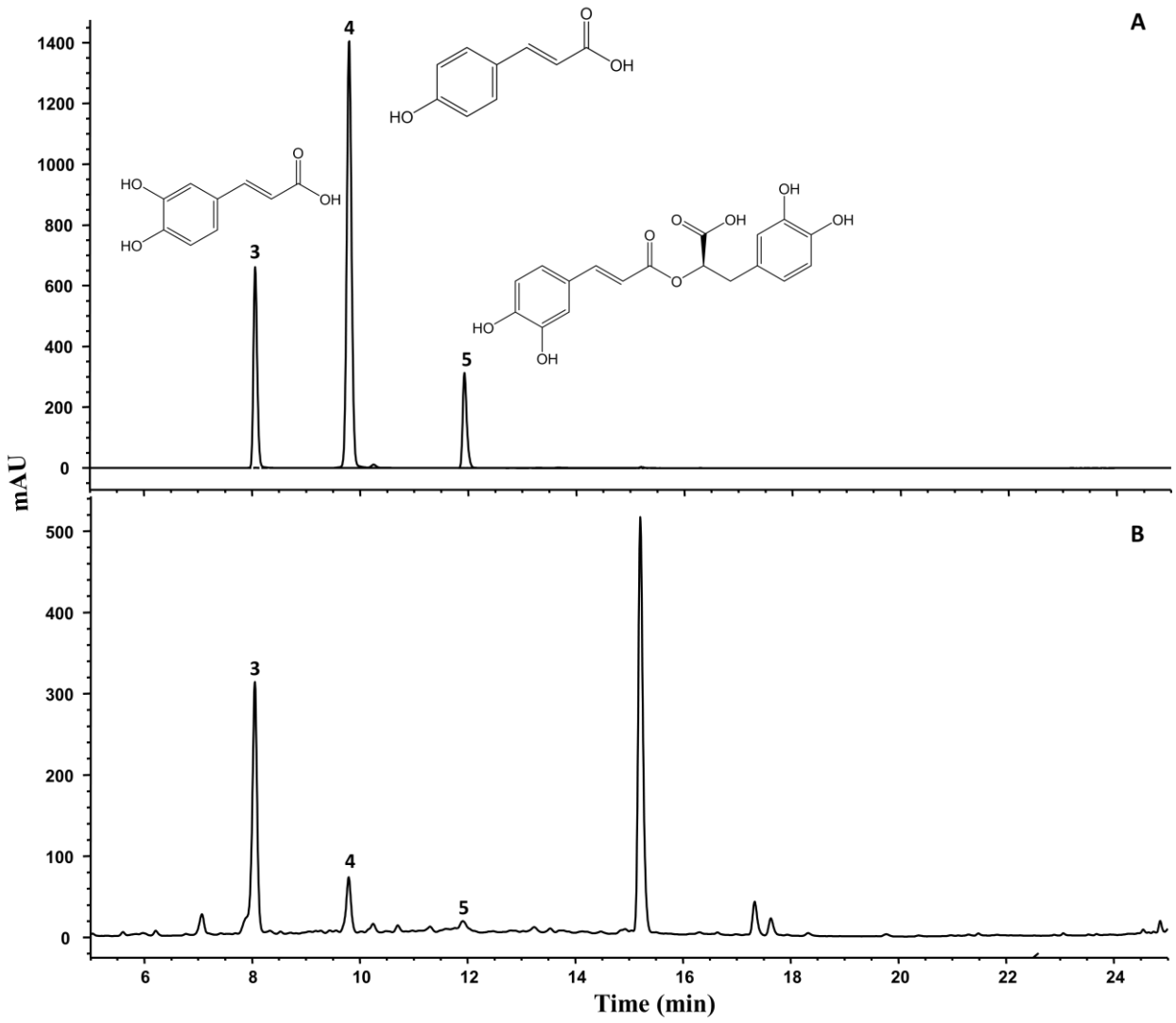

Figure 5.1 HPLC–DAD analysis at 280 nm for the identification of caffeic acid (**3**), *p*–coumaric acid (**4**), and rosmarinic acid (**5**) in the crude extract of *Cestrum schlechtendahlii* leaves (B) compared with the standard mix (A).

Table 5.1 Antifungal activity of *Cestrum schlechtendahlii* G.Don leaves extract (2 mg/disc), fractions (1 mg/disc) and compound 1 (0.5 mg/disc) against yeast-like fungi (disc diameter = 7 mm; N = 3) compared with berberine (0.5 mg/disc).

	Zone of inhibition (mm)		
Fraction or compound	*S. cerevisiae* S288C	*C. albicans* D10	*C. neoformans*
Crude extract	15.1 ± 0.2	11.4 ± 0.5	11.7 ± 0.8
CSE–XVII	15.8 ± 0.2	10.4 ± 0.2	10.2 ± 0.2
CSE–XVIII	20.7 ± 0.2	17.2 ± 0.3	17.3 ± 0.2
CSE–XVIX	19.6 ± 0.8	10.2 ± 0.4	10.9 ± 0.8
CSE–XX	17.6 ± 0.8	11.1 ± 1.0	10.7 ± 0.6
CSE–XXI	16.3 ± 0.4	12.6 ± 0.7	14.3 ± 0.1
CSE–XXII	14.3 ± 0.2	14.0 ± 0.4	12.7 ± 0.4
1	21.4 ± 0.7	16.2 ± 0.6	17.3 ± 0.3
berberine	23.2 ± 0.4	25.3 ± 1.2	22.4 ± 0.7

CSE–XVIII–19 was further chromatographed with a Sephadex LH–20 column to yield compound **2** (3.4 mg). Due to the low yield of **2**, this compound was not tested in the disc diffusion assay. A small set of minimum inhibitory concentrations (MICs) were done subsequently with compounds **1** and **2** (Table 5.2).

Table 5.2 Minimum inhibitory concentrations (μM) of saponins **1** and **2**, sapogenin 1A, and positive control berberine against *Saccharomyces cerevisiae* strains and *Fusarium graminearum* ZTE–2A.

Fungal strain (culture type)	**1**	**2**	1A	berberine
S. cerevisiae BY4741 (liquid)	16.5	> 95	> 105	600
S. cerevisiae BY4743 (liquid)	16.5	> 95	> 105	600
F. graminearum ZTE–2A (liquid)	132	NT[a]	NT	807
S. cerevisiae BY4741 (1.5% agar)	20	NT	NT	223
S. cerevisiae BY4743 (1.5% agar)	20	NT	NT	223

[a] NT - not tested

The chemical composition of the active fraction CSE–XVIII was initially assessed by thin layer chromatography (TLC) on silica gel using as an eluent EtOAc:MeOH (80:20). The results of this analysis indicated the absence of UV active chromophores and the presence of oxidizable groups in the components of CSE–XVIII, shown by the formation of a broad band after the development of the plate with a ceric sulfate solution. Moreover, CSE–XVIII composition was also analyzed by high performance liquid chromatography diode array detection (HPLC–DAD). During the analysis, no signal was detected using any of the chosen monitoring UV wavelengths (210, 254, 280 and 330 nm), confirming that the compound has no UV absorption, and supporting the initial TLC findings.

Compound **1** was obtained as an amorphous white powder. High Resolution Electrospray Ionization Mass Spectrometry (HRESIMS) (negative mode) showed a quasimolecular ion $[M–H]^-$ at a mass charge ratio (*m/z*) of 755.3107 ($C_{39}H_{63}O_{14}$), $[\alpha]_D$ -53.5. The analysis of the MS fragments along with the strong IR absorptions values (3360, 2905, 1235 and 1050 cm^{-1}) allowed

preliminary identification of the amphipathic (glycosidic and aliphatic) nature of **1** (Appendix IIIc). ESI–MS showed that compound **1** contained two sugar moieties, one hexopyranose [*m/z* 593 (M – $C_6H_{11}O_5$)] and one deoxyhexopyranose [*m/z* 449 (M – $C_6H_{11}O_5$ – $C_6H_{11}O_4$)]. (Appendix IIId).

Moreover, the analysis of the 1D (^{1}H, ^{13}C and DEPT) and 2D (HMQC, COSY, NOESY and HMBC) NMR experiments led to the identification of the major compound **1** as a known spirostanol saponin (25R)–1β,2α–dihydroxy–5α–spirostan–3–β–yl–*O*–α–L–rhamnopyranosyl–(1→2)–β–D–galactopyranoside (Figure 5.2, Appendices IIIe to IIIh). The spectroscopic values (Table 5.3) were in agreement with those previously reported by Haraguchi et al. (2000) and the identities of sugar moieties were confirmed by comparison with authentic monosaccharide standards (Sigma-Aldrich, St. Louis, MI, USA) using acid hydrolysis and TLC methods to be L–rhamnose and D–galactose (see Experimental Part). Chemical structure of the aglycone portion (1A) was confirmed by ^{1}H NMR and ESI–MS (Appendices IIIa and IIIb) and was further evaluated for antifungal activity (Table 5.2).

Compound **2** was obtained as an amorphous white powder. HRESIMS (negative mode) showed a quasimolecular ion $[M–H]^-$ at *m/z* of 609.2575 ($C_{33}H_{53}O_{10}$), $[\alpha]_D$ -50.5. IR, optical rotation and MS data of compound **2** were closely related to those displayed by compound **1**. The analysis of ESI–MS fragments showed that **2** contained the same aglycone portion and hexopyranose moiety present in **1** [m/z 449 (M-162)] (Appendices IIIi and IIIj). The absence of the deoxyhexopyranosyl moiety in **2** was further confirmed by the analysis of 1D NMR spectral data (Table 5.3). The ^{13}H and ^{13}C NMR of compound **2** displayed again characteristic signals for a spiro-steroidal saponin: one acetalic carbon at δ_C 110.6 (C-22); two quaternary methyl groups at δ_H/δ_C 0.79/17.5 (C-18), and 0.9/8.7 (C-19); two tertiary methyl groups at δ_H/δ_C 0.95/14.9 (C-

21) and 0.79/17.0 (C-27); four hydroxymethine of the aglycone skeleton at δ_H/δ_C 3.13/82.5 (C-1), 3.39/77.0 (C-2), 3.52/81.6 (C-3), and 4.37/82.2 (C-16); four hydroxymethine groups assignable to an hexopyranosyl moiety at δ_H/δ_C 3.49/76.9 (C-2'), 3.52/72.4 (C-3'), 3.48/74.5 (C-4'), and 3.80/70.4 (C-5'); one anomeric group at δ_H/δ_C 4.31/103.2 (C_1) and two hydroxymethylene groups, one belonging to the aglycone portion [δ_H/δ_C 3.45(26α), 3.32 Hβ/67.9 (C-26)] and the second to the sugar moiety [δ_H/δ_C 3.80 (Hα), 3.67 (Hβ)/62.7 (C-6')] (Appendices IIIl and IIIm). In addition, detailed interpretation of 2D NMR experiments such as DEPT, COSY, NOESY, HMQC (Appendices IIIk to IIIn) and the key long range correlations displayed in the HMBC (Figure 5.3) led to the determination that compound **2** is (25R)–1β,2α–dihydroxy–5α–spirostan–3–β–yl–*O*–β–D–galactopyranoside**,** a monosaccharide derivative of **1** and a novel natural product (Figure 5.2).- The sugar moiety in **2** was assessed again using acidic hydrolysis, TLC methods and the measurement of its optical rotation, which identified it to be D–galactose.

Compound **1** showed promising growth inhibition against both yeast-like and filamentous fungi with higher activity than the positive control berberine. In *Saccharomyces cerevisiae* strains, the MIC of **1** (16.5 μM) in liquid culture is 30-fold lower than that of berberine (600 μM) and 10-fold lower in solid culture (20 μM and 223 μM). Compound **1** was less effective against *Fusarium graminearum* ZTE–2A but was still more active than berberine with an MIC that was 6-fold lower. The MIC of **1** against *F. graminearum* ZTE–2A was estimated to be 132 μM and berberine to be 807 μM. Compound **2** and 1A (sapogenin) showed little or no activity against *S. cerevisiae,* even at the highest concentration tested (Table 5.2). The growth inhibition of **1** against *F. graminearum* ZTE-2A was much less effective against spores than the mycelia mass (Figure 5.4). Even at the highest tested concentration of 400 μg/mL (529 μM), *F. graminearum*

growth was only moderately inhibited when compound **1** was added at the beginning at the same time with the spores (Figure 5.4A); however, once the spores have germinated (after 24 hours), there was a dose-dependent inhibition of mycelia growth where **1** severely affected growth at the highest concentration (Figure 5.4B).

Table 5.3 ^{1}H and ^{13}C NMR spectroscopic data for compounds **1** and **2**.

	1		**2**	
position	δ_C[a]	δ_H[b]	δ_C[a]	δ_H[b]
1	82.6	3.12, d (9.0)	82.5	3.13, d (9.2)
2	77.2	3.38, t (9.2)	77.0	3.39, t (9.0)
3	81.1	3.59, dd (6.0, 9.0)	81.6	3.52, dd (6.2, 9.0)
4	33.6	H-4a, 1.69, m H-4b, 1.49, m	34.0	H-4a, 1.71, m H-4b, 1.45, m
5	43.0	1.08, m	42.9	1.11, m
6	32.5	H-6a, 1.67, m H-6b, 1.58, m	32.5	H-6a, 1.64, m H-6b, 1.59, m
7	33.3	H-7a, 1.69, m H-7b, 0.92, m	33.3	H-7a, 1.70, m H-7b, 0.90, m
8	36.6	1.54, m	37.0	1.54, m
9	56.7	0.89, m	56.7	0.85, m
10	42.7			
11	29.2	H-11a, 1.49, m H-11b, 1.42, m	29.3	H-11a, 1.40, m H-11b, 1.40, m
12	41.6	H-12a, 1.67, m, H-12b, 1.13, m	41.3	H-12a, 1.70, m H-12b, 1.13, m
13	41.3			
14	57.7	1.17, dd (3.5, 12.5)	57.6	1.12, m
15	32.9	H-15a, 1.97, m H-15b, 1.23, m	33.0	H-15a, 1.97, m H-15b, 1.25, m
16	82.1	4.36, dd (7.5, 14.5)	82.2	4.37, dd (7.8, 14.6)
17	64.0	1.72, dd (7.0, 8.5)	64.0	1.71, m
18	17.5	0.78, s	17.5	0.79, s
19	8.7	0.90, s	8.7	0.90, s
20	43.0	1.89, qui (7.0)	42.8	1.90, qui (6.8)
21	14.9	0.95, d (7.0)	14.9	0.95, d (7.2)
22	110.6		110.6	
23	29.9	H-23a, 1.62, m H-23b, 1.40, m	29.9	H-23a, 1.60, m H-23b, 1.42, m
24	25.1	H-24a, 2.32, dd (3.5, 14.5) H-24b, 1.44, m	25.1	H-24a, 2.31, dd (2.8, 14.4) H-24b, 1.42, m
25	31.5	1.62, m	31.5	1.57, m
26	67.9	H-26a, 3.44, m H-26b,3.30, m	67.9	H-26a, 3.45, m H-26b,3.32, m
27	17.0	0.79, d (6.0)	17.0	0.79, d (5.0)
1’	101.1	4.42, d (8.0)	103.2	4.31, d (7.6)
2’	77.1	3.65, dd (5.2, 7.2)	76.9	3.49, m
3’	76.1	3.60, dd (3.5, 9.5)	72.4	3.52, dd (4.0, 8.5)
4’	76.7	3.51, dd (4.6, 7.5)	74.5	3.48, dd (4.0, 7.0)
5’	71.0	3.75, brd (3.0)	70.4	3.80, brd (2.0)
6’	62.7	H-6’a, 3.77, dd (7.0, 11.5) H-6’b, 3.67, dd (5.0, 11.0)	62.7	H-6’a, 3.80, dd (8.0, 11.6) H-6’b, 3.67, dd (4.4, 11.0)
1’’	102.4	5.16, d (1.5)		
2’’	72.2	3.91, dd (1.7, 3.2)		
3’’	72.4	3.64, dd (1.7, 4.0)		
4’’	74.0	3.38, t (9.2)		
5’’	69.8	4.08, dd (6.0, 9.5)		
6’’	18.0	1.24, d (6.0)		

[a] Measured in 500 MHz for ^{1}H and 125 MHz for ^{13}C, in CD_3OD. [b] Coupling constants values (*J*) in Hz in parentheses.

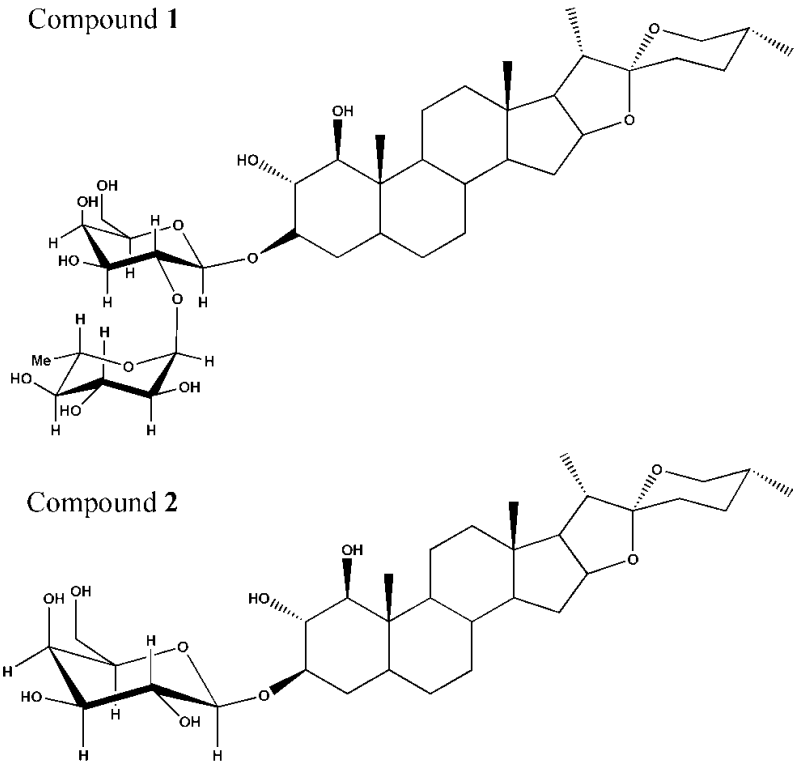

Figure 5.2 Structures of isolated saponins from the leaves of *Cestrum schlechtendahlii*: (25R)–1β,2α–dihydroxy–5α–spirostan–3–β–yl–*O*–α–L–rhamnopyranosyl–(1→2)–β–D–galactopyranoside (**1**) and (25R) –1β,2α–dihydroxy–5α–spirostan–3–β–yl–*O*–β–D–galactopyranoside (**2**).

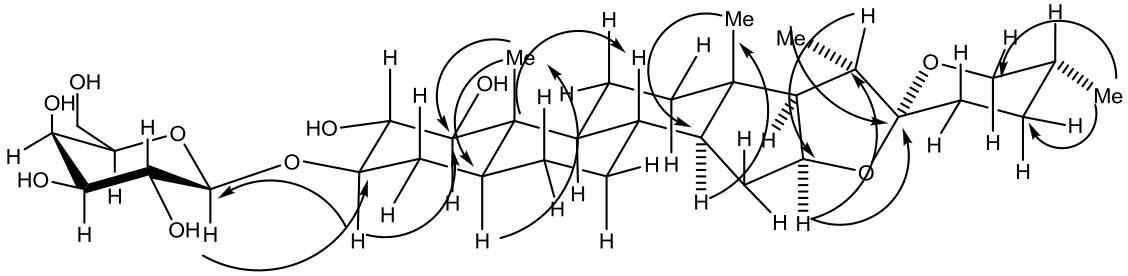

Figure 5.3 Key HMBC correlations in compound **2**.

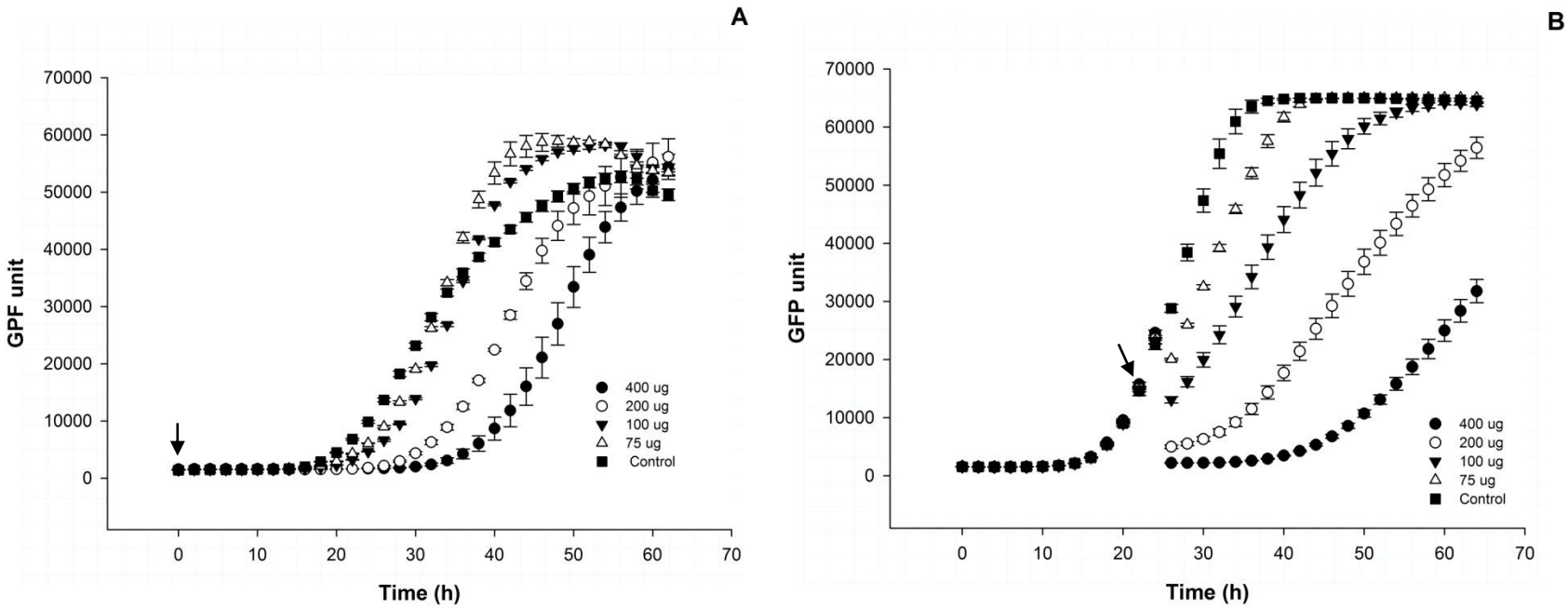

Figure 5.4 Effect of compound **1** on the growth of *Fusarium graminearum* ZTE–2A spores (A) and mycelia (B). Arrows indicate the times at which the compound was added. Mean growth (±SEM) was measured by the expression of green fluorescent protein (GFP). N = 3.

5.4 Discussion

The antifungal activity of compound **1** (isolated here from *Cestrum schlechtendahlii*) provides some validation for the traditional use of this plant by the Q'eqchi' Maya healers of Belize. *Cestrum schlechtendahlii* is another plant used by Maya healers in the treatment of fungal infections in addition to *Solanum torvum, Solanum chrysotrichum,* and *Cestrum dumetorum.* All of these Solanaceae species contain spirostanol saponins that may have the potential to be developed as antifungal agents, which target *Candida albicans* and *Cryptococcus neoformans* (Alvarez et al. 2001; Lu et al. 2009). Since **1** is active against *F. graminearum*, a major cereal pathogen, further experiments could show potential agricultural applications as well.

Even though **1** has been isolated in another Solanaceae by Haraguchi et al. (2000), we are the first to report the antifungal activity of this compound. This compares with other reports of spirostanol saponins that have been isolated from the Solanaceae as well as from other plant families and have been reported to have antifungal activities (Alvarez et al. 2001; González et al. 2004; Haraguchi et al. 2000; Lu et al. 2009; Shen et al. 2003; Yang et al. 2006). Compound **1** showed promising growth inhibition against both yeast-like and filamentous fungi with higher activity than the positive control berberine. In *S. cerevisiae*, the MIC of **1** in liquid culture is 30-fold lower than that of berberine and 10-fold lower in solid culture, suggesting that this compound may have good potential as an antifungal agent. Antifungal activity against *C. albicans* D10 is of particular interest since this clinical isolate is resistant to commercial antifungals such as amphotericin B and ketoconazole (Ficker et al. 2003). This indicates that mode of action is not related to ergosterol biosynthesis and that **1** may be useful in treating recalcitrant mycoses. Compound **1** was less effective against *F. graminearum*, a filamentous

plant pathogenic fungus, but was still more active than berberine with an MIC that was 6-fold lower. Compound **2** and 1A (sapogenin) showed little or no activity against *S. cerevisiae*, even at the highest concentration tested of 95 to 105 μM.

Since **1** and **2** have the same sapogenin, this suggests that the presence of the two sugar moieties is important for antifungal activity. Although 1A has not been tested previously, its low activity is consistent with studies of diosgenin (a structurally similar sapogenin) which showed little or no antifungal activity (Chalenko et al. 1977; Imai et al. 1967). In a structure-activity relationship study by Yang et al. (2006), spirostanol saponins containing two or more sugar moieties exhibited inhibition against various pathogenic fungi, suggesting that the number of sugar residues present is important to the antifungal activity. Crude extract of *Cestrum nocturnum* L. leaves was reported by Hernández-Albiter et al. (2007) to inhibit the germination of conidiospores in two isolates of the plant pathogenic fungus *Colletotrichum gloeosporioides*. Phytochemical analyses of this species identified spirostanol saponins with three or more sugar moieties (Ahmad et al. 1995; Mimaki et al. 2001).

Chapter 6 – Mechanisms of action of the major saponin isolated from *Cestrum schlechtendahlii* G.Don

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Statement of author contribution

JTA and CAT conceived and designed this study. Bioassays were performed by CAT with technical assistance from CDM and RS. CDM assisted in results and statistical analyses. CDM, RS, and JTA contributed in manuscript preparation.

Will be submitted to *Phytomedicine*

6.1 Introduction

Saponins are a class of naturally occurring plant glycosides known for their soap-like or 'foam-forming' properties in water (Man et al. 2010; Osbourn 1996). These compounds are structurally diverse and have been reported in at least 100 different plant families (Man et al. 2010). The structural complexity is due to the numerous variations in the aglycone backbones (sapogenin), sugar chains composition, and attachments between the individual sugar residues. There are three main types of saponins: triterpenoid, steroidal, and steroidal alkaloid; the type is determined by the sapogenin structure. These can be classified into monodesmosidic (one sugar chain consisting of one to five moieties attached at position C-3) and bidesmosidic (an additional sugar chain at either C-26 for steroids or C-28 for triterpenoids) (Osbourn 1996).

The main role of saponins in plants has been proposed to be for defense against pathogens either as 'phytoanticipins' or 'phytoprotectorants' (Morrissey and Osbourn 1999). 'Phytoanticipins' are activated upon tissue damage and/or attacks by pathogens whereas 'phytoprotectorants' are constitutively produced and have broad spectrum antimicrobial and/or insecticidal activities (Morrissey and Osbourn 1999). The biological activities of many saponins have been well documented to include antifungal, hypoglycaemic, anticancer, and immunostimulatory effects (Francis et al. 2002 and references therein). All three types of saponins isolated from various species have been shown to inhibit the growth of molds, yeasts, and filamentous fungi (Cho J et al. 2013; Miyakoshi et al. 2000; Polacheck et al. 1991; Sandrock and vanEtten 1998; Schwartz et al. 2000; Zhang et al. 2006). The main mechanism of antifungal action for saponins has been attributed to their membrane permeabilising properties; however, the exact mechanism is not fully known (Morrissey and Osbourn 1999 and references therein).

In the present study, we examined the antifungal mode of action of a steroidal saponin (**1**) isolated from the leaves of a Q'eqchi' Maya medicinal plant *Cestrum schlechtendahlii* G.Don (Solanaceae) (Figure 6.1). This saponin has been shown to inhibit the growth of *Saccharomyces cerevisiae*, *Candida albicans* D10, *Cryptoccocus neoformans*, and *Fusarium graminearum* ZTE–2A (Chapter 5). Using the diploid heterozygous knockout library of *Saccharomyces cerevisiae* (Giaever et al. 2002), growth profiles under treatment with saponin and vehicle control was compared in order to identify potential targets for antifungal action (haploinsufficiency profiling).

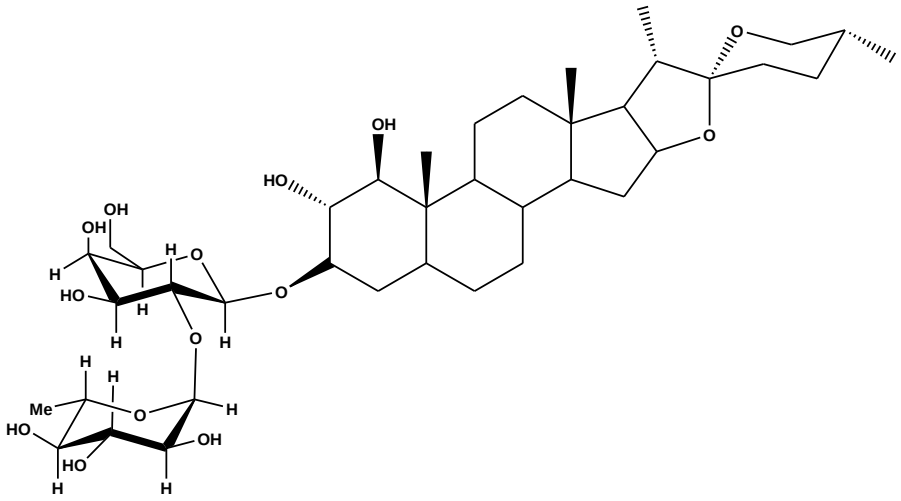


Figure 6.1 Structure of the major spirostanol saponin (**1**) isolated from the leaves of *Cestrum schlechtendahlii*.

6.2 Materials and Methods

6.2.1 Chemicals

The major saponin (**1**) was isolated from the leaves of *Cestrum schlechtendahlii* as detailed in Chapter 5. This compound was stored in the dark at 4°C until needed for subsequent experiments. All ingredients for growth media were purchased from Fisher Scientific (Ottawa, ON, Canada) and VWR (Missisauga, ON, Canada).

6.2.2 Saccharomyces cerevisiae storage and maintenance

Saccharomyces cerevisiae strain BY4743 (YSC1050, Open Biosystems, Ottawa, ON, Canada) was used as the wild type for all growth inhibition experiments. This is the diploid parental strain from which the Yeast Knockout Collection was derived. For long term storage, yeast cells were cultured in YPD broth (1% yeast extract, 2% peptone, 2% dextrose w/v) with shaking at 200 rpm and at 30°C. Overnight culture was adjusted to an OD_{600} of 0.5, incubated for another 2 hours at the same conditions, and then suspended in 15% glycerol and stored at -80°C. When needed, this frozen stock was used as a source for streak culture on 2% YPD agar, which was incubated at 30°C for 24 hours and then stored at 4°C for up to one month. Single colonies from this streak culture were used as the source inoculation for subsequent experiments.

6.2.3 Saccharomyces cerevisiae gene deletion library

The Yeast Heterozygous Collection (YSC1055, Open Biosystems, Ottawa, ON, Canada) was used to determine the mechanisms of action of the isolated saponin. This library is part of the Yeast Knockout Collection (YKO), available frozen in 96-well plates, and was developed by the *Saccharomyces* Genome Deletion Project. This collection was provided by R. Subramaniam at Agriculture and Agri-Food Canada. Approximately 6000 genes are represented in the library;

the ORF (open reading frames) of each gene is replaced by a KanMx cassette to give antibiotic resistance (to kanamycin) and a unique molecular barcode of 20 base pairs (Giaever et al. 2002).

The library was copied onto an OmniTray single-well plate (Nunc 242811) containing 2% YPD agar with a 96-pin replicator (Nunc 250520) and condensed into 18 plates of 384 colonies per plate using a Microwell® Plate Copier (Nunc 250539). From then on, all transfers were done using a 384-pin replicator (Nunc 242765) for maintenance and experimental purposes. The library was maintained and stored at 4^{o}C. Prior to each experiment, cells were transferred to a new OmniTray single-well plate with 2% YPD agar and incubated over night at 30^{o}C. The library replication method was done according to manufacturer's protocols (Nunc® Replication System). Under sterile conditions, a 384-pin replicator was used to inoculate the receiving plate from the source plate with the aid of the OmniTray Copier. Before each inoculation of different source plates, the 384-pin replicator was sterilized by rinsing the pins in the following order: 10% bleach solution, autoclaved distilled water, and 95% EtOH. The pins were immersed in each solution for at least 10 seconds; magnetic plates and stir bars were used for the bleach and water solution to facilitate with the cleaning process. In cases where there were persistently "sticky" cells, a toothbrush was used to gently scrub the pins with the bleach solution. After immersion in 95% EtOH, the replicator was flamed with a Bunsen burner to facilitate drying and left to cool for 1 to 2 minutes prior to the inoculation of the next source plate from the library.

6.2.4 Screening of saponin against the diploid library in solid media

Based on the growth inhibition results from the serial dot dilution assay in 1.5% YPD agar (Chapter 5), a source plate from the library was chosen to test the growth responses of the deletion mutants to saponin at concentrations ranging from 10 to 20 μg/mL (13.2 to 26.5 μM) in

2% YPD agar. From the results of these test plates, a saponin concentration of 15 µg/mL (19.8 µM) was selected as the experimental concentration for the screening of the diploid library in solid media. All 6000 mutants contained in 18 plates were tested using control plates of 2% YPD agar and experimental plates of 15 µg/mL of saponin in 2% YPD agar. Plate inoculation was done using the library replication method as detailed in section 6.2.3.

6.2.5 ScreenMill analysis of screening in solid media

All control and experimental plates were scanned using an Epson Perfection V750 Pro scanner running the Epson Scan Version 3.24A software. Each plate was scanned individually in positive film mode at 8-bit grayscale and 300 dpi resolution. Analysis of the difference in growth between control and experimental plates was done using the *ScreenMill* software suite according to Dittmar et al. (2010); this software suite contains Colony Measurement Engine (*CM Engine*), Data Review Engine (*DR Engine*), and Statistics Visualization Engine (*SV Engine*). *CM Engine* is an open-source macro for *ImageJ*, a free program from National Institute of Health (NIH) and is available at http://rsbweb.nih.gov/ij. Once scanned, each plate is analyzed with *CM Engine*, which calculates the area of each colony based on its size and circularity. The key file used was Hetero_Diploid_Key.txt, which is provided by *ScreenMill.* The results from *CM Engine* are saved to a text file and then inputted into *DR Engine*, which normalizes the raw data to allow comparison between plates and calculates the associated *p*-values. Here, the raw data is normalized to the "Plate Median" where each assigned value is divided by the median growth value of each plate and the t-test is used for *p*-value calculation since the data set is parametric. This normalized data is then used by *SV Engine* to determine which mutants are significant based on a predetermined *p*-value threshold (default is $p < 0.05$) and provide visualization in the form of cartoon representations.

6.2.6 Diploid library verification

From the initial diploid library screening, 10 verification plates were constructed to contain 874 'hits' or mutants of interest for saponin and another compound (antofine). Briefly, 100 µL of YPD media containing 50 µg/mL of spectinomycin (Fisher Scientific 15206701) and 50 µg/mL of chloramphenicol (Fisher Scientific 19032105) was added to each well of U-shaped 96-well plates (Nunc 262162). Individual colonies were selected from the source plates in solid media and inoculated using sterile toothpicks with the help of a Gilson™ Trackman (Gilson F70301) to illuminate the desired colonies and corresponding wells. Wild type colonies (BY4743) were also included in each plate. The plates were covered with lids (Nunc 264122) and sealed with parafilm before incubation overnight at 30^{o}C with shaking at 200 rpm. Following overnight incubation, these plates were frozen at -80^{o}C, then sealed with aluminum sheets (Axygen PlateMax PCR-AS-200) for long term storage at the same temperature, and became the new source plates. A second screening of saponin against the verification plates in solid media was attempted several times using the same library transfer method; however, the results were not conclusive due to potential pooling and/or solubility issues. Thereafter, the screening was switched to liquid media.

6.2.7 Screening of saponin against verification plates in liquid media

Based on the growth inhibition results in liquid media (Chapter 5), a source plate from the verification set was chosen to test the growth responses of the deletion mutants to saponin at concentrations ranging from 0.45 to 22.5 µg/mL (0.6 to 29.8 µM) in YPD media. From the results of these test plates, a saponin concentration of 12.5 µM (9.45 µg/mL) was selected as the experimental concentration for the screening of verification plates in liquid media. All 10 plates

were tested using 0.5% MeOH vehicle control and 12.5 μM of saponin, both in YPD media. Prior to each experiment, 100 μL of YDP media amended with spectinomycin and chloramphenicol (each at 50 μg/mL) was added to each well of a flat bottom 96-well plate (Costar 3596). This plate was then inoculated from a verification source plate using a 96-pin replicator, sealed with parafilm, and incubated overnight at 30ºC with shaking at 750 rpm to become the replication source plate. After overnight incubation, 15 μL of YPD media was added to each well of the replication source plate to compensate for evaporation using a repeating pipette. This plate was then mixed at 1200 rpm for 2 minutes to eliminate all pellets in the wells and then placed into a plate reader. Cell density was measured for all wells at 620 nm using a Polarstar Optima Microplate Reader (BMG Labtech, Ortenberg, Germany) running FLUOstar OPTIMA Ver. 2.20R2. The recorded cell densities were entered into a spreadsheet to calculate the equivalent densities if the measurement were done in a standard spectrophotometer at 600 nm and then each well was diluted with the appropriate amount of YPD media to the desired OD_{600} of 5.0. Next, 10 μL from each well of this adjusted replication source plate was transferred to another 96-well plate containing 190 μL of YPD media and mixed to bring this inoculum equivalent to an OD_{600} of 0.25 (~1.0×10^6 CFU/mL). After 1 hour incubation at 30ºC with shaking at 200 rpm, the cells were allowed to equilibrate and reach an OD_{600} of 0.5 (~1.0×10^7 CFU/mL); this was now the inoculation source plate. For each inoculation source plate, two experimental plates were required (A and B) with A receiving mutants from the first half (columns 1 to 6) of the inoculation source plate and B from the second half (column 7 to 12). The layout was set up so that columns 1 to 6 of experimental plate A contained 90 μL of 0.5% MeOH (vehicle control) in YPD media plus 10 μL the corresponding inoculum from columns 1 to 6 of the inoculation source plate and columns 7 to 12 of experimental plate A contained 90 μL

of 12.5 μM of saponin in YPD media plus 10 μL the corresponding inoculum from columns 1 to 6 of the inoculation source plate. Similarly, columns 1 to 6 of experimental plate B contained 90 μL of 0.5% MeOH (vehicle control) in YPD media plus 10 μL the corresponding inoculum from columns 7 to 12 of the inoculation source plate and columns 7 to 12 of experimental plate B contained 90 μL of 12.5 μM of saponin in YPD media plus 10 μL the corresponding inoculum from columns 7 to 12 of the inoculation source plate. This was done using a multi-channel pipette and a Gilson™ Trackman. Once the experimental plates were inoculated, yeast growth was monitored using a Biotek Powerwave XS2 microplate reader (Biotek, Winooski, Vermont, USA), running the Gen5® Microplate Data Collection & Analysis software ver. 2.00. The plate was incubated at 30°C shaking at 600 rpm, with absorbance readings taken at 600 nm every 10 minutes for 24 hours.

6.2.8 Analysis of screening in liquid media

Yeast growth difference was calculated using the area under the growth curve (AUC) at OD_{600}. For all mutants, the growth difference between experimental and control conditions were determined by subtracting the AUC of the saponin treatment from the AUC of the vehicle control treatment. This growth difference of each mutant was then compared to the growth difference of the wild type. For each plate, the mutant with the maximum growth difference (either negative or positive) from the wild type was considered to have 100% change. All other mutants on that plate were normalized to this value. The % change can be calculated using the following equation:

$$\%\ change = \frac{|\Delta AUC\ (WT_{con} - WT_{exp}) - \Delta AUC\ (M_{con} - M_{exp})|}{\max\ \Delta AUC} \times 100\%$$

where $AUC\ WT_{con}$ is the *AUC* of the wild type under vehicle control treatment, $AUC\ WT_{exp}$ is the *AUC* of the wild type under saponin treatment, $AUC\ M_{con}$ is the AUC of the mutant under vehicle control treatment, $AUC\ M_{exp}$ is the *AUC* of the mutant under saponin treatment, and max ΔAUC is the maximum growth difference for a given plate. The mutants displaying at least 50% of the maximum growth difference in the liquid screening were entered into GeneMANIA (http://www.genemania.org/) to search for relationships between them. GeneMANIA is a program that finds related genes based on a set of query genes using functional association data from the Saccharomyces Genome Database (SGD). Association data can be defined by protein interactions, genetic interactions, pathways, co-expression, co-localization and protein domain similarity.

6.3 Results

The initial screening of saponin (**1**) against the yeast diploid heterozygous deletion library in solid media at a sub-lethal concentration of 15 μg/mL (19.8 μM) yielded 244 mutants of the 6000 tested from the *ScreenMill* analysis, which were considered to have their growth significantly reduced ($p < 0.05$). The complete list of these mutants along their annotated gene names and functions are listed in Appendix IV. Based on annotation from the *Saccharomyces* Genome Database (SGD), the mutants of interest can be grouped into 13 target types (Figure 6.2). Of the 244 mutants, the most prominent target type was UNK (proteins of unknown functions) with approximately 18% of the hits (45). The next highest categories were DNA (DNA transcription, replication, repairs, and regulation) followed by BIOSYN (biosynthesis, metabolism, and signalling), and PROT (protein synthesis, transport, and localization) representing 15% (36), 14% (34), and 13% (32) of hits, respectively. MEMB (membrane

proteins) and DUB (dubious ORFs) were next with approximately 8%, followed by MITO (mitochondrial proteins) and CYC/BUD (cell cycle, budding, and sporulation) both at 6%. The remaining categories each represented 3% of hits or less. From these preliminary results, it was clear that target types were widespread and further verification experiments were needed to provide further insights to the mechanisms of action of saponin.

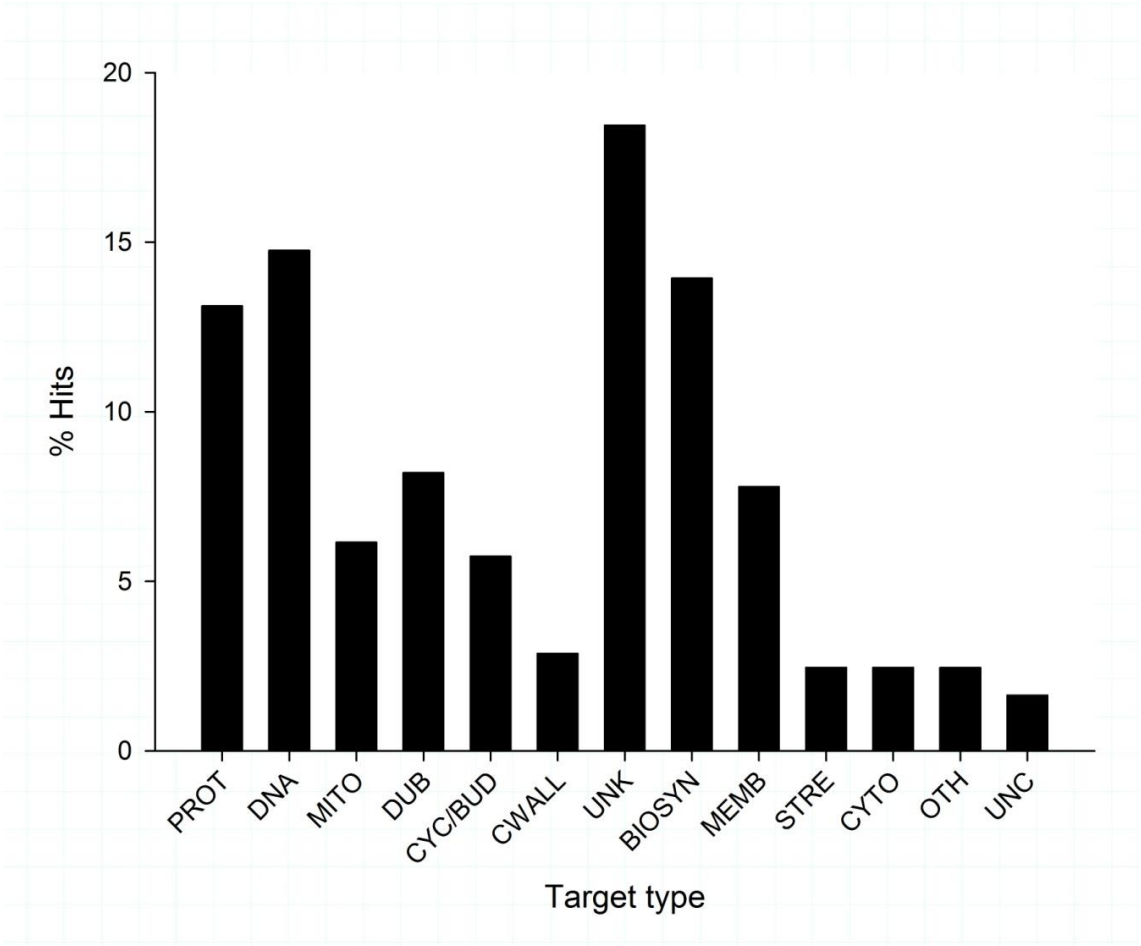


Figure 6.2 Summary of the screening of saponin (**1**) at 15 μg/mL against *Saccharomyces cerevisiae* diploid heterozygous deletion library in 2% YPD agar. Total hits = 244 (considered significantly different from the control, $p < 0.05$). Target type: PROT = protein synthesis, transport, and localization, DNA = DNA transcription, replication, repairs, and regulation, MITO = mitochondrial proteins, DUB = dubious ORFs, CYC/BUD = cell cycle, budding, and sporulation, CWALL = cell wall, UNK = known function, BIOSYN = biosynthesis, metabolism, and signalling, MEMB = membrane protein, STRE = stress response, CYTO = cytoskeleton, OTH = ions transport and other processes, UNC = uncharacterized.

Subsequent screening of saponin (**1**) against the same library was done in liquid media at a sub-lethal concentration of 12.5 μM in order to reduce the number of identified targets. Based on the normalized % change calculated, 77 of the 874 tested mutants showed at least 50% growth difference when compared to the wild type. These mutants along with their associated genes and functions (if known) are listed in Table 6.1. Based on SGD annotation, the highest target type was PROT (protein synthesis, transport, and localization) with ~23 % of the hits (18) (Figure 6.3). The next highest categories are BIOSYN (biosynthesis, metabolism, and signalling) followed by DNA (DNA transcription, replication, repairs, and regulation) at ~16% (12) and UNK (proteins of unknown functions) both with ~12% (9) each. The remaining categories each contained 7% or less. Furthermore, 12 genes (*PDR3, AST1, DER1, PER1, SWF1, CAB5, HTD2, PAN6, PAH1, CUE1, MDM32,* and *MCT1*) were either membrane proteins or associated with lipid synthesis and/or metabolism. According to GeneMANIA, 53% of the 77 ORFs had physical interactions with SSA1, an ATPase involved in protein folding, signaling, and transmembrane transport.

Table 6.1 Diploid heterozygous *Saccharomyces cerevisiae* mutants with >50% growth difference compared to wild type BY4743. Growth difference was calculated by comparing treatments to vehicle control (0.5% MeOH) and to 12.5 μM saponin (**1**) in YPD media.

ORF[a]	Gene[b]	Gene function[c]
YAL005C	*SSA1*	ATPase involved in protein folding and NLS-directed nuclear transport
YAL035W	*FUN12*	GTPase, required for general translation
YAR018C	*KIN3*	Nonessential serine/threonine protein kinase; possible role in DNA damage response; influences tolerance to high levels of ethanol
YBL001C	*ECM15*	Non-essential protein of unknown function; may have a role in yeast cell-wall biogenesis
YBL005W	*PDR3*	Transcriptional activator of the pleiotropic drug resistance network, regulates expression of ATP-binding cassette (ABC) transporters
YBL014C	*RRN6*	Component of the core factor (CF) rDNA transcription factor complex
YBL069W	*AST1*	Peripheral membrane protein that interacts with the plasma membrane ATPase Pma1p; involved in targeting to the plasma membrane, possibly by influencing incorporation into lipid rafts
YBR097W	*VPS15*	Myristoylated serine/threonine protein kinase involved in vacuolar protein sorting
YBR190W	N/A[d]	Dubious open reading frame unlikely to encode a functional protein
YBR201W	*DER1*	ER membrane protein that promotes export of misfolded polypeptides
YCR044C	*PER1*	Protein of the endoplasmic reticulum; required for GPI-phospholipase A2 activity; remodels GPI anchor proteins for association with lipid rafts
YDL160C-A	N/A	Protein of unknown function
YDR044W	*HEM13*	Coproporphyrinogen III oxidase; part of heme biosynthetic pathway
YDR126W	*SWF1*	Palmitoyltransferase that acts on transmembrane proteins
YDR196C	*CAB5*	Subunit of the CoA-Synthesizing Protein Complex (CoA-SPC)
YDR382W	*RPP2B*	Ribosomal protein P2 beta; involved in elongation process
YDR454C	*GUK1*	Guanylate kinase; converts GMP to GDP; required for growth and mannose outer chain elongation of cell wall N-linked glycoproteins
YDR507C	*GIN4*	Protein kinase involved in bud growth and assembly of the septin ring
YEL043W		Predicted cytoskeleton protein involved in intracellular signaling
YEL059W	*HHY1*	Dubious open reading frame unlikely to encode a functional protein
YER011W	*TIR1*	Cell wall mannoprotein
YER012W	*PRE1*	Beta 4 subunit of the 20S proteasome
YER056C-A	*RPL34A*	Ribosomal 60S subunit protein L34A
YER116C	*SLX8*	Subunit of Slx5-Slx8 SUMO-targeted ubiquitin ligase (STUbL) complex
YER117W	*RPL23B*	Ribosomal 60S subunit protein L23B
YER121W	N/A	Putative protein of unknown function; may be involved in phosphatase regulation and/or generation of precursor metabolites and energy
YFL063W		Dubious open reading frame,
YGL006W	*PMC1*	Vacuolar Ca2+ ATPase involved in depleting cytosol of Ca2+ ions
YGL190C	*CDC55*	Non-essential regulatory subunit B of protein phosphatase 2A (PP2A); involved in mitosis
YGL235W	N/A	Putative protein of unknown function
YGR027C	*RPS25A*	Protein component of the small (40S) ribosomal subunit
YGR054W	N/A	Eukaryotic initiation factor (eIF) 2A; associates specifically with both 40S subunits and 80 S ribosomes
YGR142W	*BTN2*	v-SNARE binding protein that facilitates specific protein retrieval from a late endosome to the Golgi
YGR186W	*TFG1*	TFIIF (Transcription Factor II) largest subunit; involved in both transcription initiation and elongation of RNA polymerase II

Table 6.1 Continued

ORF	Gene	Gene function
YGR188C	*BUB1*	Protein kinase involved in the cell cycle checkpoint into anaphase
YHL034C	*SBP1*	Protein that binds eIF4G and has a role in repression of translation
YHR035W	*NEL1*	Activator of Sar1p GTPase activity; not an essential gene
YHR067W	*HTD2*	Mitochondrial 3-hydroxyacyl-thioester dehydratase; involved in fatty acid biosynthesis
YHR151C	*MTC6*	Protein of unknown function
YIL110W	*HPM1*	AdoMet-dependent methyltransferase
YIL145C	*PAN6*	Pantothenate synthase, required for pantothenic acid biosynthesis
YIL153W	*RRD1*	Peptidyl-prolyl cis/trans-isomerase; involved in G1 phase progression, microtubule dynamics, bud morphogenesis and DNA repair
YJL008C	*CCT8*	Subunit of the cytosolic chaperonin Cct ring complex; related to Tcp1p, required for the assembly of actin and tubulins in vivo
YJL189W	*RPL39*	Ribosomal 60S subunit protein L39; required for ribosome biogenesis
YJR024C	*MDE1*	5'-methylthioribulose-1-phosphate dehydratase; acts in the methionine salvage pathway
YKL087C	*CYT2*	Cytochrome c1 heme lyase; involved in maturation of cytochrome c1
YKL104C	*GFA1*	Glutamine-fructose-6-phosphate amidotransferase; part of chitin biosynthesis
YKL109W	*HAP4*	Transcription factor; subunit of the heme-activated, glucose-repressed Hap2p/3p/4p/5p CCAAT-binding complex
YKL173W	*SNU114*	GTPase component of U5 snRNP involved in mRNA splicing via spliceosome
YLL019C	*KNS1*	Nonessential putative protein kinase of unknown cellular role
YLR055C	*SPT8*	Subunit of the SAGA transcriptional regulatory complex
YLR100W	*ERG27*	3-keto sterol reductase; part of ergosterol biosynthesis
YLR212C	*TUB4*	Gamma-tubulin, involved in nucleating microtubules from both the cytoplasmic and nuclear faces of the spindle pole body
YLR249W	*YEF3*	Gamma subunit of translational elongation factor eEF1B
YLR297W	N/A	Protein of unknown function
YMR141C	N/A	Dubious open reading frame; unlikely to encode a functional protein
YMR165C	*PAH1*	Mg2+-dependent phosphatidate (PA) phosphatise; responsible for de novo lipid synthesis and formation of lipid droplets
YMR170C	*ALD2*	Cytoplasmic aldehyde dehydrogenase; involved in ethanol oxidation and beta-alanine biosynthesis
YMR174C	*PAI3*	Cytoplasmic proteinase A (Pep4p) inhibitor
YMR175W	*SIP18*	Phospholipid-binding hydrophilin with a role in dessication resistance
YMR263W	*SAP30*	Component of Rpd3L histone deacetylase complex; involved in silencing at telomeres, rDNA, and silent mating-type loci; involved in telomere maintenance
YMR264W	*CUE1*	Ubiquitin-binding protein; ER protein involved in protein degradation
YOL010W	*RCL1*	Endonuclease that cleaves pre-rRNA at site A2 for 18S rRNA biogenesis
YOR098C	*NUP1*	Nuclear pore complex (NPC) subunit, involved in protein import/export and in export of RNAs
YOR133W	*EFT1*	Elongation factor 2 (EF-2), catalyzes ribosomal translocation during protein synthesis
YOR135C	*IRC14*	Dubious open reading frame; unlikely to encode a functional protein
YOR142W	*LSC1*	Alpha subunit of succinyl-CoA ligase
YOR144C	*ELG1*	Subunit of an alternative replication factor C complex; important for DNA replication and genome integrity
YOR147W	*MDM32*	Mitochondrial inner membrane protein required for normal mitochondrial morphology and inheritance

Table 6.1 Continued

ORF	Gene	Gene function
YOR199W	N/A	Dubious open reading frame; unlikely to encode a functional protein
YOR220W	*RCN2*	Protein of unknown function
YOR221C	*MCT1*	Predicted malonyl-CoA:ACP transferase produces intermediates for phospholipid remodeling
YOR223W	N/A	Protein of unknown function found in the ER and vacuole lumen
YOR251C	*TUM1*	Rhodanese domain sulfur transferase
YOR350C	*MNE1*	Protein involved in splicing Group I aI5-beta intron from COX1 mRNA; mitochondrial matrix protein
YOR375C	*GDH1*	NADP(+)-dependent glutamate dehydrogenase
YOR379C	N/A	Dubious open reading frame; unlikely to encode a functional protein

[a] code of ORF (open reading frame) which has been deleted from the mutant
[b] gene name associated with a particular ORF annotated from *Saccharomyces* Genome Database (SGD)
[c] function of the associated gene
[d] no known gene associated with this ORF

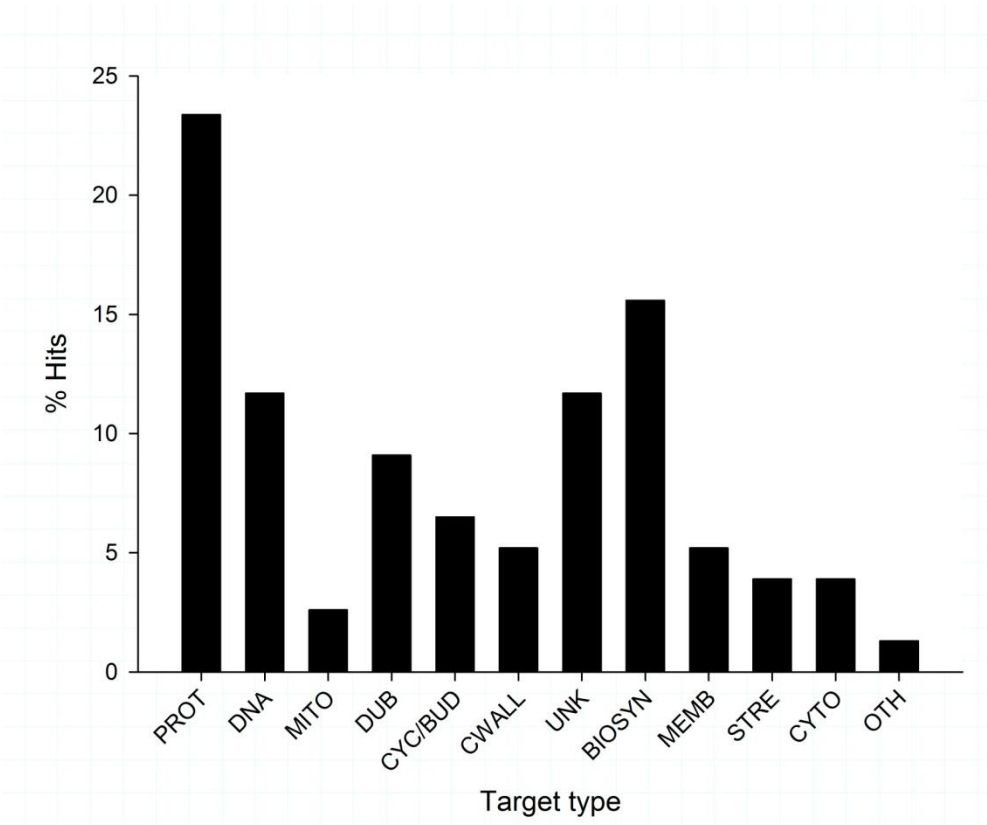


Figure 6.3 Summary of the screening of saponin (**1**) at 12.5 μM against *Saccharomyces cerevisiae* diploid heterozygous deletion library in YPD media. Total hits = 77 (at least 50% growth difference compared to the wild type BY4743). Target type: PROT = protein synthesis, transport, and localization, DNA = DNA transcription, replication, repairs, and regulation, MITO = mitochondrial proteins, DUB = dubious ORFs, CYC/BUD = cell cycle, budding, and sporulation, CWALL = cell wall, UNK = known function, BIOSYN = biosynthesis, metabolism, and signaling, MEMB = membrane protein, STRE = stress response, CYTO = cytoskeleton, OTH = ions transport and other processes.

6.4 Discussion

Using induced haploinsufficiency profiling, many potential targets for the isolated saponin (**1**) were identified. From the two library screens, it was clear that these target proteins were responsible for various cellular processes such as DNA transcription, protein synthesis, and biosynthetic pathways. Despite previous work (Morrissey and Osbourn 1999) that suggests membrane disruption as a major mode of action of saponins, in the solid media screening results presented here, membrane proteins and lipid synthesis/metabolism were only a minor portion of % hits when compared to DNA processes, protein synthesis, and unknown functions. This suggested that there may be other antifungal mechanisms involved for saponins that may not be related to membrane permeabilisation. Since **1** has a steroidal sapogenin, this part of the molecule may have a broad range of targets including inhibition of enzymes and receptor binding. Conversely, in the liquid screening, 12 of the 77 mutants with growth differences of at least 50% from the wild type were either missing membrane proteins or proteins associated with lipid synthesis and/or metabolism. These screenings were preliminary and further verification experiments involving different concentrations of **1** are needed to provide further understanding of its antifungal mechanism of action.

The proposed model for membrane disruption activity of saponins is through interaction with sterols, leading to loss of membrane integrity and ion leakage (Morrissey and Osbourn 1999 and references therein). The interaction between saponins and the fungal membrane involves forming saponin-sterol complexes and changing the phospholipid organization (Yamasaki et al. 1987). Once formed, the saponin-sterol complexes aggregate and cause either formation of membrane pores or tubular/spherical complexes outside the membrane, extracting the sterols from the fungal phospholipid bilayer (Armah et al. 1999; Keukens et al. 1992, 1995; Morrissey

and Osbourn 1999). Steroidal glycoalkaloids are believed to extract membrane sterols (Keukens et al. 1992, 1995). The aggregation of saponin-sterol complexes is facilitated by the interaction between the sugar chains (Armah et al. 1999; Keukens et al. 1995). Through electron microscopy, many studies have shown that exposure of fungal cells to various saponins resulted in formation of transmembrane pores and/or membrane damage (Armah et al. 1999; Bangham and Horne 1963; Glauert et al. 1962; Seeman et al. 1973; Zhang et al. 2006).

Other spirostanol saponins structurally related to **1** have been demonstrated to cause increased membrane permeability and disruption of membrane potential in fungal cells (Cho J et al. 2013; Zhang et al. 2006). Dioscin (Figure 6.4), an antifungal spirostanol saponin isolated from the roots of *Dioscorea nipponica* Mikano, was shown to disrupt membrane integrity and caused leakage of dyes in the propidium iodide assay and a membrane model (Cho J et al. 2013). In another study, Zhang et al. (2006) isolated two saponins TTS-12 and TTS-15 from *Tribulus terrestris* L. Both compounds were potent inhibitors of clinical strains of *Candida albicans* and *Cryptococcus neoformans*. Examination under transmission electron microscopy showed severe damage to fungal cell membrane after treatment with TTS-12. These saponins differ from **1** in that they are unsaturated (one double bond) and that they contain three or more sugar moieties.

Not all saponins have membrane lytic activities and/or interact with membrane sterols. Fukuda et al. (1985) and Woldemichael and Wink (2001) reported that saponins with two or more sugar moieties have less membrane permeabilisation capability than those with only one sugar residue. In another study, Escalante et al. (2008) showed that phytolaccoside B, an antifungal triterpenoid saponin isolated from the berries of *Phytolacca tetramera* Hauman, did not bind to ergosterol. Thus, the antifungal mechanism of action for phytolaccoside B is not related to loss of membrane integrity (although some damage to the membrane was observed).

Similarly, enfumafungin (a structurally related triterpenoid saponin isolated from the fermentation of *Hormonema* sp.) interfered with fungal growth via specific inhibition of (1→3)–β–D–glucan synthase, a key enzyme in cell wall polymer synthesis (Schwartz et al. 2000).

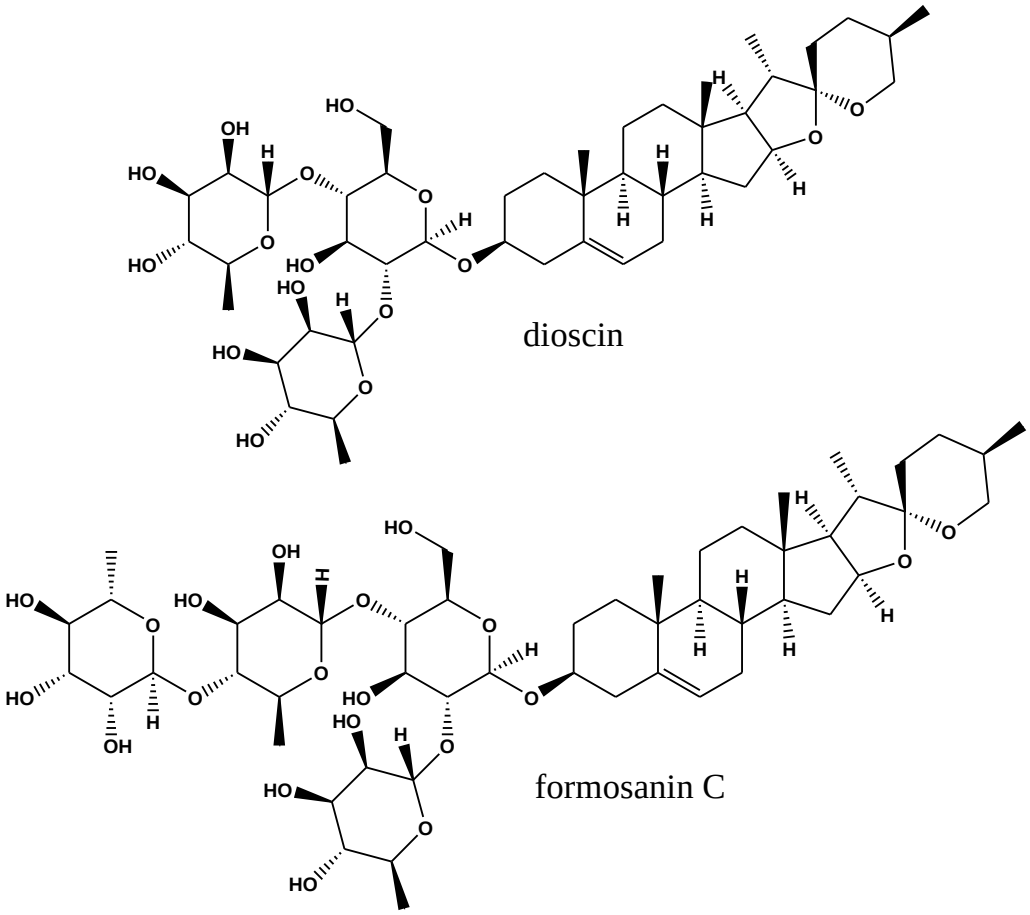


Figure 6.4 Structures of other spirostanol saponins with antifungal activity.

To our knowledge, this is one of the few antifungal mechanistic studies of any saponin using the yeast knockout library. Recently, another spirostanol saponin formosanin C (Figure 6.4) was screened using both the diploid homozygous and heterozygous *Saccharomyces cerevisiae* knockout collections (Lee AY et al. 2014). Formosanin C is present in the roots of *Paris polyphylla* var. *yunnanensis* (Franch.) Hand.-Mazz. and shares the same sapogenin as

dioscin (Man et al. 2009). In the study by Lee AY et al. (2014), treatment with formosanin C at 406 nM resulted in an average 33.8% growth inhibition for heterozygous mutants and 22.6% for homozygous mutants. Furthermore, 33 heterozygous mutants had significant fitness defect (FD) scores ($p < 0.01$); of these, four related genes (*PDR3*, *SPT8*, and *NUP1*) also showed up as hits in the present study with compound **1**. PDR3 is a transcription activator of the pleiotropic drug resistance network, which regulates the expression of ATP-binding cassette (ABC) transporters. SPT8 is a subunit of the SAGA transcription regulatory complex and NUP1 is a component of the nuclear pore complex involved in protein and RNA transport. These fitness defect scores were generated from a ratio of a molecular tag's signal (amplified via PCR followed by microarray hybridization) in the treatment samples and its signal in the control samples. The significant interactions in the Lee AY et al. (2014) study were identified as FD scores that deviated significantly from all other scores in a given screen (or the outliers). In the present study, growth difference (measured by colony size and area under the curve) is compared between the treatment of compound **1** and vehicle control. Global analysis of all fitness scores of formosanin C showed a major response signature corresponding to 'sphingolipid biosynthesis and PDR1'. Similarly, it can be inferred that **1** would also have the same response signature as formosanin C (since they are both spirostanal saponins and are structurally similar) and that its antifungal mechanism of action is through inhibition of membrane lipid synthesis and ABC transporter expression. Future experiments using different concentrations of compound **1** and yeast overexpression library could provide additional information on the mechanism of action. Furthermore, an enrichment analysis would eliminate false positives and reduce the number of targets, which could be confirmed via secondary assays. Clearly, the number of targets suggests

that **1** is pleiotropic and has a number of potential targets in fungi, which may confer an ability to avoid rapid evolution of resistance in fungal pests of the plant.

Chapter 7 – General discussion

7.1 Summary of results and novel contributions

The focus of this thesis was to examine the antimicrobial activity of select neotropical plant extracts and to identify novel phytochemicals that can interfere with the formation of bacterial biofilm and fungal growth. In particular, two targeted discovery methods were used to select potential plant species: a botanical survey of neotropical families and study of ethnobotanical materials. In collaboration with the Universidad Nacional Autonoma (UNA) of Costa Rica and the Q'eqchi' Maya Healers Association (QMHA) of Belize, prominent and rare plant families of the neotropics and traditional medicines were assessed for biofilm inhibitory and antifungal activities using model organisms.

Biological activities of neotropical plants

In Chapter 2, 126 extracts belonging to 71 species collected from different regions of Costa Rica were assessed for their abilities to inhibit bacterial quorum sensing (QS) and biofilm formation. Preliminary assessments showed that there was a broad range of QS and biofilm inhibitory activities with a third of the plant species showing significant inhibition. These species belonged to the Lepidobotryaceae, Melastomataceae, Meliaceae, Sapindaceae, and Simaroubaceae. Piperaceae species showed little or no activity. A comparison of the inhibitory activities of the three main plant families examined (Meliaceae, Melastomataceae, and Piperaceae) showed there was no statistical difference in average QS inhibition between the families. However, Melastomataceae and Meliaceae bark and wood showed significantly more inhibitory activities against biofilm formation than Piperaceae. For many Meliaceae species, bark and wood showed more inhibitory activities than leaves. Of the 19 Melastomataceae species

tested, nine displayed more QS inhibition than the alga *Delisea pulchra*, a positive control containing known QS inhibitors halogenated furanones (Giskov et al. 1996; Manefield et al. 1999; Raffa et al. 2005; Shiner et al. 2005). These include *Aciotis rubricaulis*, *Clidemia dentata*, *Conostegia rhodopetala*, *Leandra melanodesma*, *Miconia longifolia*, *Monochaetum floribundum*, *Monochaetum vulcanicum*, *Ossaea brenesii*, and *Topobea maurofernandeziana*. For the 30 Meliaceae species, a third showed stronger anti-QS activities than *D. pulchra* (*Cedrela fissilis* leaves, *Cedrela salvadorensis* leaves, *Chukrasia tabularis* leaves/bark/wood, *Guarea guidonia* leaves, *Guarea pterorhachis* leaves and bark, *Guarea pyriformis* bark, *Swietenia macrophylla* bark, *Swietenia mahogani* wood, *Trichilia pleeana* leaves, and *Trichilia septentrionalis* leaves and wood). In other families, all parts of *Ruptiliocarpon caracolito* (Lepidobotryaceae) except bark and *Simarouba glauca* (Simaroubaceae) leaves and bark also displayed more QS inhibition from the positive control *D. pulchra.* Many plant extracts also had growth inhibition of 1 to 5 mm beyond the disc while *Ruptiliocarpon caracolito* and *Chukrasia tabularis* showed inhibitory zones of 10 to 12 mm.

For biofilm growth, 40% of extracts showed either no inhibition or actually enhanced the formation of the biofilm mass when compared to the vehicle control (50% methanol). Thirteen Meliaceae and Melastomataceae species showed more significant biofilm inhibition than the positive control allicin (74.4 ± 8.1% growth). These species include *Aciotis rubricaulis*, *Clidemia dentata*, *Conostegia pittieri*, *Leandra melanodesma*, *Monochaetum floribundum*, *Topobea maurofernadeziana*, *Cedrela guianensis* bark and wood, *Cedrela odorata* bark, *Chukrasia tabularis* all parts, *Guarea pterorhachis* leaves, *Swietenia macrophylla* bark, *Trichilia martiana* leaves, and *Trichilia septentrionalis* wood. In other families, all parts of *Ruptiliocarpon caracolito* (Lepidobotryaceae) and the leaves and bark of *Cupania glabra* (Sapindaceae) also

showed significant inhibition when compared to allicin. IC_{50}s for biofilm inhibition some of the most active species ranged from 45 to 266 µg/mL.

This is the first report of anti-QS and anti-biofilm activities in the Lepidobotryaceae, Sapindaceae, and Simaroubaceae. Adonizio et al. (2006) showed QS inhibition by *Tetrazygia bicolour* leaves, a Melastomataceae. Interestingly, all of the active plant species from the Melastomataceae reported here have little or no previous phytochemical information and biological activities reported. Although the phytochemistry of the active Meliaceae species such as *Chukrasia tabularis* A. Juss, *Carapa guianensis* Aubl., and *Swietenia mahogani* (L.) Jacq. has been studied extensively, no active compounds have been reported for QS and/or biofilm inhibition. The majority of the Meliaceae extracts tested here have little or no phytochemical information available. The most active plant in our bioassays was *Ruptiliocarpon caracolito* L. All parts tested of *R. caracolito* except the bark showed significant QS and biofilm inhibition.

In Chapter 4, extracts of 12 Marcgraviaceae species (a family endemic to the neotropics) from Costa Rica and Brazil were assessed for the same biological activities: inhibition of quorum sensing, biofilm formation, and fungal growth. Quorum sensing (QS) inhibitory activities ranged from 7.7 ± 0.3 to 21.9 ± 0.8 mm. In particular, three extracts showed significantly more QS inhibition than the positive control *D. pulchra* extract (12.8 ± 0.7 mm). These include *Marcgravia nervosa* Triana & Planch. leaves and stems and *Marcgravia schippii* Standl. leaves. The roots of *Schwartzia brasiliensis* (Choisy) Bedell ex Gir-Cañas and stems of *Schwartzia costaricensis* (Gilg.) Bedell displayed similar anti-QS activities to that of *D. pulchra*. *Sarcopera* and *Souroubea* species showed little to no QS inhibition.

For biofilm formation, a large range of inhibition was observed. Both the stems and roots of *Marcgravia polyantha* Delp. significantly inhibited biofilm growth of *Pseudomonas*

aerguinosa PA14 at 45.4 ± 3.1% and 30.3 ± 3.2 % of growth of vehicle control, respectively, when compared to the positive control allicin (1 μL or 1.08 mg) with 74.8 ± 8.1 % growth. However, *M. polyantha* leaves only showed moderate inhibition at 78.5 ± 3.0 % growth. Furthermore, five extracts displayed from 30% to 40% biofilm inhibition: *Marcgraviastrium subsessile* (Benth.) Bedell stems, *Schwartzia brasiliensis* leaves and root, and *Schwartzia costaricensis* leaves and stems. *Marcgravia nervosa, Sarcopera,* and *Souroubea* species had little or no activity in this bioassay.

All tested Marcgraviaceae extracts showed little to no inhibition against the growth of *Saccharomyces cerevisiae* S288C except *Marcgravia nervosa.* When compared to the positive control berberine at 1 mg/disc (19.3 ± 0.7 mm), both leaves and stems extracts showed inhibitory zones of 23.9 ± 0.2 and 23.6 ± 0.2 mm, respectively.

HPLC–APCI–MS analysis of the crude extract of *Marcgravia nervosa* leaves identified the presence of ursolic acid, betulinic acid, α–amyrin, β–amyrin, and lupeol. However, all of these compounds were inactive in the QS and fungal bioassays at 250 μg/disc. The leaves extract was then fractionated using liquid-liquid extraction with hexane, ethyl acetate (EtOAc), and ethanol (EtOH) to give three fractions. These fractions were tested using the same bioassays, which identified the EtOH fraction as the most active. Both EtOAc and EtOH fractions showed antifungal activities comparable to the positive control berberine. For QS inhibition, the hexane fraction showed little or no activity while both EtOH and EtOAc fractions were more active than the *D. pulchra* extract.

The ^{1}H NMR spectra of the three fractions showed the presence of the same metabolite which featured a prominent O-CH_3 group in the 3.9 ppm range and two low field aromatic multiplets. The key difference among the extracts was the concentration of this compound with

the highest concentration residing in the EtOH fraction. This fraction was further fractionated using silica gel open glass column to give 18 secondary fractions. Fraction 4 (85–90% hexane) showed the same activity as the EtOH fraction; however, NMR analysis showed that the bioactive component has co-eluted with betulinic acid. A second column was performed on this fraction to obtain a pure sample of the active phytochemical. ^{1}H and ^{13}C spectra of the isolated phytochemical were obtained. Comparison of these spectra with published values identified this compound as 2–methoxy–1,4–naphthoquinone. The identification and mass were verified via UPLC–PDA–MS analysis.

2–methoxy–1,4–naphthoquinone showed potent QS inhibition at the tested concentration of 20 μg/disc. The minimum inhibitory concentration (MIC) for this compound was determined to be 85–100 μM against *Saccharomyces cerevisiae* BY4741 (haploid) and BY4743 (diploid). These MICs are at least fivefold lower than that of berberine (positive control) against the same strains (600 μM for both).

To our knowledge, this is the first study of the bacterial biofilm and quorum sensing inhibition by Marcgraviaceae and only the second type of the biological activity reported for Marcgraviaceae species. The anxiolytic activity of *Souroubea* species has been documented in rats (Mullally et al. 2011; Puniani et al. 2014) and dogs (Villalobos et al. 2014). Interestingly, none of active species (*Marcgravia nervosa*, *Marcgravia polyantha*, *Marcgravia schippii*, *Marcgraviastrum subsessile*, *Schwartzia brasiliensis*, and *Schwartzia costaricensis*) has been previously studied in terms of biological activities and phytochemistry. The presence of triterpenoids such as ursolic acid and betulinic acid is characteristic for this plant family. However, these compounds do not appear to be responsible for the observed anti-QS, antifungal, and biofilm inhibitory activities as they were inactive at tested concentrations of 5 μg/mL for

biofilm and 250 µg/disc for QS and antifungal. Preliminary fractionation of *Marcgravia polyantha* stems showed biofilm inhibition in the polar region, suggesting that the active phytochemicals could be polyphenols or other polar compounds.

The isolation of 2–methoxy–1,4–naphthoquinone from the leaves of *M. nervosa* is the first report of a naphthoquinone in the Marcgraviaceae. Quinones are known for their antibacterial and antifungal activities (Savoia 2012). Ding et al. (2011) found that the anthroquinone emodin inhibited biofilm formation of *P. aeruginosa* and *S. maltophilia* at 20 µM. 2–methoxy–1,4–naphthoquinone was first isolated from *Impatiens balsamina* L. (Panichayupakaranant et al. 1995; Panichayupakaranant 2001; Yang et al. 2001), a widely used plant in Traditional Chinese Medicine (Ding et al. 2008). The antimicrobial activity of this naphthoquinone against several bacterial strains such as S*taphylococcus aureus*, *Bacillus cereus, Aeromonas salmonicida* and fungi such as *Fusarium oxysporum* has been reported (Fostel and Lartey 2000). More recently, this compound showed promising activity in the treatment of multiple antibiotic resistant strains of a bacterium responsible for stomach ulcers, *Helicobacter pylori* (Wang et al. 2011) and the mechanism of action was proposed to occur via oxidative stress. A comparison of the antifungal activity of chimaphilin, another naphthoquinone previously isolated in our research group, shows that 2–methoxy–1,4–naphthoquinone is more active by a factor of 30 (Galv án et al. 2008).

The occurrence of quinones in the order Ericales, to which the Marcgraviaceae family belongs, has been estimated to be approximately 10% (Rocha et al. 2014). In particular, naphthoquinones such as plumbagin and derivatives have been isolated from the ironwood family, Ebenaceae, a related plant family in the Ericales (Higa et al. 2002; Kuo et al. 1996; Lee and Lee 2008). The occurrence of naphthoquinone secondary metabolite in *M. nervosa*, and

some Ericales species and its absence in the other Marcgraviaceae and Ericales species evaluated could be the result of specific microbial evolutionary pressure for the napthoquinone production in these plants. Secondary metabolites can evolve in a specific species or genus as they originate as a strategy for survival and to overcome threats in a specific environment (Kennedy and Wightman 2011). The environment from which *Marcgravia nervosa* has been collected is a high humidity and temperature ecosystem, making this the perfect climate for the development of microorganisms, including plants and animal pathogens. The evolution of a biosynthetic pathway of a compound like the naphthoquinone with a broad spectrum of antimicrobial activity provides a selective advantage for these plants to survive in this climate. We suggest that napthoquinone evolution has occurred independently in plants that are not closely related, but could possibly start with a common shikimate derivative common to Ericales.

Biological activities of Q'eqchi' Maya medicines

In Chapter 3, extracts of 61 Q'eqchi' Maya medicinal plant species collected over the last 5 years for general and focused ethnobotanicals purposes, belonging to 35 families and 22 orders, were tested for their ability to interfere with bacterial quorum sensing (QS) and biofilm formation as well as fungal growth inhibition. These species are used by the healers to treat many conditions. When the ethnobotanical use reports were classified according to the Cook (1995) use categories, the top four categories with the largest number of species were infections/infestations (INF), digestive system disorders (DIG), pain (PAI), and nervous system disorders (NER) with 40 species, 35 species, 31 species, and 30 species cited, respectively. Muscular skeletal system disorders (MUS), circulatory systems disorders (CIR), poisonings (POI), and skin/subcutaneous cellular tissue disorders (SKI) are the next highest categories with 23 species, 20 species, 17 species, and 17 species used, respectively. Many plants are used by the

Q'eqchi' Maya healers for multiple treatments and thus appear in more than one category. When the top eight use categories were considered, SKI was the category with the highest percentage of active species (53%); INF and MUS were the next highest with 48%, followed by PAI, POI, and DIG with 45%, 41%, and 40%, respectively. Considering the taxonomic affiliation of the tested plants, the orders with the highest activity were Malpighiales, Myrtales, and Cucurbitales with 100%, 83%, and 75%, respectively. The Malpighiales includes families such as Euphorbiaceae and Passifloraceae; the Myrtales includes the Melastomataceae and Combretaceae while the Cucurbitales includes the Begoniaceae and Cucurbitaceae.

Preliminary assessments showed that QS inhibitory activities in *Chromobacterium violaceum* ATCC 12472 ranged from 7.0 ± 0.1 mm to 26.1 ± 0.3 mm. In particular, four species displayed significant QS inhibition when compared to the positive control *D. pulchra* extract (12.8 ± 0.7 mm): *Combretum fruticosum* (Loefl.) Stuntz. leaves (Combretaceae), *Chamaesyce hyssopifolia* (L.) Small. leaves (Euphorbiaceae), *Euphorbia lancifolia* L. leaves (Euphorbiaceae), and *Blakea cuneata* Standl. leaves (Melastomataceae). The leaves of *Hyptis capitata* Jacq. (Lamiaceae), stems of *B. cuneata*, and leaves of *Petiveria alliacea* L. (Phytolaccaceae) showed similar QS inhibitory activities of that of *D. pulchra*.

For biofilm growth, most species showed either little to no inhibition or actually enhanced the formation of the biofilm mass when compared to the vehicle control (50% methanol). However, the leaves of *Croton schiedeanus* Schtldl. (Euphorbiaceae) and *Blakea cuneata* Standl. (Melastomataceae) significantly inhibited the growth of *P. aeruginosa* PA14 biofilm more than the positive control allicin (74.4 ± 8.1% control) with growths of 49.1 ± 2.9 % and 27.1 ± 3.1 %, respectively. Five species showed similar inhibitory activities to that of allicin: *Begonia heracleifolia* Schltdl. & Cham. (Begoniaceae), *Momordica charantia* L.

(Cucurbitaceae), *Hyptis capitata* Jacq. (Lamiaceae), *B. cuneata* stems (Melastomataceae), and *Miconia gracilis* Triana (Melastomataceae).

Many tested extracts showed little to no growth inhibition against *Saccharomyces cerevisiae* S288C except *Passiflora oerstedii* Mast. var. *choconiana* S. Watson (Passifloraceae), *Campyloneurum brevifolium* (Lodd. ex Link) Link (Polypodiaceae), and *Cestrum schlechtendahlii* G.Don. *Campyloneurum brevifolium* and *Cestrum schlechtendahlii* showed significant activities against S288C, similar to that of berberine (19.3 ± 0.7 mm) with inhibitory zones of 17.7 ± 0.3 mm and 15.1 ± 0.6 mm, respectively. These two species also inhibited the growth of *Candida albicans* D10 and *Cryptococcus neoformans* but to a lesser extent.

To our knowledge, this is the first report of biological activities for *Blakea cuneata* Standl (Melastomatacaea), *Miconia gracilis* Triana (Melastomataceae), *Campyloneurum brevifolium* (Lodd. ex Link) Link (Polypodiaceae), and *Cestrum schlechtendhalii* G.Don (Solanaceae). Interestingly, there also have been no phytochemical studies for any of these species. *Blakea cuneata* (Oxlaho chajom) is used by the Q'eqchi' Maya healers for fever, swelling, insect bites and treatment of digestive system troubles such as stomach cramps and ulcers. Both the leaves and stems of this species showed inhibitory activities against bacterial QS and biofilm growth with the leaves having stronger inhibition. This suggests that the active principles are present in both the stems and leaves. *Miconia gracilis*, another Melastomataceae, also showed anti-QS and biofilm inhibition.

Combretum fruticosum (Loefl.) Stuntz. (K'an shan k'aham in Q'eqchi') leaves are used by healers to treat diarrhoea. *C. fruticosum* (Combretaceae) leaves showed strong QS inhibitory activities and minor interference to biofilm formation (~17% inhibition). Extracts of the leaves

and stems of this species have been reported by Braga et al. (2009) to show potent inhibition of angiotensin-converting enzyme (ACE). Leaves extract also showed antimicrobial activity against both Gram-positive and Gram-negative bacteria *S. aureus*, *E. coli*, *P. mirabilis*, *P. aeruginosa* (Smith et al. 2000). Furthermore, *C. fruticosum* leaves extract was also effective against different strains of methicillin-resistant *S. aureus* (MRSA) with minimum inhibitory concentrations (MICs) ranging from 250 to 500 μg/mL (Barneche et al. 2011). However, no phytochemical investigation has been reported for this species.

From these results, it is clear that the healers use a very wide assemblage of plant biodiversity and some pharmacological validation for the use of traditional Q'eqchi' Maya medicinal plants for the treatment of infections and related symptoms are provided. Further investigation such as bioassay-guided fraction of the active species (Appendix I) could lead to the development of botanical therapies for treatment of biofilm-associated infections. Moreover, pharmacological validation of traditional knowledge could increase interest in and conservation of Maya traditional medicine for future generations.

In Chapter 5, the leaf extract of an active plant species *Cestrum schlechtendahlii* were further assessed for phytochemical composition and biological activities. HPLC–DAD analysis of the crude extract identified the presence of caffeic acid, *p*–coumaric acid, and rosmarinic acid, all of which showed no antifungal activity in the current assays. The crude extract was fractionated using chromatography using silica gel and Sephadex to yield two spirostanol saponins (one major and one minor). High resolution MS/MS, 1D and 2D NMR spectroscopy experiments confirmed the identification of these saponins to be (25R)–1β,2α–dihydroxy–5α–spirostan–3–β–yl–*O*–α–L–rhamnopyranosyl–(1→2)–β–D–galactopyranoside (major) and (25R)–1β,2α–dihydroxy–5α–spirostan–3–β–yl–*O*–β–D–galactopyranoside (minor).

The major saponin showed promising growth inhibition against both yeast-like and filamentous fungi with higher activity than the positive control berberine. In *Saccharomyces cerevisiae* strains, the MIC of this compound (16.5 μM) in liquid culture is 30-fold lower than that of berberine (600 μM) and 10-fold lower in solid culture (20 μM and 223 μM). This saponin was less effective against *Fusarium graminearum* ZTE–2A but was still more active than berberine with an MIC that was 6-fold lower. The MIC of this compound against *F. graminearum* ZTE–2A was estimated to be 132 μM and berberine to be 807 μM. The minor saponin and the sapogenin showed little or no activity against *S. cerevisiae*, even at the highest concentration tested. The growth inhibition of the major saponin against *F. graminearum* ZTE–2A was much less effective against spores than the mycelia mass. Even at the highest tested concentration of 400 μg/mL (529 μM), *F. graminearum* growth was only moderately inhibited when this compound was added at the beginning at the same time with the spores; once the spores have germinated (after 24 hours), there was a dose-dependent inhibition of mycelia growth where it severely affected growth at the highest concentration.

This is the first phytochemical report for *Cestrum schlechtendahlii*. The major saponin has been isolated in another Solanaceae species by Haraguchi et al. (2000); however, this is the first report of its antifungal activity. The minor saponin is a monosaccharide derivative of the major saponin and is a novel natural product.

In Chapter 6, the antifungal mechanisms of action for the isolated major saponin were studied using yeast knockout libraries. The initial screening of this saponin against the yeast diploid heterozygous deletion library in solid media at a sub-lethal concentration of 15 μg/mL (19.8 μM) yielded 244 mutants of the 6000 tested from the *ScreenMill* analysis, which were considered to have their growth significantly reduced ($p < 0.05$). Based on annotation from the

Saccharomyces Genome Database (SGD), the mutants of interest can be grouped into 13 target types. Of the 244 mutants, the most prominent target type was UNK (proteins of unknown functions) with approximately 18% of the hits (45). The next highest categories were DNA (DNA transcription, replication, repairs, and regulation) followed by BIOSYN (biosynthesis, metabolism, and signalling), and PROT (protein synthesis, transport, and localization) representing 15% (36), 14% (34), and 13% (32) of hits, respectively. MEMB (membrane proteins) and DUB (dubious ORFs) were next with approximately 8%, followed by MITO (mitochondrial proteins) and CYC/BUD (cell cycle, budding, and sporulation) both at 6%. The remaining categories each represented 3% of hits or less. From these preliminary results, it was clear that target types were widespread and further verification experiments were needed to provide further insights to the mechanisms of action of this saponin.

Subsequent screening of the isolated saponin against the same library was done in liquid media at a sub-lethal concentration of 12.5 μM in order to reduce the number of identified targets. Based on the normalized % change calculated, 77 of the 874 tested mutants showed at least 50% growth difference when compared to the wild type. Based on SGD annotation, the highest target type was PROT (protein synthesis, transport, and localization) with ~23 % of the hits (18). The next highest categories are BIOSYN (biosynthesis, metabolism, and signalling) followed by DNA (DNA transcription, replication, repairs, and regulation) at ~16% (12) and UNK (proteins of unknown functions) both with ~12% (9) each. The remaining categories each contained 7% or less. Furthermore, 12 genes (*PDR3, AST1, DER1, PER1, SWF1, CAB5, HTD2, PAN6, PAH1, CUE1, MDM32,* and *MCT1*) were either membrane proteins or associated with lipid synthesis and/or metabolism. According to GeneMANIA, 53% of the 77 ORFs physical

interactions with SSA1, an ATPase involved in protein folding, signaling, and transmembrane transport.

To our knowledge, this is one of the few antifungal mechanistic studies of any saponin using the yeast knockout library. Recently, another spirostanol saponin formosanin C was screened using both the diploid homozygous and heterozygous *Saccharomyces cerevisiae* knockout collections (Lee AY et al. 2014). Formosanin C is present in the roots *Paris polyphylla* var. *yunnanensis* (Franch.) Hand.-Mazz.(Man et al. 2009). In the study by Lee AY et al. (2014), treatment with formosanin C at 406 nM resulted in an average 33.8% growth inhibition for the heterozygous mutants and 22.6% for the homozygous mutants. Furthermore, 33 heterozygous mutants had significant fitness defect scores ($p < 0.01$); of these, four related genes (*PDR3, SPT8,* and *NUP1*) also showed up as hits in the present study with the major saponin. PDR3 is a transcription activator of the pleiotropic drug resistance network, which regulates the expression of ATP-binding cassette (ABC) transporters. SPT8 is a subunit of the SAGA transcription regulatory complex and NUP1 is a component of the nuclear pore complex involved in protein and RNA transport. Global analysis of all fitness scores of formosanin C showed a major response signature corresponding to 'sphingolipid biosynthesis and PDR1'. Similarly, it can be inferred that the isolated saponin would also have the same response signature as formosanin C and that its antifungal mechanism of action is through inhibition of membrane lipid synthesis and ABC transporter expression. Clearly, this suggests that the isolated saponin is pleiotropic and has a number of potential targets in fungi, which may confer an ability to avoid rapid evolution of resistance in fungal pests of the plant.

7.2 Comparisons between major findings and literature

The presence of quorum sensing and biofilm inhibitors in plants has been documented in numerous species using various model organisms. Recently, there have been many studies reporting QS and biofilm inhibitory activities by natural products against both bacteria and fungi. Table 7.1 lists a representative sample of these phytochemicals and their activities. The comprehensive list can be found in the Appendix V. A comparison between Table 7.1 and results from this thesis showed that QS and biofilm inhibitors belong to diverse chemical classes. Sulfur-containing compounds such as allicin, ajoene, and thiocyanates have been shown to interfere with biofilm formation and quorum sensing of both Gram-positive and Gram-negative bacteria (Borges et al. 2013; Ganin et al. 2013; Jakobsen et al. 2012; Lin et al. 2013; Pérez-Giraldo et al. 2003). Allicin (from garlic), used as a positive control in the biofilm assay, inhibited *Staphylococcus epidermidis* and *Pseudomonas aeruginosa* PAO1 biofilm adhesion as well as the expression of QS-regulated virulence factors in *P. aeruginosa* PAO1 (Pérez-Giraldo et al. 2003; Lin et al. 2013). Similarly, ajoene (also from garlic) downregulated virulence factors expression (elastase, rhamnolipid, enterotoxins) in the same strain (Jakobsen et al. 2012). In a mouse pulmonary infection model, subcutaneous treatment of ajoene at 25 mg/kg, significantly improved bacteria clearance after 3 days (Jakobsen et al. 2012). Furthermore, thiocyanates such as sulforaphane and allyl isothiocyanate (from Brassicaceae species) inhibited QS in *Escherichia coli* and *Chromobacterium violaceum* and reduced biofilm formation in *Pseudomonas aeruginosa* and *Listeria monocytogenes* (Ganin et al. 2013; Borges et al. 2013; Borges et al. 2014).

Different types of terpenes such as monoterpenes, limonoids, and triterpenes have also been reported to have anti-biofilm and anti-QS activities. Thymol and carvacrol were effective

against new and existing biofilms of *L. monocytogenes* and *P. aeruginosa* (Soumya et al. 2011; Upadhyay et al. 2013). These monoterpenoids also downregulated genes critical to *L. monocytogenes* biofilm formation (Upadhyay et al. 2013). Sesquiterpenoids acanthospermolides and salvipisone reduced biofilm growth in *P. aeruginosa, S. aureus,* and *S. epidermidis* (Cartegena et al. 2007; Kuźma et al. 2007; Walencka et al. 2007). Limonoids isolated from *Citrus* × *aurantium* (Rutaceae) showed 40 to 70% inhibition of bioluminescence in *Vibryo harveyi* at 6.25 μg/mL (Vikram et al. 2011). Betulinic acid, a lupane triterpene, showed no activity at 5 μg/mL in our bioassay with *P. aeruginosa* PA14; at 100 μg/mL biofilm formation is greatly enhanced in the same strain (Cho HS et al. 2013). Ursane triterpenoids, on the other hand, have been documented to have good inhibitory activities. Ursolic acid has been shown to inhibit *P. aeruginosa* PAO1, *E. coli* JM109, and *V. harveyi* BB120 biofilms by 50 to 87% at 10 μg/mL (Ren et al. 2005). This compound was not active against *P. aeruginosa* PA14 biofilm in our bioassay at a concentration of 5 μg/mL. Furthermore, gymnemic acids (ursane triterpene glycosides) isolated from *Gymnema sylvestre* (Apocynaceae) have been reported by Vediyappan et al. (2013) to inhibit yeast-to-hypha transition in *Candida albicans* SC5314. These compounds also inhibited conidial germination and hyphal growth in *Aspergillus fumigates.* Treatment of a gymnemic acids mixture, at 40 μg/mL, significantly improved the survival rate of *Candida albicans*-infected *Caenorhabditis elegans*.

By far, phenolics have the highest number of active compounds reported in term of their effects on quorum sensing and biofilm formation when compared to all other classes. A literature search showed 66 active compounds belonging to the phenolics chemical class, with the next highest class (terpenes) having 19 and the rest (alkaloids, coumarins, organosulfur compounds,

Table 7.1 Phytochemicals affecting microbial quorum sensing (QS) and/or biofilm formation**.**

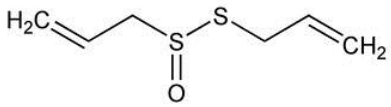
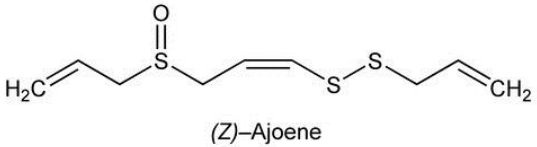
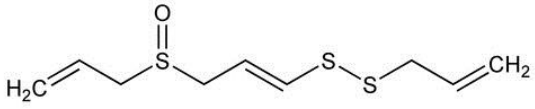
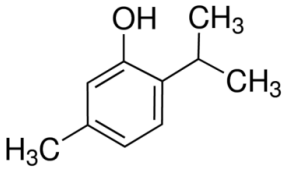
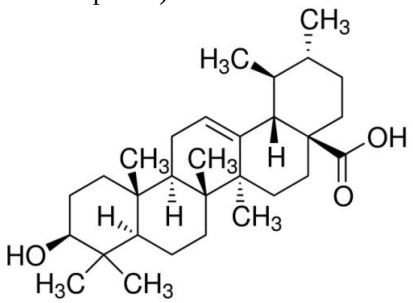
Active constituents (source plants)	Biological activities	Reference
Sulfur-containing compounds **allicin** (*Allium sativum* L. – Amaryllidaceae) 	• Inhibited *P. aeruginosa* PA14 biofilm (74% at 1 μL) • Inhibited biofilm formation by >90 % in *S. epidermidis* strains at 4 mg/mL • Reduced adhesion of GFP-transformed *P. aeruginosa* PAO1, EPS production (70%), biofilm thickness (50%), and expression of virulence factors at 128 μg/mL	This work Pérez-Giraldo et al. 2003 Lin et al. 2013
ajoene (*Allium sativum* L. – Amaryllidaceae)  *(Z)*–Ajoene  *(E)*–Ajoene	• Inhibited QS in *P. aeruginosa lasB-gfp* (IC_{50} = 15 μM) , *rhlA-gfp* (IC_{50} = 50 μM), *E. coli luxI-gfp* (IC_{50} = 100 μM) reporter strains • Downregulated QS-regulated virulence factors (5-fold at 80 μg.mL) and improved bacteria clearance in mouse infection model (subcutaneous treatment of 25 mg/kg body weight)	Jakobsen et al. 2012
Terpenoids **thymol** (*Thymus vulgaris* L. – Lamiaceae) 	• Inhibited formation of new and inactivated preformed biofilms in *L. monocytogenes* (0.5 mM and 5 mM, respectively) • Downregulated genes critical to *L. monocytogenes* biofilm formation (0.5 mM) • Downregulated expression of enterotoxin genes in *S. aureus* at 64μg/mL (>5-fold) • Inhibited formation of biofilm in *P. aeruginosa* ATCC 27853, CIP A22, and IL5 at 0.1% by 86%, 54%, and 70%, respectively	Upadhyay et al. 2013 Qiu et al. 2010a Soumya et al. 2011
ursolic acid (various species) 	• Inhibited biofilm formation in *P. aeruginosa* PAO1 (>87%), *E. coli* JM109 (50%), and *V. harveyi* BB120 (57%) at 10 μg/mL • Induced expression of chemotaxic and motility genes and repressed sulphur metabolism in *E. coli* K-12 at 10 μg/mL • Inhibited biofilm formation in *P.aeruginosa* PAO1 by 35% at 10 μg/mL	Ren et al. 2005 Hu et al. 2006

Table 7.1 Continued.

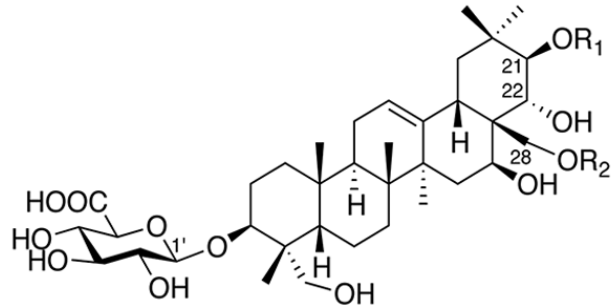
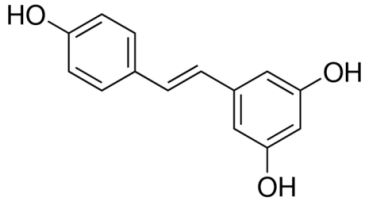
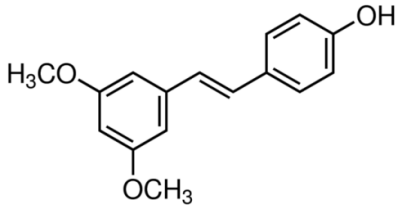
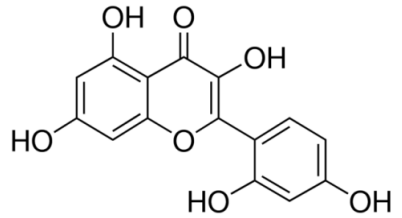
Active constituents (source plants)	Biological activities	Reference
gymnemic acids (*Gymnema sylvestre* (Retz.) R.Br. ex Sm. – Apocynaceae and Asclepiadaceae species)  GA-III, R1 = (S)-2-methylbutyroyl, R2 = H; GA-IV, R1 = tigloyl, R2 = H GA-XIII, R1 = H, R2 = (S)-2-methylbutyroyl; GA-XIV, R1 = H, R2 = tigloyl	• Mixture of 4 acids at 40 μg/mL inhibited yeast-to-hypha transition in *C. albicans* SC5314 and induced conversion of hyphae back to yeast form (100% after 11 hours) • Inhibited conidial germination and hyphal growth in *Aspergillus fumigates* by 74% at 40 μg/mL • Treatment of 40 μg/ml mixture improved survival in *C. albicans*-fed *C. elegans* infection model (100% rescue) by inhibiting formation of invasive hyphae	Vediyappan et al. 2013
Flavonoids and stilbenoids **resveratrol** (various species)  	• Inhibited *S. aureus* biofilm formation (30%) and enhanced *S. epidermidis* biofilm formation (1.5-fold) at 100 μg/mL • Inhibited *P. acnes* biofilm formation by 80% at 0.32% • Inhibited biofilm formation in *P. aeruginosa* PA14 and *E. coli* O157:H7 at 50 μg/mL	Morán et al. 2014 Coenye et al. 2012 Cho HS et al. 2013
pterostilbene (*Vitis* sp. – Vitaceae and Eriaceae species)  	• Inhibited formation of new and mature *C. albicans* SC5314, Y0109, 0304103, and 01010 biofilms at 16 μg/mL • Inhibited hyphal formation in *C. albicans* at 4 μg/mL • Treatment of 16 μg/mL altered expression of genes involved in morphological transition, ergosterol biosynthesis, filamentation, and cell surface proteins; also effective in rat central venous catheter infection model	Li DD et al. 2014
quercetin (various species)  	• Inhibited AI-induced bioluminescence in *V. harveyi* BB886, MM32 (75% at 6.25 μg/mL) and biofilm formation in *E. coli* O157:H7, *V. harveyi* BB120 (60% at 6.25 μg/mL) • Inhibited biofilm formation in MRSA (>80%) and MSSA (>50%) strains at 1 μg/mL • Reduced expression of genes involved in QS and virulence in *S. aureus* at 10 μg/mL	Vikram et al. 2010 Lee et al. 2013

Table 7.1 Continued

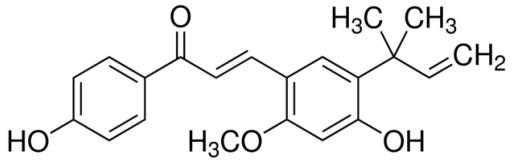
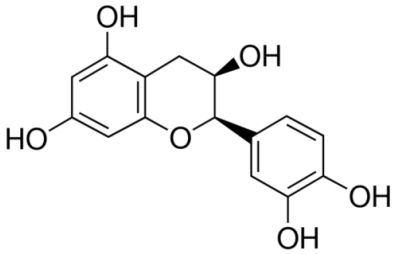
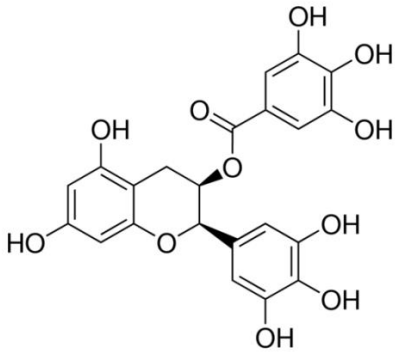
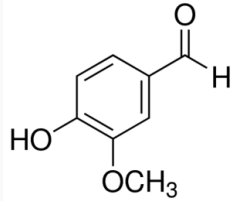
Active constituents (source plants)	Biological activities	Reference
licochalcone A (*Glycyrrhiza* sp. – Fabaceae) 	• Downregulated expression of enterotoxin genes in *S. aureus* ATCC 29213 and MRSA 2985 at 2 μg/mL after 4 hour growth	Qiu et al. 2010b
	• Treatment of 64 μg/mL to mature *S. aureus* ATCC 29213 biofilms downregulated expression of pathogenic factors, cell wall and biofilm-related proteins	Shen et al. 2014
(-)–epicatechin (*Camellia sinensis* (L.) Kuntze – Theaceae and others) 	• Inhibited elastase activity in *P. aeruginosa* PAO1 by 40% at 4 mM	Vandeputte et al. 2010
	• Enhanced biofilm formation in *P. aeruginosa* PAO1 (2-fold at 200 μg/mL) and *A. tumefaciens* C58 (2-fold at 400 μg/mL)	Plyuta et al. 2013
	• Increased AHL production in *E. coli* JDL271/pAL105 at 40 to 200 μg//mL	
	• Inhibited violacein production in *C. violaceum* ATCC 12472 (33% at 1 mg/mL)	Borges et al. 2014
	• Inhibited biofilm formation in *E. coli* JM109 by 40% at 1 mg/mL	
(-)–epigallocatechin gallate (*Camellia sinensis* (L.) Kuntze – Theaceae) 	• Inhibited QS in *E. coli* MT102 (pSB403) and *P. putida* (pKR-C12) (>50% and 40% at 40 μg/mL, respectively)	Huber et al. 2003
	• Reduced biofilm formation (30%) and swarming motility in *B. cepacia* (100%) at 40 μg/mL	
	• Inhibited biofilm formation in *E. corrodens* by >60% at 1 mM (MIC = 0.1 mM)	Matsunaga et al. 2010
Other phenolics and acid derivatives		
vanillin (*Vanilla planifolia* Jacks. ex Andrews – Orchidaceae) 	• Inhibited violacein production in *C. violaceum* CV026 (69%) and biofilm formation in *A. hydrophila* (50%) at 0.25 mg/mL	Ponnusamy et al. 2009
	• Inhibited biofilm formation in *A. hydrophila* on membrane filters by 90% with pre-treatment of 0.18 mg/mL	Kappachery et al. 2010
	• Enhanced biofilm formation in *P.aeruginosa* PAO1 (3-fold at 200 μg/mL) and *A. tumefaciens* C58 (2-fold at 25 μg/mL) and AHL production in *E. coli* JDL271/pAL105 at 40 μg/mL	Plyuta et al. 2013

Table 7.1 Continued

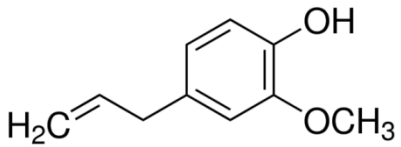
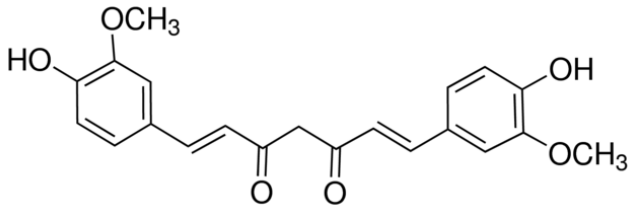
Active constituents (source plants)	Biological activities	Reference
eugenol (various species) 	• Inhibited *las* QS-mediated elastase production (32% at 200 μM) and *pqs* QS-mediated pyocanin production (56% at 50 μM) in *P. aeruginosa* , QS-mediated violacein production in *C. violaceum* (48% at 150 μM)	Zhou et al. 2013
	• Decreased biofilm formation in *P. aeruginosa* PAO1 (43% at 400 μM)	
	• Inhibited formation of new and inactivated preformed biofilms in *L. monocytogenes* (2.5 mM and 25 mM, respectively)	Upadhyay et al. 2013
	• Downregulated genes critical to *L. monocytogenes* biofilm formation (2.5 mM)	
	• Inhibited biofilm formation in *K. pneumoniae* clinical isolates (MIC = 63.5 μg/mL)	Magesh et al. 2013
curcumin (*Curcuma longa* L. – Zingiberaceae) 	• Inhibited biofilm formation in *S. epidermidis* (MIC = 25 μg/mL), *P. aeruginosa* PAO1and AHLs production at 1 μg/mL	Sharma et al. 2014 Ruddrappa and Bais 2008
	• Altered expression of QS-related genes, reduced virulence factors production (60 to 80%) and mortality in infection models (28 to 80%) with treatment of 3 μg/mL in *P. aeruginosa* PAO1	
	• Inhibited biofilm formation (50%), alginate production (20 to 70%), and motility in *V. harveyi, V. parahaemolyticus*, and *V. vulnificus* at 75 μg/mL	Packiavathy et al. 2013
	• Inhibited violacein production in *C. violaceum* CV026, alginate (63%) and rhamnolipid (56%) production in *P. aeruginosa* PAO1, prodigiosin production (58%) in *S.marcescens* FJ584421 at 100 μg/mL	Packiavathy et al. 2014
	• Inhibited swimming motility by 50% in *E. coli* ATCC 10536 (50 μg/mL), *P. aeruginosa* PAO1 (50 μg/mL), *P. mirabilis* ATCC 7002 (75 μg/mL), and *S. marcescens* FJ584421 (75 μg/mL)	
	• Inhibited biofilm formation in *E. coli* (52%), *P. aeruginosa* PAO1 (89%), *P. mirabilis* (52%), and *S. marcescens* (76%) at 100 μg/mL	
	• Inhibited sortase A activity (IC_{50} = 10 μM) and biofilm formation in *S.mutans* UA159 (at 15 μM)	Hu et al. 2013
	• Completely eradicated 48-hour and 14-day biofilms and reduced biomass of 8-week biofilm in *E. faecalis* ATCC 29212 (625 μg/mL)	Neelakantan et al. 2013
	• Completely inhibited biofilm formation in *H. pylori* ATCC 43504 and other clinical isolates at 8 μg/mL for up to 10 days	Pattiyathanee et al. 2009
	• Inhibited biofilm formation (50%) and surface adhesion (15%) in *C. albicans* at 50 μg/mL	Shahzad et al. 2014

Table 7.1 Continued

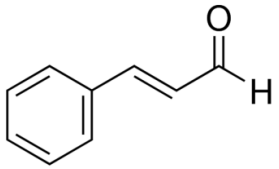
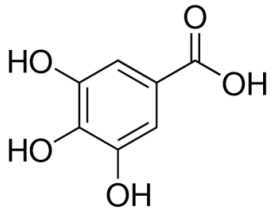
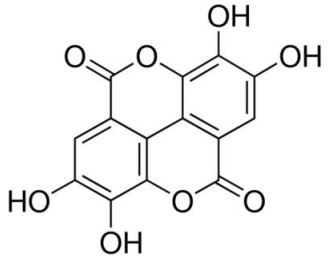
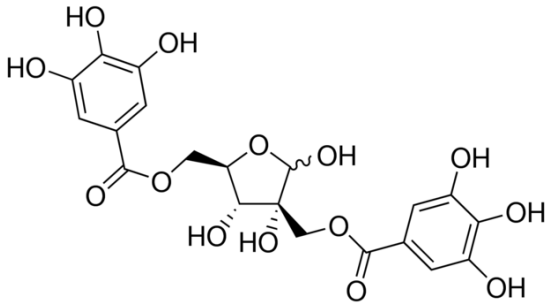
Active constituents (source plants)	Biological activities	Reference
cinnamaldehyde (*Cinnamomum* sp. – Lauraceae) 	• Inhibited AI-2-mediated QS in *Vibrio* spp. (65% at 100 μM) • Inhibited formation of new and inactivated preformed biofilms in *L. monocytogenes* (0.75 mM and 10 mM, respectively) • Downregulated genes involved in *L. monocytogenes* biofilm formation (0.75 mM) • Inhibited biofilm formation in *S. epidermidis* (MIC = 125 μg/mL) • Inhibited formation of new and inactivated mature biofilms in *C. sakazakii* (750 μM and 38 mM, respectively) and downregulated expression biofilm-related genes	Brackman et al. 2008 Upadhyay et al. 2013 Sharma et al. 2014 Amalaradjou and Venkitanarayanan 2011
gallic acid (various species) 	• Increased biofilm formation in *S. epidermidis* by 3-fold at 188 μg/mL • Inhibited biofilm formation in *E. corrodens* by 80% at 1 mM • Enhanced biofilm formation in *P. aeruginosa* PAO1 (2-fold at 25 μg/mL) and *A. tumefaciens* C58 (2-fold at 100 μg/mL); reduced biofilm formation in *P.aeruginosa* PAO1 by 30% at 200 μg/mL • Inhibited violacein production in *C. violaceum* ATCC 12472 (59% at 1 mg/mL)	Morán et al. 2014 Matsunaga et al. 2014 Plyuta et al. 2013 Borges et al. 2014
ellagic acid (various species) 	• Inhibited violacein production in *C. violaceum:* 18.3 mm at 36 μg • Inhibited QS in *E. coli* MT102 (pSB403) by 40% at 40 μg/mL and *P. putida* (pKR-C12) by 40% at 30 μg/mL • Inhibited biofilm formation (50% at 40 μg/mL) and swarming motility (100% at 20 μg/mL) in *B. cepacia* • Reduced biofilm formation in *S. dysgalactiae* strains by 70% at 4 μg/mL • Inhibited biofilm formation in *S. aureus* ATCC 11632 (60% at 15 μg/mL), MRSA ATCC 33591 (70% at 20 μg/mL), *E. coli* ATCC 10536 (60% at 15 μg/mL), and *C. albicans* ATCC 90028 (50% at 20 μg/mL)	This work Huber et al. 2003 Dürig et al. 2010 Biakkiyaraj et al. 2013
hamamelitannin (*Hamamelis virginiana* L. – Hamamelidaceae) 	• Inhibited *agr* QS regulator RNAIII and δ-hemolysin production at 50 μg/mL • Reduced cell attachment of MRSA to polystyrene plate at 4 μg/mL • In mice infection model, treatment of grafts with 30 mg/mL showed no detectable MRSA and MRSE load after 7 days	Kiran et al. 2008

Table 7.1 Continued

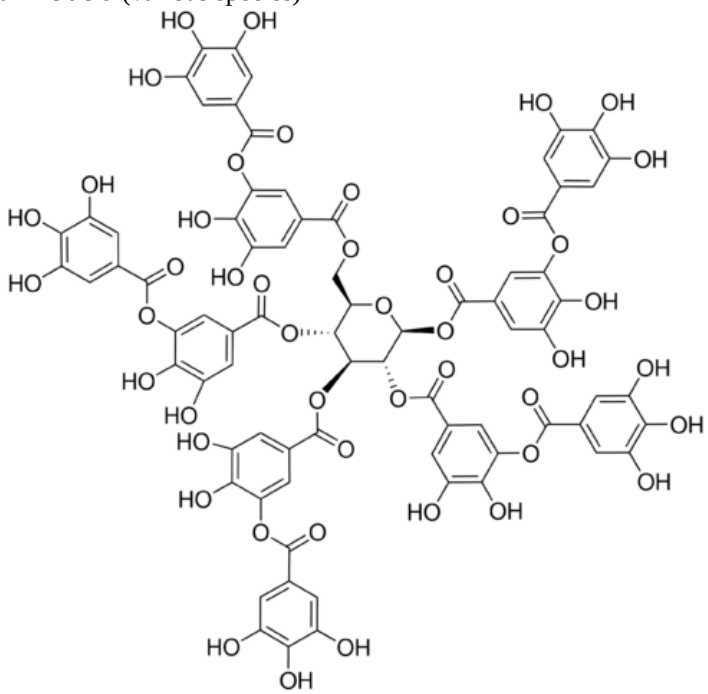
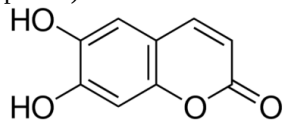
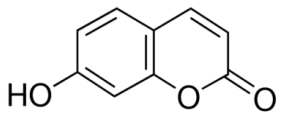
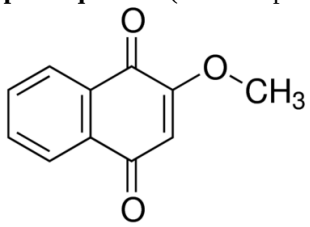
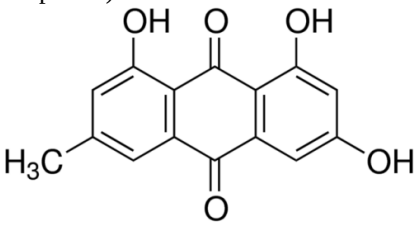
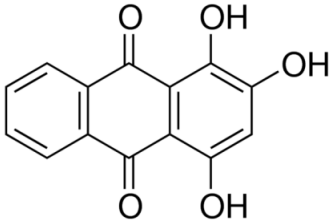
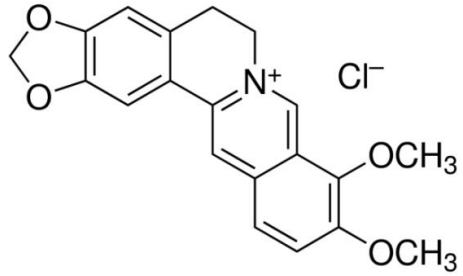
Active constituents (source plants)	Biological activities	Reference
tannic acid (various species) 	• Inhibited violacein production in *C.violaceum* (21.8 mm at 500 μg) • Inhibited biofilm formation in *P. aeruginosa* PA14 (72% at 200 μg/mL) • Enhanced biofilm formation in *P. aeruginosa* PA14 at 100 μg/mL • Inhibited QS in *P. putida* (pKR-C12) by 40% at 30 μg/mL and *E. coli* MT102 (pSB403) by 20% at 60 μg/mL • Inhibited biofilm formation in *S. aureus* by >50% at 20 μg/mL • Reduced expression of genes responsible for QS and virulence in *S. aureus* at 20 μg/mL • Inhibited *S. aureus* biofilm formation (60% at 2 μM) and increased *isaA* expression (a transglycosylase)	This work Huber et al. 2003 Cho HS et al. 2013 Payne et al. 2013
<u>Coumarins and derivatives</u> **aesculetin** (various species) 	• Inhibited QS in *C. violaceum* CV026, *P. aeruginosa*, *E. coli* JB523 (30 to 60% at 100 μM) • Inhibited biofilm formation in *S. aureus* strains (53 to 77% at 128 μg/mL) • Reduced expression of biofilm-related genes (motility, adhesion, virulence) in *E. coli* O157:H7 at 50 μg/mL	Brackman et al. 2009 Dürig et al. 2010 Lee J et al. 2014
umbelliferone (Apiaceae species) 	• Inhibited biofilm formation in *E. coli* O157:H7 (90% inhibition at 50 μg/mL • Reduced expression of biofilm-related genes (motility and adhesion) in *E. coli* O157:H7 at 50 μg/mL • Inhibited formation of *S. aureus* CECT 976 biofilm by 50% at 800 μg/mL	Lee J et al. 2014 Monte et al. 2014

Table 7.1 Continued

Active constituents (source plants)	Biological activities	Reference
Quinones		
2–methoxy–1,4–naphthoquinone (various species) 	• Inhibited violacein production in *C. violaceum* ATCC 12472 (21.8 mm at 20 μg/disc)	This work
emodin (various species) 	• Inhibited biofilm formation in *P. aeruginosa* PAO1 (75%) and *S. maltophilia* (43%) at 20 μM	Ding et al. 2011
purpurin (*Rubia tinctorum* L. – Rubiaceae) 	• Inhibited yeast-to-hypha transition in *C. albicans* SC5314 at 3 μg/mL • Inhibited formation of new and preformed *C. albicans* biofilms (50% at 5 μg/mL and 30% at 10 μg/mL, respectively) • Downregulated expression of hypha-specific genes in *C. albicans* at 5 to10 μg/mL	Tsang et al. 2012
Alkaloids		
berberine (Berberidaceae species and others) 	• Inhibited biofilm formation in *S. epidermidis* ATCC 35984 (50% at 30 μg/mL) and SE243 (50% at 45 μg/mL) • Inhibited biofilm formation in *K. pneumoniae* clinical isolates (MIC = 63.5 μg/mL)	Wang et al. 2009 Magesh et al. 2013

and quinones) each consisting of 9 or less compounds (see Appendix V). Eugenol, a simple phenolic present in many plants, has been shown to inhibit the production of QS-mediated violacein in *C. violaceum* and virulence factors in *P. aeruginosa* PAO1 by 32 to 56% at concentrations of 50 to 200 μM (Zhou et al. 2013). This compound was also effective against biofilms of *P. aeruginosa, L. monocytogenes,* and *Klebsiella pneumoniae* clinical isolates (Magesh et al. 2013; Upadhyay et al. 2013; Zhou et al. 2013). Other simple phenolics such as vanillin and gallic acid showed mixed effects depending on the organism and concentration tested. In a study by Ponnusamy et al. (2009), 250 μg/mL of vanillin inhibited QS in *C. violaceum* and biofilm formation in *Aeromonas hydrophila.* In another study, at 200 μg/mL vanillin enhanced AHL production in *E. coli* JDL271/pAL105 and biofilm formation in *P. aeruginosa* PAO1 and *Agrobacterium tumefaciens* C58 by at least 2-fold (Plyuta et al. 2013). Similarly, gallic acid at 200 μg/mL had no effect on *P. aeruginosa* PA14 biofilms in our bioassay but inhibited *P. aeruginosa* PAO1 biofilm formation by 30% (Plyuta et al. 2013) and enhanced *S. epidermidis* biofilms by 3-fold at a similar concentration (Morán et al. 2014). Stilbenes such as resveratrol and pterostilbene have been reported to interfere with the formation of both fungal and bacterial biofilms. In a study by Cho HS et al. (2013), 50 μg/mL of resveratrol inhibited *P. aeruginosa* PA14 and *E. coli* O157:H7 biofilms. In another study, Coenye et al. (2012) showed that resveratrol at 0.32% also inhibited biofilm formation in *Propionibacterium acnes*. At a higher concentration of 100 μg/mL, this compound was inhibitory to *S. aureus* biofilms but actually enhanced biofilm formation in *S. epidermidis* (Morán et al. 2014). For pterostilbene, Li DD et al. (2014) showed that treatment of 16 μg/mL inhibited new and mature biofilms in various *C. albicans* strains. In the same study, at a concentration of 4 μg/mL pterostilbene prevented hyphal formation in the same fungal strains. Transcriptomic analyses

showed that this compound altered the expression of genes involved in morphological transition, ergosterol biosynthesis, filamentation, and cell surface proteins. Furthermore, in a rat central venous catheter infection model, treatment of pterostilbene showed antibiofilm effects in a dose-dependent manner (Li DD et al. 2014).

Flavonoids are another type of phenolics that showed inhibitory activities in quorum sensing and biofilm formation. In a study by Vikram et al. (2010), quercetin was shown to inhibit bioluminescence in *Vibryo harveyi* strains by 75% at a concentration of 6.25 μg/mL. Lee et al. (2013) reported antibiofilm activities of this compound against *E. coli* O157:H7 and *V. harveyi* BB120 at the same concentration as well as inhibition of *S. aureus* biofilms at 1 μg/mL. Microarray analyses by the same authors showed that quercetin reduced the expression of genes involved in QS and virulence of *S. aureus.* Other flavonoids such as catechins from green tea (*Camellia sinensis* L.) are also showed similar effects. In a study by Matsunaga et al. (2010), (-)–gallocatechin, (-)–epigallocatechin, (-)–catechin gallate, (-)–epicatechin gallate, (-)–gallocatechin gallate, and (-)–epigallocatechin gallate all inhibited biofilm formation in *Eikenella corrodens* at 1 mM. (-)–catechin, a related compound, also inhibited violacein and virulence factors production in *C. violaceum* and *P. aeruginosa* PAO1, respectively (Vandeputte et al. 2010). However, (-)–epicatechin (another related compound) showed different activity depending on the tested organism. At 200 μg/mL, (-)–epicatechin enhanced biofilm formation in *P. aeruginosa* PAO1 and *A. tumefaciens* C58 (Plyuta et al. 2013). At higher concentrations of 1 mg/mL, this compound inhibited *E. coli* JM109 biofilms by 40% (Borges et al. 2014). In terms of QS, (-)–epicatechin inhibited violacein production in *C. violaceum* at 1 mg/L (Borges et al. 2014) but increased AHL production in *E. coli* DL271/pAL105 at concentrations of 40 to 200 μg/mL (Plyuta et al. 2013). (-)–epigallocatechin gallate also showed QS inhibitory activities against *E.*

coli MPT102 and *Pseudomonas putida* as well as swarming motility in *Burkholderia cepacia* at 40 μg/mL (Huber et al. 2003).

Another noteworthy phenolic in terms of QS and biofilm activities is curcumin. This compound has been well studied and showed various biological effects. In our bioassay, curcumin at 10 μg/mL had no effect against *P. aeruginosa* PA14 biofilms. In another study, Karaman et al. (2013) showed that this compound enhanced biofilm formation in *Staphylococcus aureus* at 16 μg/mL. The opposite effect was seen by Ruddrappa and Bais (2008) and Pattiyathanee et al. (2009) where curcumin inhibited AHL production and biofilm growth in *P. aeruginosa* PAO1 at 1 μg/mL and completely inhibited biofilm formation in *Helicobacter pylori* clinical isolates at 8 μg/mL, respectively. At higher concentrations from 25 to 625 μg/mL, this compound displayed antibiofilm activities against *Staphylococcus epidermidis, Vibrio* sp., *Escherichia coli, Pseudomonas aeruginosa* PAO1, *Proteus mirabilis, Serratia marcescens, Klebsiella pneumoniae, Enterococcus faecalis, Streptococcus mutans,* and *Candida albicans* (Hu et al. 2013; Magesh et al. 2013; Neelakantan et al. 2013; Packiavathy et al. 2013; Packiavathy et al. 2014; Shahzad et al. 2014; Sharma et al. 2014). In terms of QS, curcumin inhibited violacein production in *C. violaceum* as well as virulence factors production in *Vibrio* sp., *P. aeruginosa* PAO1, *S. marcescens* at concentrations ranging from 3 μg/mL to 100 μg/mL (Packiavathy et al. 2013; Packiavathy et al. 2014; Ruddrappa and Bais 2008).

Phenolic acids and their derivatives have also been reported to have anti-QS and antibiofilm effects. In our bioassay, ellagic acid at 36 μg/disc showed greater QS inhibition in *C. violaceum* when compared to the positive control *D. pulchra* extract. This activity is confirmed by Huber et al. (2003) where QS inhibition was observed in *E. coli* MT102 and *P. putida* at concentrations of 40 μg/mL and 30 μg/mL, respectively. This compound was not effect against

P. aeruginosa PA14 biofilms at 10 μg/mL in our bioassay. In another study, ellagic acid inhibited biofilm formation in various *S. dysgalactiae* strains by 70% at 4 μg/mL (Dürig et al. 2010). At higher concentrations of 15 to 40 μg/mL, ellagic acid was inhibitory against *B. cepacia*, *S. aureus, E. coli*, and *C. albicans* biofilms (Biakkiyaraj et al. 2013; Huber et al. 2003). Similarly, 1,2,3,4,6–penta–*O*–galloyl–β–D–glucopyranose, punicalagin, and hamamelitannin also exhibited inhibitory activities (Kiran et al. 2008; Li et al. 2013; Li G et al. 2014; Lin et al. 2011; Payne et al. 2013). At a concentration of ~4 μM, 1,2,3,4,6–penta–*O*–galloyl–β-D–glucopyranose inhibited biofilm formation in *S. aureus* by 60% (Lin et al. 2011; Payne et al. 2013). Punicalagin and hamamelitannin are tannins that interfered with QS in Gram-negative and Gram-positive bacteria, respectively. In a study by Li G et al. (2014), punicalagin (an ellagitannin) inhibited violacein production in *C. violaceum* as well as swimming and swarming motility in *Salmonella typhimurium* SL1344 at 15.6 μg/mL. Further analyses by these authors showed a downregulation of QS and motility-related genes in *S. typhimurium* at the same concentration. Similarly, hamamelitannin (a gallotannin) was shown to reduce cell attachment of methicillin-resistant *S. aureus in vitro* at 4 μg/mL (Kran et al. 2008). At a higher concentration of 50 μg/mL, δ–hemolysin production and QS regulator RNAIII in *S. aureus* were inhibited by hamamelitannin. Furthermore, this decrease in virulence was confirmed *in vivo* with a graft infection model using rats. At pre-treatment of 30 mg/mL, implanted grafts showed no detectable MRSA and MRSE loads after 7 days (Kiran et al. 2008). Tannic acid was active against both Gram-negative at Gram-positive bacteria. At 100 μg/mL, Huber et al. (2003) showed an enhancement of biofilm formation in *P. aeruginosa* PA14; however, in our bioassay a 72% inhibition was observed at 200 μg/mL against PA14 biofilms. In other studies, antibiofilm activities were shown at lower concentrations from 3.4 to 20 μg/mL against *S. aureus* (Cho HS et al. 2013; Payne et al. 2013).

In terms of QS, inhibition was observed in *P. putida* at 30 μg/mL (Huber et al. 2003) and *S. aureus* at 20 μg/mL (Payne et al. 2013).

Coumarins, quinones, and alkaloids are other chemical classes with QS and biofilm inhibitory activities. Aesculetin (a coumarin) was reported to inhibit QS in *C. violaceum, P. aeruginosa,* and *E. coli* JB523 by 30 to 60% at 100 μM (Brackman et al. 2009; Dürig et al. 2010). In another study, Lee et al. (2014) showed that aesculetin was effective against *S. aureus* biofilms (>50% inhibition at 128 μg/mL) and reduced the expression of biofilm-related genes (at 50 μg/mL) in *E. coli* O157:H7. Furthermore, aesculetin decreased Shiga-like toxin production in *E. coli* O157:H7 and reduced virulence in a *C. elegans* infection model. In the same study, another coumarin – umbelliferone also inhibited biofilm formation and expression of motility and adhesion genes in *E. coli* O157:H7 and expression. The antibiofilm activity of umbelliferone was also confirmed in *S. aureus* by Monte et (2014). In a study by Ding et al. (2011), quinones such as chrysophanol, emodin, and shikonin all showed inhibitory activities against biofilms of *P. aeruginosa* PAO1 and *S. maltophilia*. Purpurin, another quinone, was shown to repress yeast-to-hypha transition in *C. albicans* SC5314 at 3 μg/mL (Tsang et al. 2011). At higher concentrations of 5 to 10 μg/mL, this compound was effective against new and existing *C. albicans* biofilms. Further analyses showed that purpurin downregulated the expression of hypha-specific genes (Tsang et al. 2011). In regard to alkaloids, a few have been reported to have inhibitory activities against bacteria biofilm. In particular, Wang et al. (2009) and Magesh et al. (2013) showed that berberine inhibited the biofilm formation in *S. eipidermidis* and *K. pneumoniae* at concentrations of 30 to 63.5 μg/mL. Similarly, chelerythrine and sanguinarine was also effective against Gram-positive biofilms of *S. aureus* and *S. epidermidis* at micromolar concentrations (Artini et al. 2012). Furthermore, resperine, an alkaloid from *Rauwolfia* sp.

(Apocynaceae), showed inhibitory activity against *K. pneumoniae* biofilms with a minimum inhibitory concentration of 15.6 μg/mL (Magesh et al. 2013).

7.3 Future studies

The results presented in this thesis showed that neotropical plant species as well as traditional Q'eqchi' Maya medicines contain phytochemicals that interfere with bacterial quorum sensing and biofilm formation as well as fungal growth. Since only Gram-negative bacteria were used, it would be worthwhile to compare the effects of these plants on QS and biofilm formation in Gram-positive bacteria such as *Staphylococcus aureus* and *Staphylococcus epidermidis.* Also, of the 206 extracts studied, the majority has not been examined in terms of their effects on fungal biofilms. Thus, it would be interesting to assess these plants for their potential to inhibit biofilm formation and related processes such as yeast-to-hypha transition in a model fungus like *Candida albicans.* This would provide more insights on the antimicrobial properties of these plants. Moreover, plant extracts should be tested with ecologically relevant species such as *Pseudomonas syringae*, *Pseudomonas putida, Bacillus subtilis* as these are typically found in the phyllosphere and the rhizosphere. Similarly, since most biofilms in nature are dual and multi-species, assessment of the top plants in these models would contribute to a better understanding of the biological activities of these extracts. Furthermore, more focused efforts should be placed on bioactive species such as *Blakea cuneata* and *Ruptiliocarpon caracolito* to identify the active principles. Preliminary bioassay-guided fractionation showed that the biofilm inhibitory activities of these plants belonged in the more polar fractions. Phytochemical analyses of the active fractions using HPLC and MS indicated the presence of gallic acid and ellagic acid derivatives. Further work is required to isolate and identify these bioactive compounds. In regard to *Cestrum schlechtendahlii*, future experiments using an over-expression library of

Saccharomyces cerevisiae and secondary assays would confirm the targets and provide further insights into the anti-fungal mechanisms of spirastanol saponins.

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Appendix I

Preliminary results from the bioassay-guided isolation of anti-quorum sensing and biofilm inhibitory principles from the Maya medicinal plant *Blakea cuneata* Standl.

Materials and Methods

General experimental procedures

NMR spectra were recorded on a Bruker Avance 400 spectrometer in CD_3OD at 400 MHz (^1H) and 100 MHz (^{13}C) using tetramethylsilane (TMS) as an internal standard. High Resolution Electrospray Ionization Mass Spectrometry (HRESIMS) was done using a Waters XEVO G2 UPLC–QTOF–ESI system. Electrospray Ionization Mass Spectrometry (ESI–MS) was done using a Shimadzu LCMS 2020 Series system. Open column chromatography was carried out on silica gel 60 (70-230 mesh, Merck) and Sephadex LH-20. TLC analyses were performed on silica gel 60 F254 plates (Merck) and visualization of the plates was carried out using a ceric sulfate (10%) solution in H_2SO_4.

Plant material

Leaves of *Blakea cuneata* were collected in 2009 and 2011 in Jalacte, Belize under ethical approval (permits #H11-11-09, #H03-070-01). Plant material was preserved in 70% ethanol immediately after collection. Plant identification was performed by M. Otárola-Rojas, P. Sanchez-Vindas, and L. Poveda from the Universidad Nacional of Costa Rica. Voucher specimens (JVR8012) were deposited at the University of Ottawa Herbarium, the Herbario Juvenal Valerio Rodriguez, and the Belize Forest Department.

Plant extraction

Leaves (300 g) from the 2009 collection were ground using a blender (Waring commercial LR 8992) and extracted with 80% EtOH in a 1:10 biomass to solvent (w/v) ratio. The plant material/ethanol mixture was shaken at room temperature overnight at 200 rpm (shaker: New Brunswick Scientific) then filtered using vacuum filtration. A second extraction was performed with the plant residue, using a 1:5 w/v ratio and the mixture was again shaken and filtered. The two extracts were combined, dried *in vacuo* using a rotary evaporator (Yamato RE 500) at 45ºC, lyophilized to remove any residual water using a freeze-dryer (EC Super Modulyo, ~ -55 ℃, 10^{-2} mbar) to yield 29 g of crude extract and then stored at -20ºC in the dark until needed. In the 2011 collection, 1.75 kg of leaves was extracted using the same method to yield 200 g of crude extract.

Bacteria strains and culture

Chromobacterium violaceum ATCC 12472 was used in the quorum sensing bioassay and *Pseudomonas aeruginosa* PA14 in the biofilm bioassay. *C. violaceum* was purchased from ATCC and *P. aeruginosa* PA14 was obtained from T. Mah (University of Ottawa). These strains were maintained as liquid culture with shaking (200 rpm) at 30ºC (incubator: New Brunswick Scientific Series 25, Edison, NJ, USA) in nutrient broth (BD, Sparks, MD, USA) and LB broth (Fisher Scientific, Fairlawn, NJ, USA), respectively.

Quorum sensing (QS) bioassay

A modified disc diffusion assay described by Adonizio et al. (2006) was used to determine whether the plant extracts can interfere with the QS of *C. violaceum. C. violaceum* produces a purple pigment, violacein, which is under QS control. The inhibition of violacein

production will indicate the disruption of QS. Briefly, sterile paper disks (Oxoid, Basingstoke, Hants, UK) loaded each with 1 mg of extract were placed onto TGY agar plates (BD, Sparks, MD, USA) inoculated with 100 µL of overnight cultures then incubated without agitation for 24 hours at 30°C (incubator: Precision Automatic CO_2 Incubator). QS inhibition was indicated by a colourless opaque halo around the disc and growth inhibition by a clear halo. Plates were examined under a dissecting microscope to confirm whether the extract has anti-QS and/or antibacterial activity. The controls used were: 70% ethanol as vehicle control and *N*–decanoyl–L–homoserine lactone (C10–HSL) (400 µg/disc, ≥96%, Cayman Chemical, Ann Arbor, MI, USA) as positive control. Extract of *Delisea pulchra* (Greville) Montagne (1 mg/disc) was also included for comparative purposes as this alga contains known QS inhibitors, the halogenated furanones (Givskov et al. 1996; Raffa et al. 2005; Shiner et al. 2005). Each sample was tested in triplicate.

Biofilm bioassay

A spectrophotometric assay was adapted from Ren et al. (2005) to assess the biofilm inhibitory activity of the plant extracts. Extracts were prepared in 50% methanol and tested at a final concentration of 400 µg/mL. Overnight culture of *P. aeruginosa* was diluted 1:50 in M63 medium broth (Amresco, Solon, OH) supplemented with 0.4% arginine and 1mM $MgSO_4$. This inoculum was grown in a 24-well flat bottom microtiter plate (Costar 3526) without agitation for 24 hours at 37°C to allow biofilm formation (incubator: Precision Automatic CO_2 Incubator). The plate was then decanted and washed with distilled water three times to eliminate non-adherent cells. The remaining biofilm was stained with 0.1% crystal violet (Fisher Scientific, Fairlawn, NJ) for 15 minutes. The excess dye was decanted, the plate was washed three times with distilled water, and then air dried overnight. The biofilm was quantified by dissolving the

dye in ethanol and reading the absorbance at 570 nm (spectrophotometer: SpectraMax M5, Molecular Devices, Sunnyvale, CA, USA). The controls used were: vehicle control with 50% methanol, negative control with M63 medium broth, and positive control with allicin (1 μL/mL or 1.08 mg/mL, 97%, AK Scientific Inc., Mountain View, CA, USA). Biofilm formation was expressed relative to the vehicle control. Samples were tested in triplicate.

Bioassay-guided fractionation using column chromatography and preparative HPLC

The crude leaf extract from the 2009 collection (10 g) was fractionated using silica gel open glass column with elution gradients hexane–EtOAc (100:0 → 0:100) and EtOAc–MeOH (100:0 → 50:50). The collected fractions were analyzed using TLC and pooled into 21 primary fractions (BC–I–A to BC–I–U). The yields and elution conditions are listed in Table 1. All fractions were assessed for QS and biofilm inhibitory activities at 0.5 mg/disc and 0.4 mg/mL, respectively. Fraction BC–I–U (eluted with 50:50 EtOAc–MeOH) was chosen for further isolation work using preparative scale HPLC. 575 mg of BC–I–U was fractionated using an Agilent 1200 Series preparative scale HPLC system (Agilent Technologies, Montreal, QC, Canada) consisting of a binary pump (flow rate range 5–100 ml/min), an autosampler with a 2 mL loop, a diode array detector with a flow cell (path length 3 mm and maximum pressure limit 120 bars) and a fraction collector (40 mL collection tubes). A reverse phase C18 Gemini Axia column 250 mm × 21.2 mm internal diameter, particle size 10 microns (Phenomenex Inc., Torrance, CA, USA) was used at room temperature with a flow rate of 31.5 mL/min. Optimized separation was achieved with the following method: initial conditions of 5% water (H_2O) and 95% acetonitrile (ACN) with an increasing gradient to 100% ACN in 25 min; the column was flushed with 100% ACN for 5 min, allowed to equilibrate for another 5 min and then set back to

the initial conditions. DAD was set to monitor wavelengths 254 nm, 280 nm and 330 nm. After pooling, 5 secondary fractions were collected (BC–I–U–1 to BC–I–U–5) with yields ranging from 1 to 99 mg (Table 2). Due to low yields, only fraction BC–I–U–1 was tested in the biofilm bioassay at a concentration of 0.2 mg/mL. HPLC–DAD analysis showed that BC–I–U–1 was still complex and thus further fractionation was required.

To increase the yields, another silica gel column was done with 19 g of crude extract from the 2009 collection using the same elution gradients listed above. The collected fractions were analyzed using TLC and pooled into 16 primary fractions (BC–II–A to BC–II–Q). The yields and elution conditions are listed in Table 3. All fractions were assessed for biofilm inhibitory activities at 0.2 mg/mL. Fraction BC–II–N (1.4 g) was further chromatographed with a Sephadex column using MeOH as the mobile phase. All fractions were analyzed using TLC and pooled to yield 21 secondary fractions (BC–II–N–1 to BC–II–N–21). The yields of these fractions are listed in Table 4. Secondary fractions with sufficient yields were tested for biofilm inhibitory activity at 0.2 mg/mL. Compound **1** was obtained from BC–II–N–9 (Table 5) with a yield of 7 mg. Fraction BC–II–Q was selected for further isolation work using preparative scale HPLC. Using the same C18 column and a 9 min isocratic method of 5% ACN, two secondary fractions were collected: BC–II–Q–1 (2.171 g) and BC–II–Q–2 (390 mg). Both fractions were tested in the QS and biofilm bioassays at 1 mg/disc and 0.2 mg/mL, respectively. HPLC–DAD analyses showed both secondary fractions were complex and the low yields prevented further fractionation work.

The crude leaf extract from the 2011 collection (108 g) was fractionated using silica gel open glass column with elution gradients hexane–EtOAc (100:0 → 0:100) and EtOAc–MeOH (100:0 → 50:50). The collected fractions were analyzed using TLC and pooled into 11 primary

fractions (BC–III–A to BC–III–K). The yields and elution conditions are listed in Table 6. All fractions were assessed for biofilm inhibitory activity at 0.2 mg/mL, respectively. Fractions BC–III–G and BC–III–H (eluted with 75:25 EtOAc–MeOH) were combined and rechromatographed with another silica gel column to give 84 secondary fractions with yields ranging from 1 to 15 mg. Secondary fractions with sufficient yields were tested for biofilm inhibition. Fraction BC–III–H (3 g) was further fractionated using preparative scale HPLC using the same C18 column at room temperature and a flow rate of 25 mL/min. Optimized separation was achieved with the following method: initial conditions 94% H_2O + 0.1% TFA (A) and 6% ACN + 0.1% TFA with an increasing gradient to 100% B in 50 min; the column was flushed with 100% ACN for 5 min, allowed to equilibrate for another 5 min and then returned to initial conditions. Monitoring wavelengths were set to 210, 254, 280, 330, and 400 nm. Time–based collection was done from 0 to 24 min and peak–based collection from 24 to 45 min. Eleven secondary fractions were collected (Table 7); these fractions and the time–based collection were analyzed using UPLC–QTOF–MS/MS.

HPLC–DAD analysis

Chromatographic analyses of the crude extract and fractions were performed on an Agilent 1100 series HPLC system consisted of a quaternary pump, a degasser, an auto-sampler with 100 μL loop, a column thermostat and a diode array detector (DAD). The identification of the phenolics was corroborated by comparing the retention time and maximum UV absorption values with authentic commercial standards (Sigma-Aldrich, St. Louis, MI, USA). The system The analyses were performed using a Luna Phenyl–Hexyl 100 mm × 2.0 mm (3μm particle size) with column temperature set at 45 ℃ and a flow rate of 0.4 ml/min. The mobile phase A is water containing 0.1% formic acid and B is acetonitrile. Optimized separation was achieved with the

following method: initial conditions of 95% A and 5% B with an increasing gradient to 100% B in 20 min; the column was flushed with 100% A for 5 min and then set back to the initial conditions. DAD was set to monitor wavelengths 210, 254 nm, 280 nm and 330 nm.

UPLC–QTOF–MS/MS analysis

UPLC conditions: Acquity BEH C18 1.7um 2.1 × 100mm column connected with a VanGuard Pre-column 2.1 × 5mm. Mobile phases are A: water + 0.1% formic acid and B: acetonitrile + 0.1% formic acid (Fisher Optima LC–MS); flow rate is 0.5 mL/min, column temperature of 50 ℃, and sample temperature 10 ℃. Mobile phase composition is as followed: 0–1 min 5% A isocratic, 1–6 min linear gradient 5-50% B, 6–8 min 50–95%B, 8.01–10 min 5% A isocratic (total run time 10 min). Sample injection conditions: 1uL injection followed by a strong wash 200uL (90% acetonitrile +1 0% water) and weak wash 600uL (10% acetonitrile + 90% water). QTOF analysis conditions: MassLynx software, MSe ESI+ mode, lock mass Leucine Enkephalin 12C 556.2615, source temperature 120 ℃, desolvation 304, temperature 400 ℃, Cone gas (N2) flow 50 L/hr, desolvation gas (N2) flow 1195 L/hr. MSe conditions, mass range 100–1500 Da, F1 CE, 6V, F2 CER 10–30V, Cone voltage 20V, Scan time 1 sec. Calibration, 50–1000 Da sodium formate. Masses and elemental composition were verified using MetLIN (http://metlin.scripps.edu/metabo_search_alt2.php).

Results

QS and biofilm inhibitory activities

The leaf extract of *Blakea cuneata* Standl (Oxlaho chajom in Q'eqchi') showed greater QS and biofilm inhibitory activities than the positive controls (Figures 1 and 2). The IC_{50} of crude extract for biofilm inhibition was calculated to be 108.5 μg/mL (Figure 3). Of the 21 primary fractions, the polar ones BC–I–O to BC–I–U were active with QS inhibition ranging from 20.3 ± 0.4 to 26.5 ± 0.4 mm at 500 μg/disc (Figure 1). These same fractions were also active in the biofilm bioassay with activity ranging from 24.4 ± 4.4% to 58.6 ± 6.5% control at 400 μg/mL (Figure 2). Fraction BC–I–U showed biofilm inhibition with an IC_{50} of 22.5 μg/mL (Figure 4). At 200 μg/mL, secondary fraction BC–I–U–1 inhibited biofilm growth by 55.0 ± 5.6% (Figure 2). Similarly, the polar primary fractions BC–II–L to BC–II–Q inhibited biofilm growth with activities ranging from 19.1 ± 0.1 to 43.9 ± 2.1% control at 200 μg/mL (Figure 5). Of the two secondary fractions, BC–II–Q–2 was more active with QS inhibition of 21.0 ± 0.4 mm at 1 mg/disc compared to 12.7 ± 0.7 mm for BC–II–Q–1. For biofilm inhibition, BC–II–Q–2 was also more active with 25.7 ± 4.7% control at a tested concentration of 200 μg/mL compared to 60.0 ± 4.4% for BC–II–Q–1 (Figure 6).

Table 1 Elution conditions and yields of BC–I primary fractions from silica gel column.

Fraction	Elution solvent	Yield (mg)
BC–I–A	85% hexane – 15% EtOAc	160
BC–I–B	85% hexane – 15% EtOAc	60
BC–I–C	80% hexane – 20% EtOAc	20
BC–I–D	80% hexane – 20% EtOAc	70
BC–I–E	80% hexane – 20% EtOAc	50
BC–I–F	80% hexane – 20% EtOAc	8
BC–I–G	50% hexane – 50% EtOAc	7
BC–I–H	50% hexane – 50% EtOAc	10
BC–I–I	50% hexane – 50% EtOAc	10
BC–I–J	50% hexane – 50% EtOAc	10
BC–I–K	30% hexane – 70% EtOAc	50
BC–I–L	90% EtOAc – 10% MeOH	8
BC–I–M	90% EtOAc – 10% MeOH	50
BC–I–N	90% EtOAc – 10% MeOH	50
BC–I–O	80% EtOAc – 20% MeOH	320
BC–I–P	80% EtOAc – 20% MeOH	260
BC–I–Q	75% EtOAc – 25% MeOH	260
BC–I–R	75% EtOAc – 25% MeOH	380
BC–I–S	75% EtOAc – 25% MeOH	1210
BC–I–T	75% EtOAc – 25% MeOH	530
BC–I–U	50% EtOAc – 50% MeOH	1610

Table 2 Retention times and yields of BC–I–U secondary fractions from preparative scale HPLC.

Fraction	Retention time (min)	Yield (mg)
BC–I–U–1	1.996	99
BC–I–U–2	2.587	4
BC–I–U–3	12.804	1
BC–I–U–4	19.390	1
BC–I–U–5	22.924	2

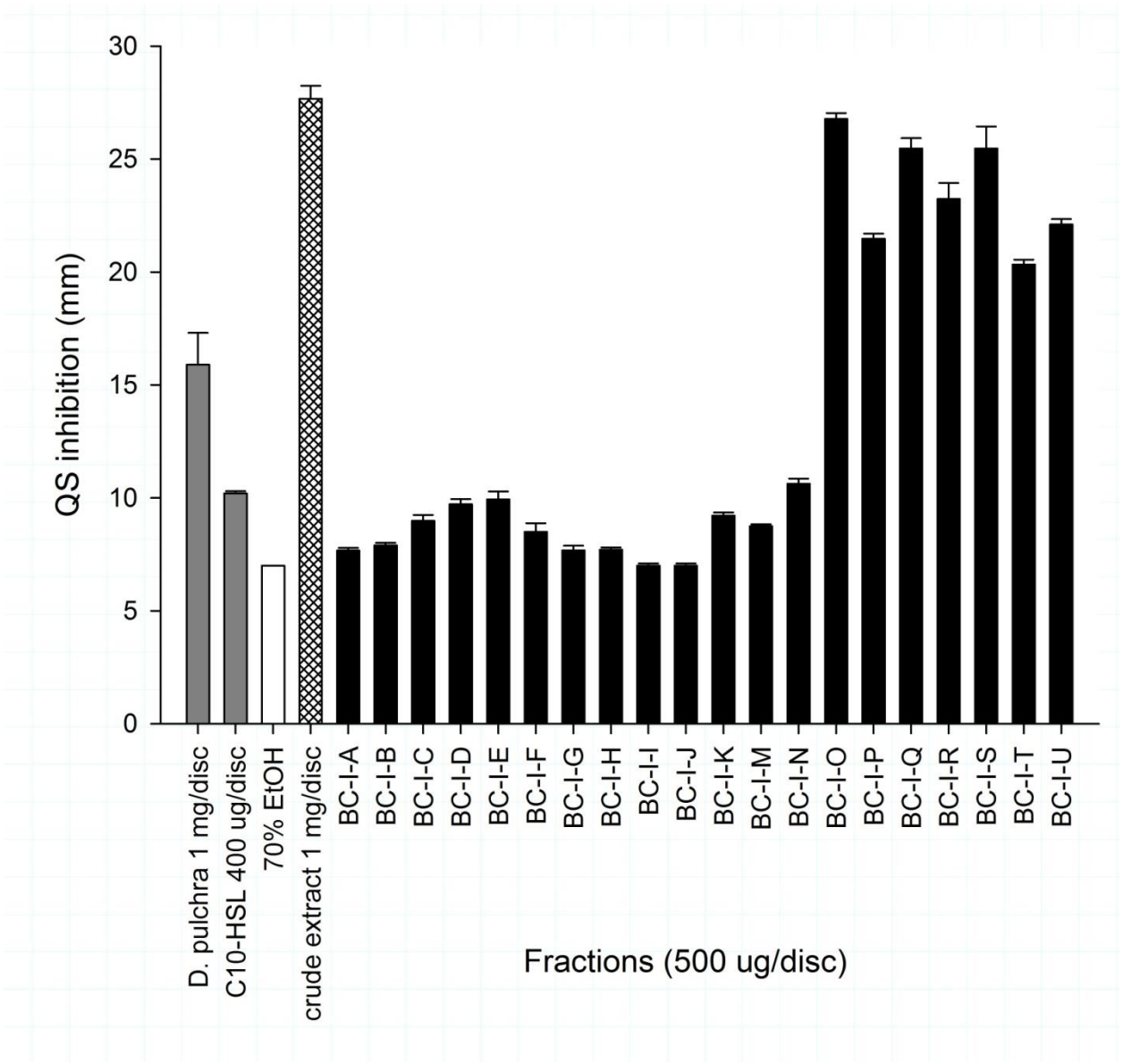


Figure 1 Average inhibition ± S.E.M. of quorum sensing (QS) in *Chromobacterium violaceum* ATCC 12472 by *Blakea cuneata* Standl. crude extract (1 mg/disc) and primary fractions (500 µg/disc) compared to the positive controls C10–HSL (400 µg/disc) and *Delisea pulchra* (Greville) Montagne extract (1 mg/disc). N = 3, disc diameter = 7 mm.

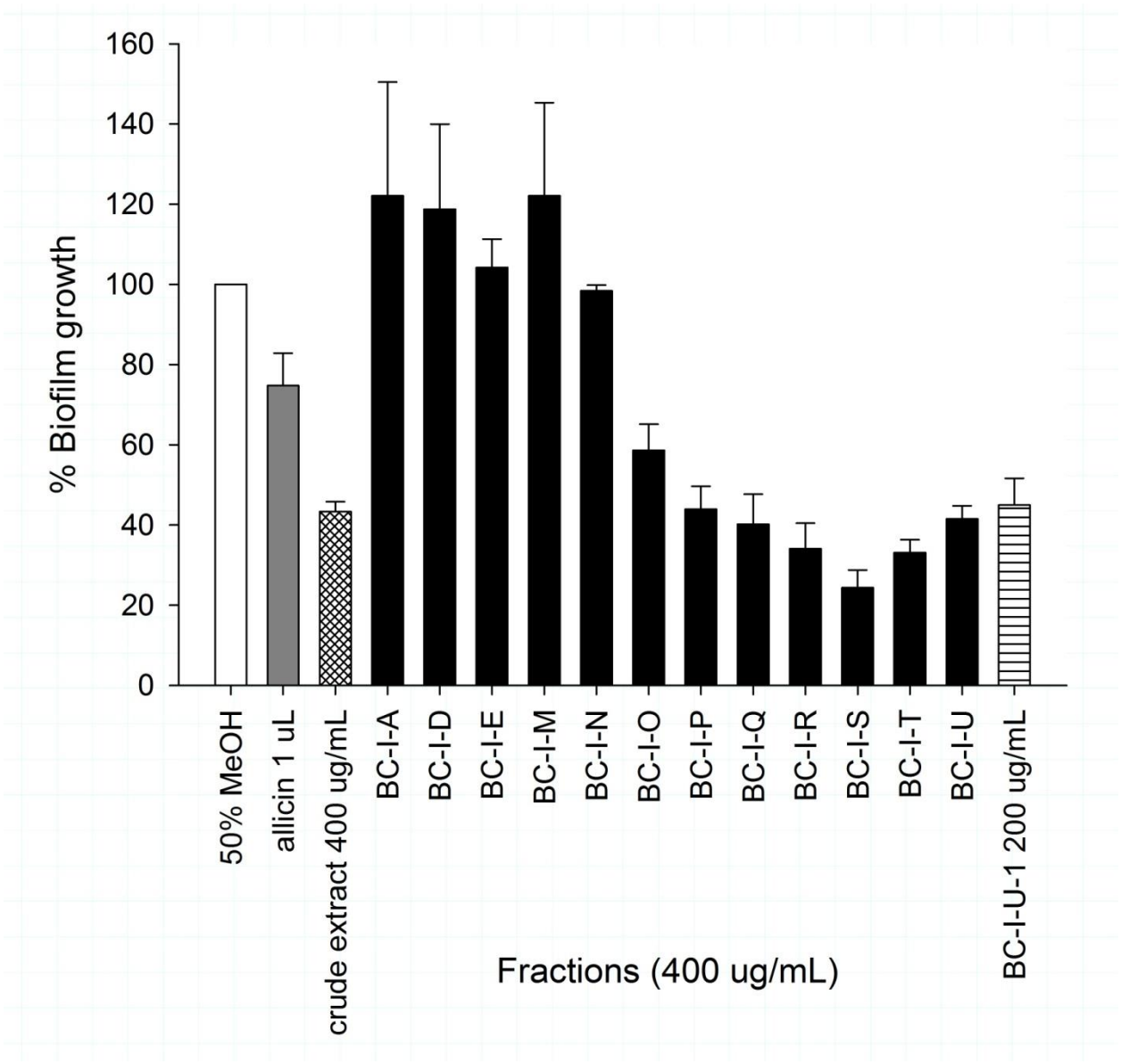


Figure 2 Average biofilm growth ± S.E.M. of *Pseudomonas aeruginosa* PA14 relative to vehicle control (50% MeOH) in the presence of *Blakea cuneata* Standl. crude extract (400 µg/mL) and BC–I primary (400 µg/mL) and secondary (200 µg/mL) fractions compared to the positive control allicin (1 µL/mL or 1.08 mg/mL). N = 3.

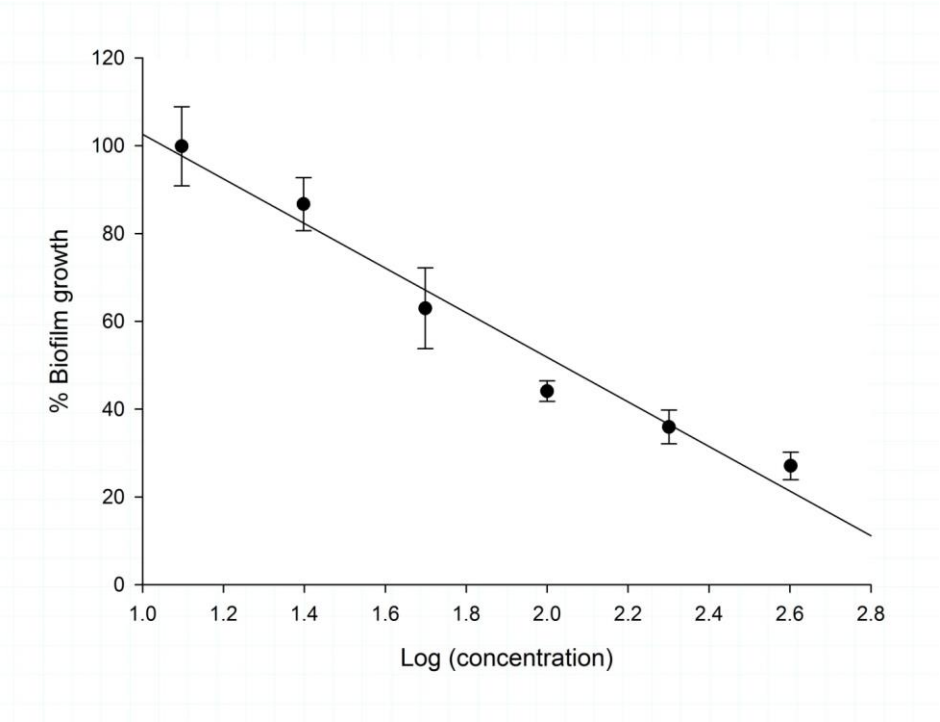


Figure 3 Inhibition (± S.E.M.) of biofilm formation in *Pseudomonas aeruginosa* PA14 at varying concentrations of *Blakea cuneata* Standl. crude extract (N = 3).

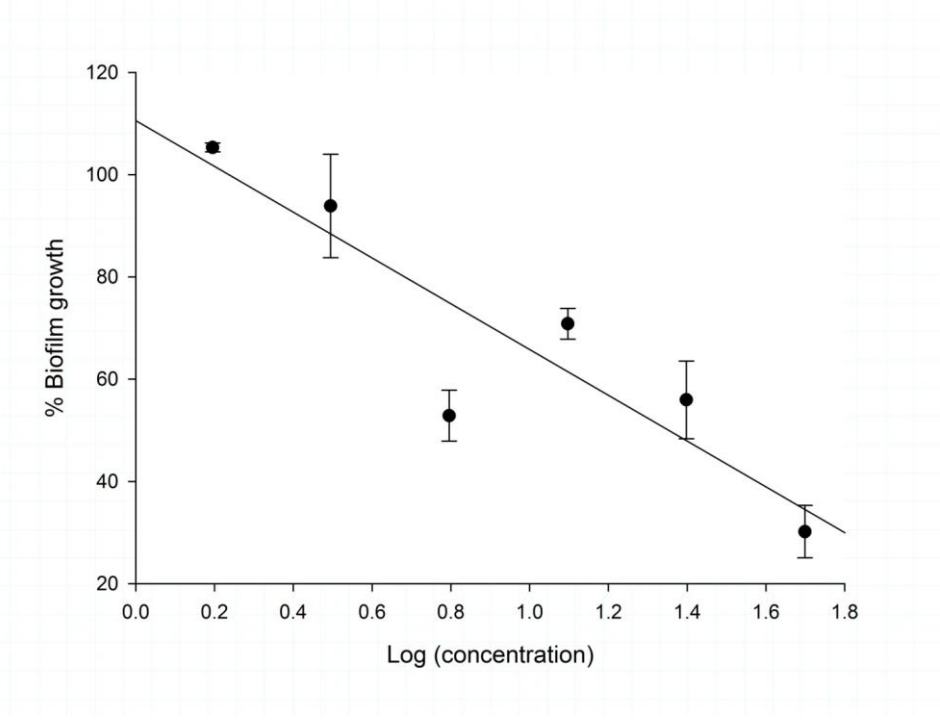


Figure 4 Inhibition (± S.E.M.) of biofilm formation in *Pseudomonas aeruginosa* PA14 at varying concentrations of *Blakea cuneata* Standl. primary fraction BC–I–U (N = 3).

Table 3 Elution conditions and yields of BC–II primary fractions from silica gel column.

Fraction	Elution solvent	Yield (mg)
BC–II–A	85% hexane – 15% EtOAc	513
BC–II–B	85% hexane – 15% EtOAc	112
BC–II–C	80% hexane – 20% EtOAc	223
BC–II–D	80% hexane – 20% EtOAc	55
BC–II–E	80% hexane – 20% EtOAc	24
BC–II–F	80% hexane – 20% EtOAc	77
BC–II–G	50% hexane – 50% EtOAc	137
BC–II–H	50% hexane – 50% EtOAc	62
BC–II–I	30% hexane – 70% EtOAc	161
BC–II–K	30% hexane – 70% EtOAc	55
BC–II–L	80% EtOAc – 20% MeOH	2067
BC–II–M	80% EtOAc – 20% MeOH	316
BC–II–N	80% EtOAc – 20% MeOH	1672
BC–II–O	75% EtOAc – 25% MeOH	1480
BC–II–P	75% EtOAc – 25% MeOH	2120
BC–II–Q	75% EtOAc – 25% MeOH	6423

Table 4 Yields of BC–II–N secondary fractions from Sephadex column.

Fraction	Yield (mg)
BC–II–N–1	2
BC–II–N–2	4
BC–II–N–3	2
BC–II–N–4	28
BC–II–N–5	170
BC–II–N–6	185
BC–II–N–7	26
BC–II–N–8	42
BC–II–N–9	31
BC–II–N–10	68
BC–II–N–11	2
BC–II–N–12	3
BC–II–N–13	9
BC–II–N–14	4
BC–II–N–15	17
BC–II–N–16	8
BC–II–N–17	61
BC–II–N–18	48
BC–II–N–19	6
BC–II–N–20	219
BC–II–N–21	394
Compound **1**	7

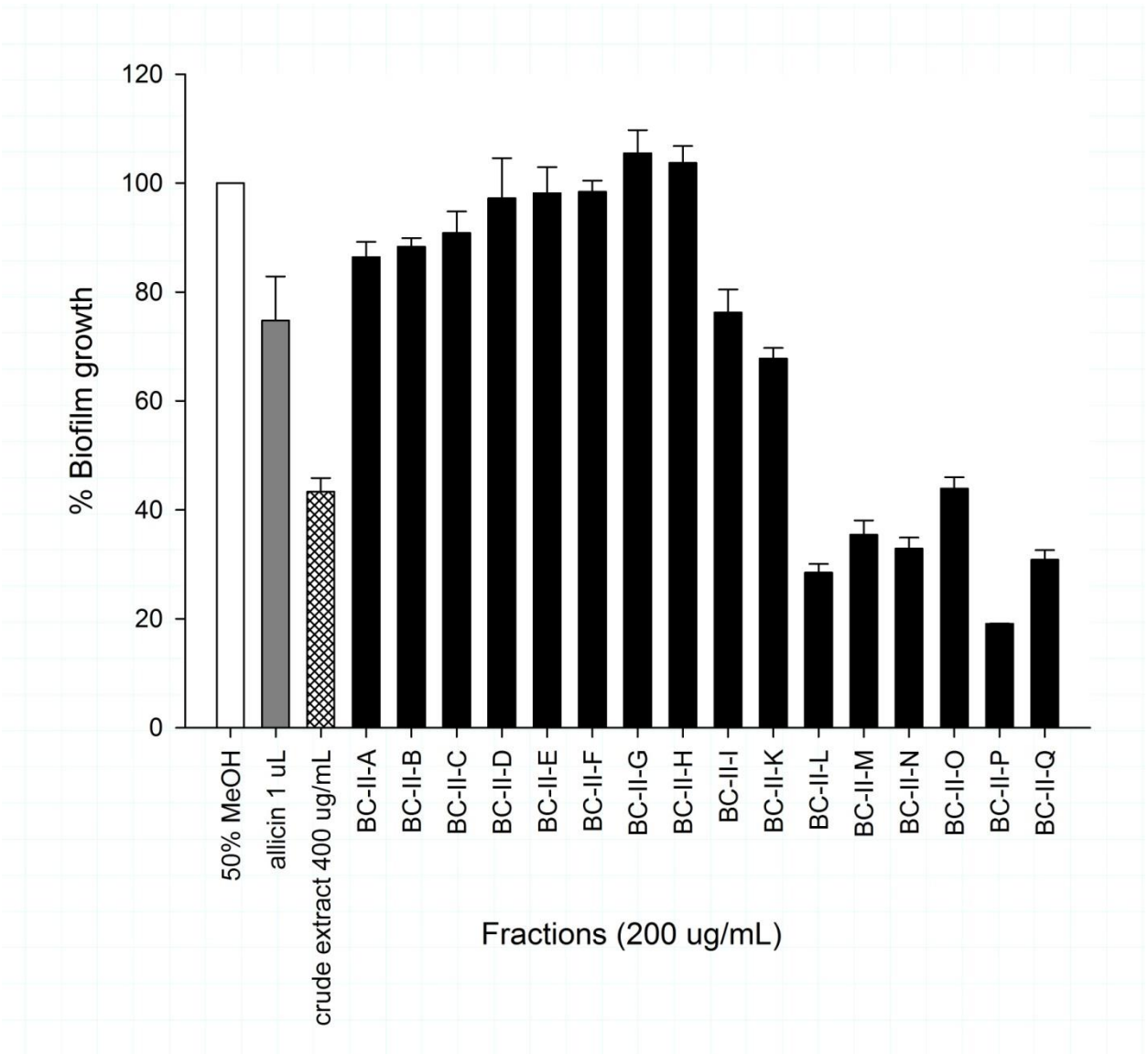


Figure 5 Average biofilm growth ± S.E.M. of *Pseudomonas aeruginosa* PA14 relative to vehicle control (50% MeOH) in the presence of *Blakea cuneata* Standl. crude extract (400 μg/mL) and BC–II primary fractions (200 μg/mL) compared to the positive control allicin (1 μL/mL or 1.08 mg/mL). N = 3.

Compound **1** isolated from secondary fraction BC–II–N–9 was confirmed by NMR to be homoplantaginin or hispidulin–7–*O*–glucoside (Figure 7 and Table 5); this C–linked glycoside is less common than other phenolics and could be a biomarker for *B. cuneata.* Spectroscopic data for this compound is similar to those reported in the literature (Jerga et al. 1990; Lu et al. 2011). At the highest tested concentration of 150 μg/mL, compound **1** was inactive in the biofilm bioassay (Figure 6). Other secondary fractions from BC–II–N showed biofilm inhibitory activities ranging from 41.2 ± 6.1 to 74.0 ± 11.4% control (Figure 6). The biological activities of the polar fractions in this plant was also confirmed in primary fractions BC–III–G to BC–III–J with 38.4 ± 4.6 to 54.1 ± 4.7% control (Figure 8). Polar secondary fractions from BC–III–G+H showed biofilm inhibition ranging from 51.1 ± 4.7 to 71.9 ± 0.5% control at a tested concentration of 100 μg/mL (Figure 8). Due to the low yields, only sub–fraction BC–III–H–1 (resulting from the time collection of 0–24 min from preparative scale HPLC) was tested for biofilm inhibition. This fraction was active with 53.8 ± 2.0% control at 200 ug/mL (Figure 8).

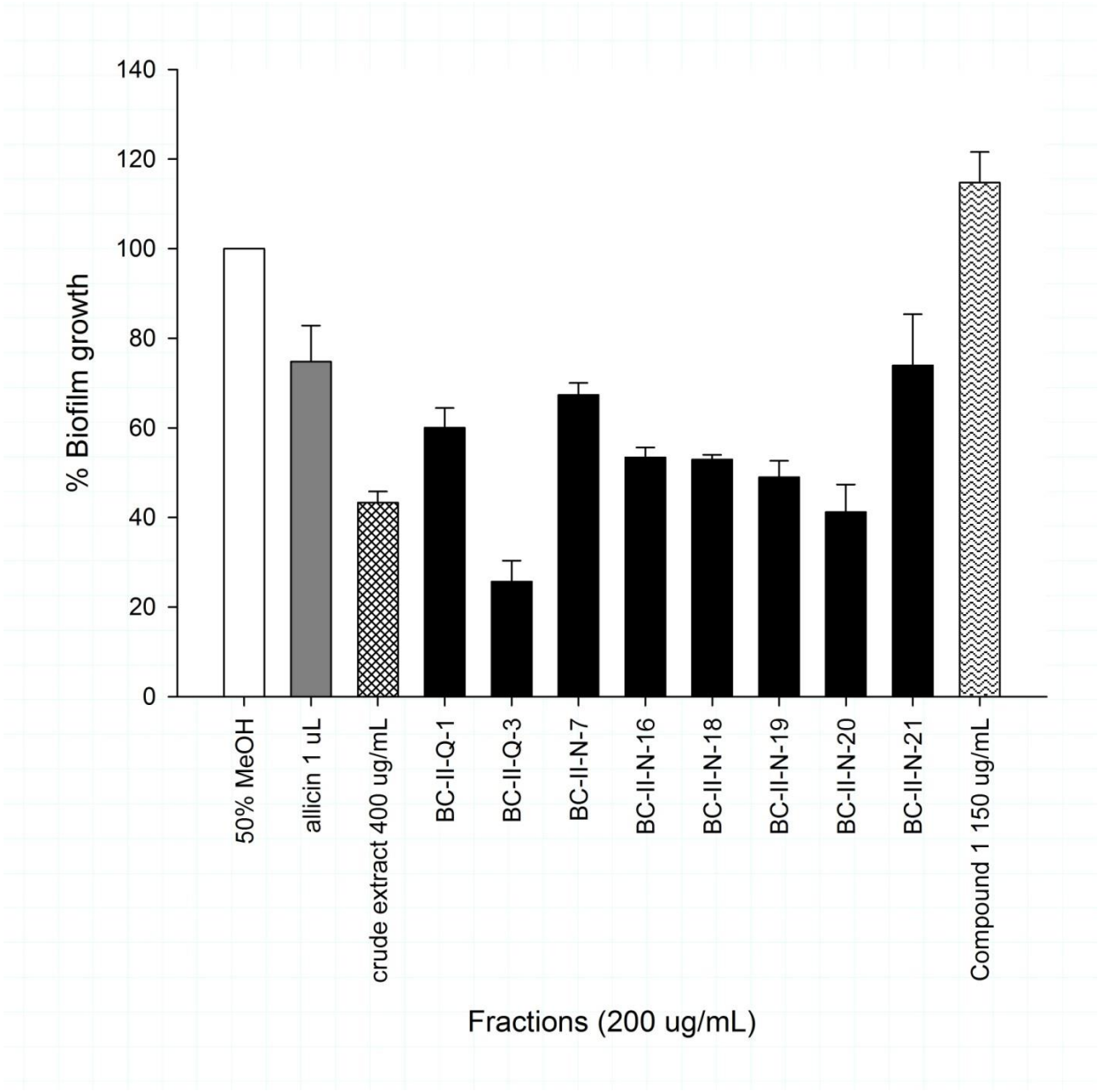


Figure 6 Average biofilm growth ± S.E.M. of *Pseudomonas aeruginosa* PA14 relative to vehicle control (50% MeOH) in the presence of *Blakea cuneata* Standl. crude extract (400 μg/mL), BC–II secondary fractions (200 μg/mL), and compound **1** (150 μg/mL) compared to the positive control allicin (1 μL/mL or 1.08 mg/mL). N = 3.

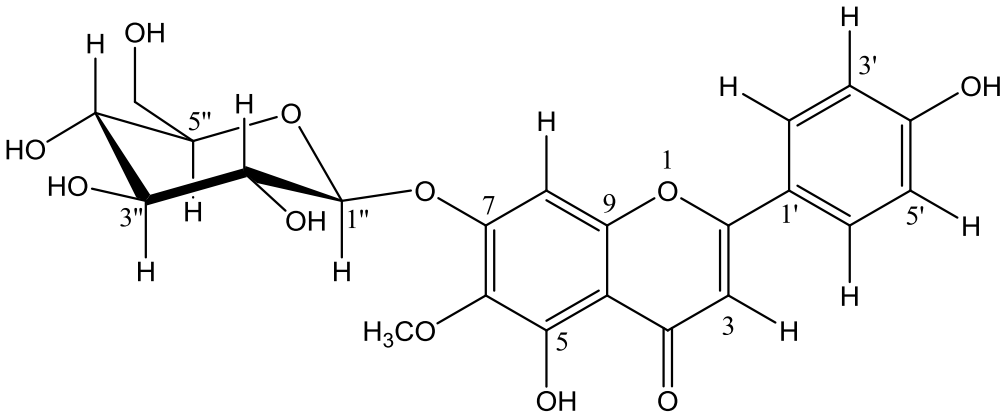


Figure 7 Chemical structure of compound **1**.

Table 5 1D and 2D NMR spectroscopic data for compound **1**.

# C	Cpos	δ_C	DEPT	δ_H	HMBC
1	*CH*$_3$O-	61.5	CH_3	3.88, s, 3H	
2	6''	62.6	CH_2	Ha, 3.96, d, *J*=2.2 Hz Hb, 3.72, dd, *J* = 6.0, 12.0 Hz	
3	4''	71.3	CH	3.42, t, *J* = 9.0 Hz	
4	2''	74.8	CH	3.55, m	H-3''
5	3''	78.0	CH	3.55, m	H-4',H-2''
6	5''	78.5	CH	3.53,t, *J* = 8.8 Hz	H-6b''
7	8	95.8	CH	6.94, s	H-2''
8	1''	102.0	CH	5.12,d, *J* = 7.2 Hz	H-2''
9	3	103.7	CH	6.64, s	H-8
10	10	107.5	C		H-8, H-3
11	3', 5'	117.1	2-CH	6.91, d, *J* = 8.8 Hz	H-3',H-5'
12	1'	123.0	C		H-3',H-5'
13	2', 6'	129.7	2-CH	7.87, d, *J* = 8.8 Hz	H-2',H-6'
14	6	134.2	C		H-8, O-CH_3
15	5	154.1	C		
16	7	154.3	C		
17	9	157.9	C		H-1'', H-8
18	4'	163.1	C		H-2', H-6'
19	2	166.9	C		H-3, H-4', H6'
20	4	184.3	C=O		H-3, H-8

Table 6 Elution conditions and yields of BC–III primary fractions from silica gel column.

Fraction	Elution solvent	Yield (g)
BC–III–A	100% hexane	0.7
BC–III–B	50% hexane – 50% EtOAc	5.593
BC–III–C	50% hexane – 50% EtOAc	1.030
BC–III–D	100% EtOAc	1.641
BC–III–E	100% EtOAc	0.287
BC–III–F	100% EtOAc	0.035
BC–III–G	75% EtOAc – 25% MeOH	7.247
BC–III–H	75% EtOAc – 25% MeOH	8.942
BC–III–I	75% EtOAc – 25% MeOH	7.720
BC–III–J	75% EtOAc – 25% MeOH	3.044
BC–III–K	50% EtOAc – 50% MeOH	49.160

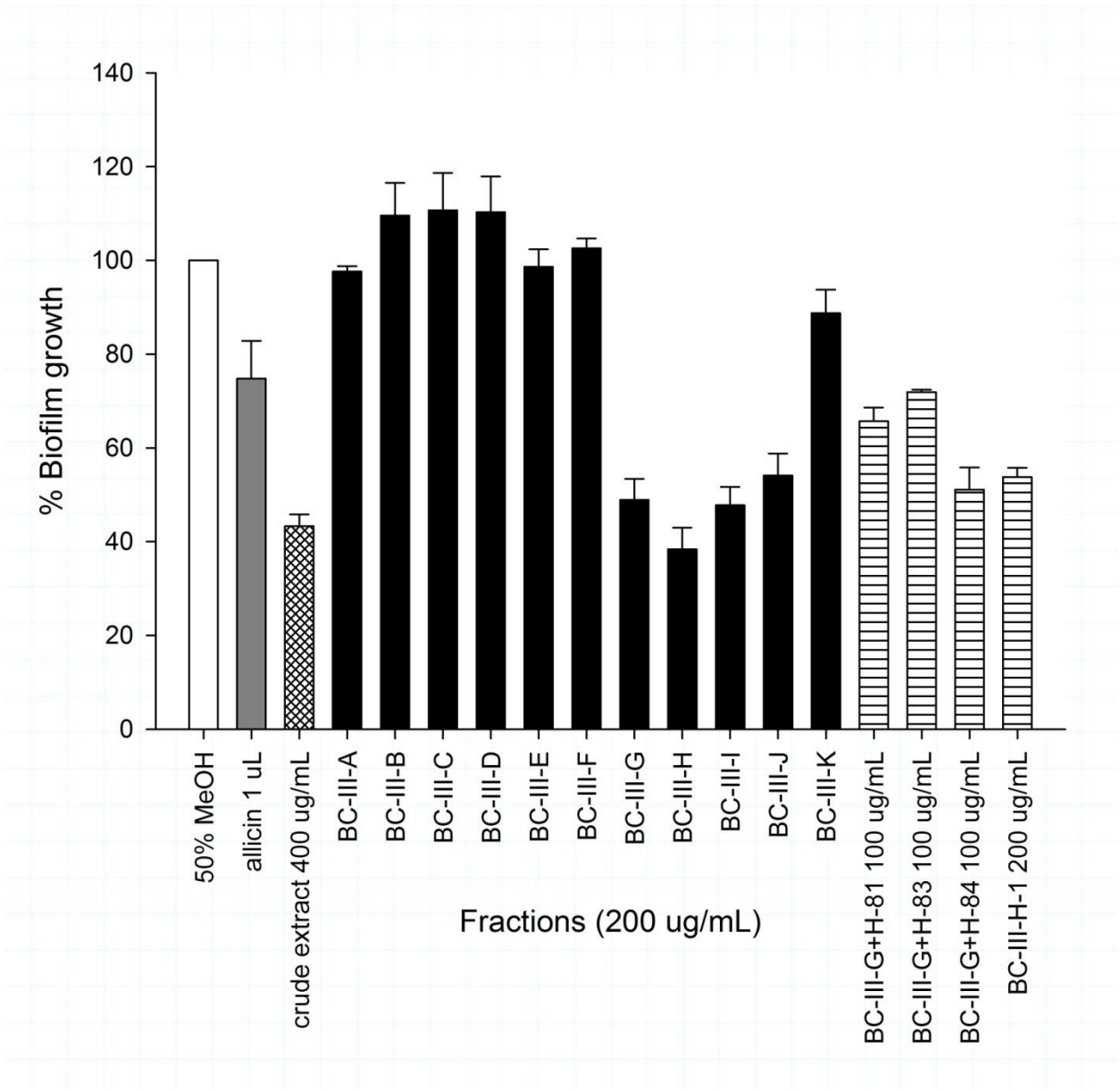


Figure 8 Average biofilm growth ± S.E.M. of *Pseudomonas aeruginosa* PA14 relative to vehicle control (50% MeOH) in the presence of *Blakea cuneata* Standl. crude extract (400 μg/mL) and BC–III primary (200 μg/mL) and secondary fractions (100 to 200 μg/mL) compared to the positive control allicin (1 μL/mL or 1.08 mg/mL). N = 3.

Phytochemical analyses

HPLC–DAD analyses of the crude extract, primary and secondary fractions showed that the composition for most was still quite complex. All active fractions contained major peaks in the polar region (Figures 9 to 14). UPLC–QTOF–MS/MS analysis of secondary fractions from BC–III–H collected from preparative scale HPLC confirmed the purity of these fractions. Analyses using MassLynx and MetLin identified masses and potential elemental composition for these compounds (Table 7). Fraction BC–III–H–5 was confirmed by MS/MS fragmentation pattern (Figure 15) and MetLIN mass search to be hispidulin–7–*O*–glucoside (compound **1**).

Table 7 Retention times, yields, masses and elemental composition of BC–III–H secondary fractions from preparative scale HPLC analyzed with UPLC–QTOF–MS/MS.

Fraction	**Retention time (min)**	**Yield (mg)**	**$[M–H]^+$**	**Elemental composition**
BC–III–H –1	0 to 24	381	N/A[a]	N/A[a]
BC–III–H–2	26.15	2.7	485.1084	$C_{24}H_{21}O_{11}$
BC–III–H–3	27.15	2.5	615.1350	$C_{29}H_{27}O_{15}$
BC–III–H–4	28.16	2.3	689.3912	$C_{38}H_{57}O_{11}$
BC–III–H–5	29.15	2.7	463.1254	$C_{22}H_{23}O_{11}$
BC–III–H–6	30.15	1.6	689.3868	$C_{45}H_{53}O_6$
BC–III–H–7	31.15	1.2	669.1870	$C_{26}H_{27}O_{20}$
BC–III–H–8	32.71	1.4	669.1824	$C_{33}H_{33}O_{15}$
BC–III–H–9	35.59	2.3	689.3859	$C_{45}H_{53}O_6$
BC–III–H–10	39.28	2.1	119.1398	$C_{12}H_{18}NO$
BC–III–H–11	43.20	1.0	527.3350	$C_{32}H_{47}O_6$

[a] complex fraction

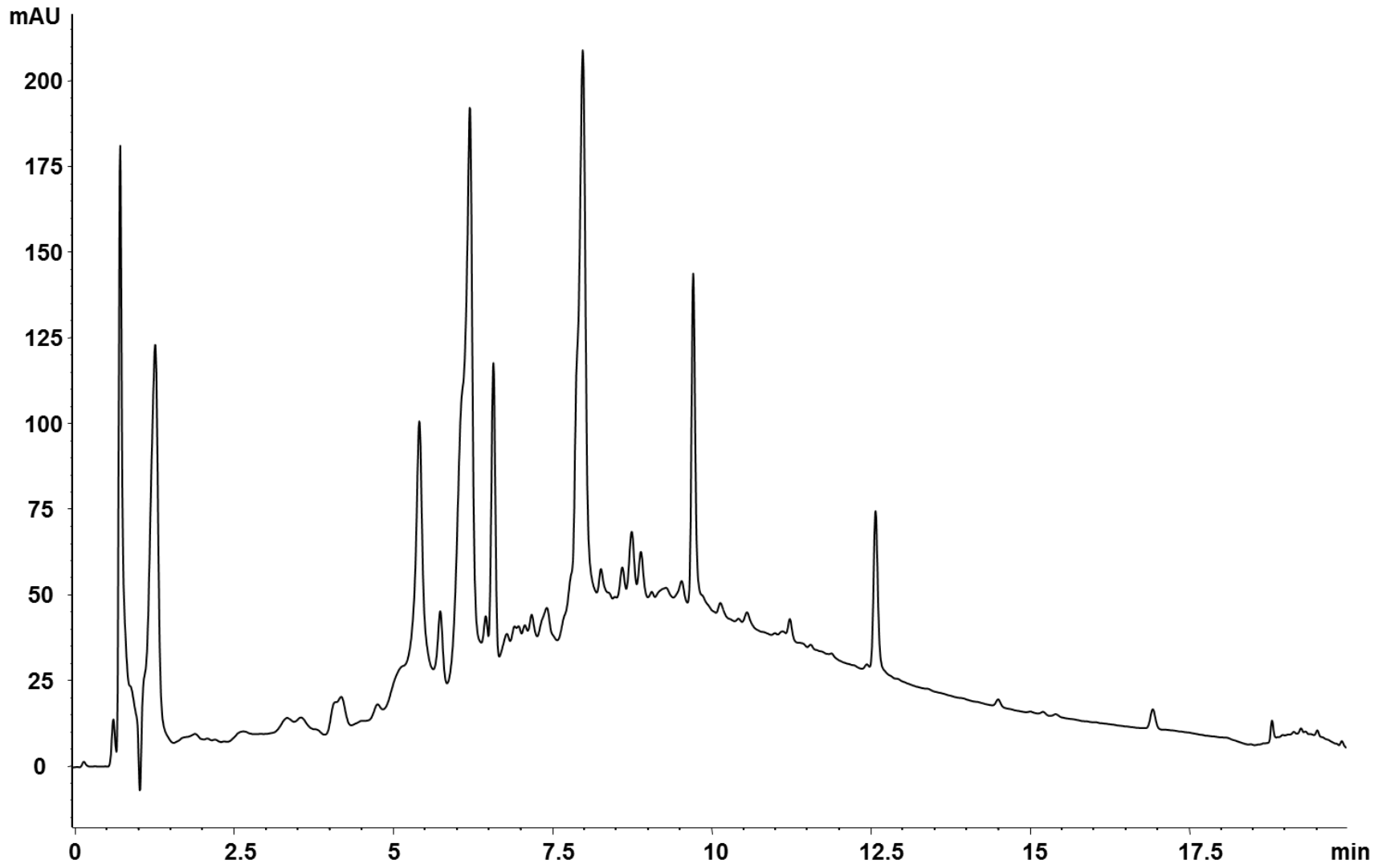


Figure 9 HPLC–DAD chromatogram of *Blakea cuneata* primary fraction BC–I–U at 280 nm.

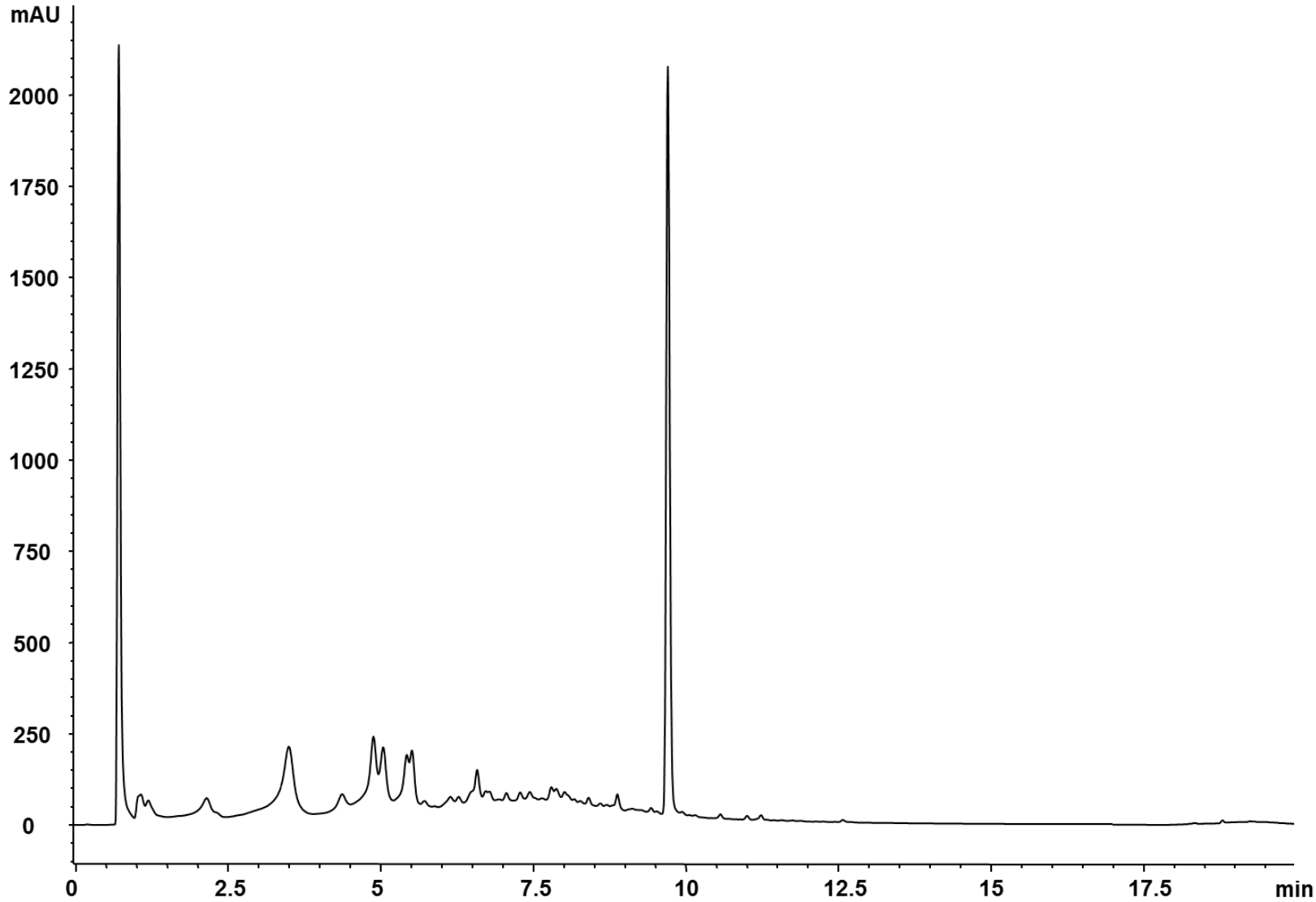


Figure 10 HPLC–DAD chromatogram of *Blakea cuneata* primary fraction BC–II–N at 280 nm.

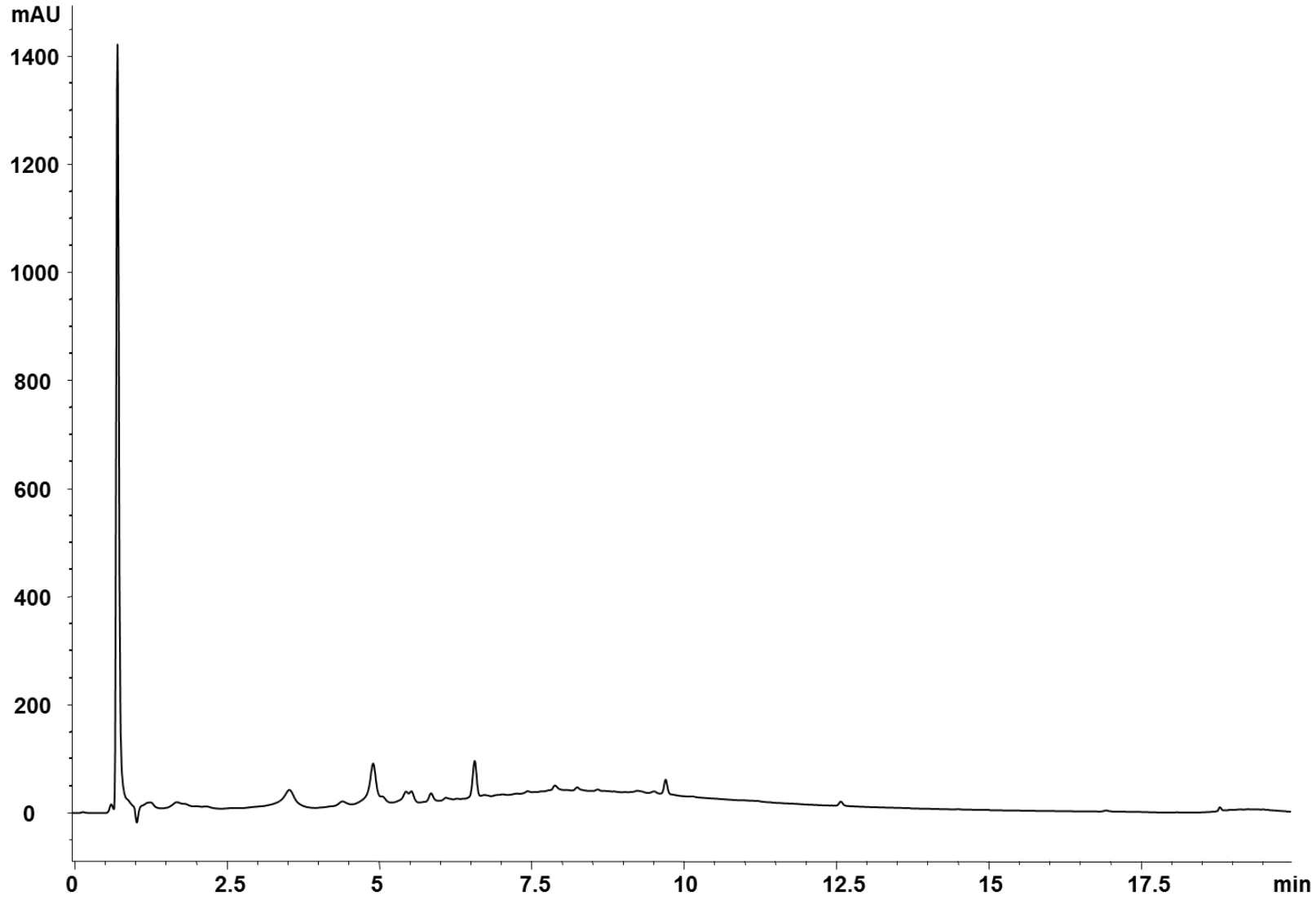


Figure 11 HPLC–DAD chromatogram of *Blakea cuneata* primary fraction BC–II–Q at 280 nm.

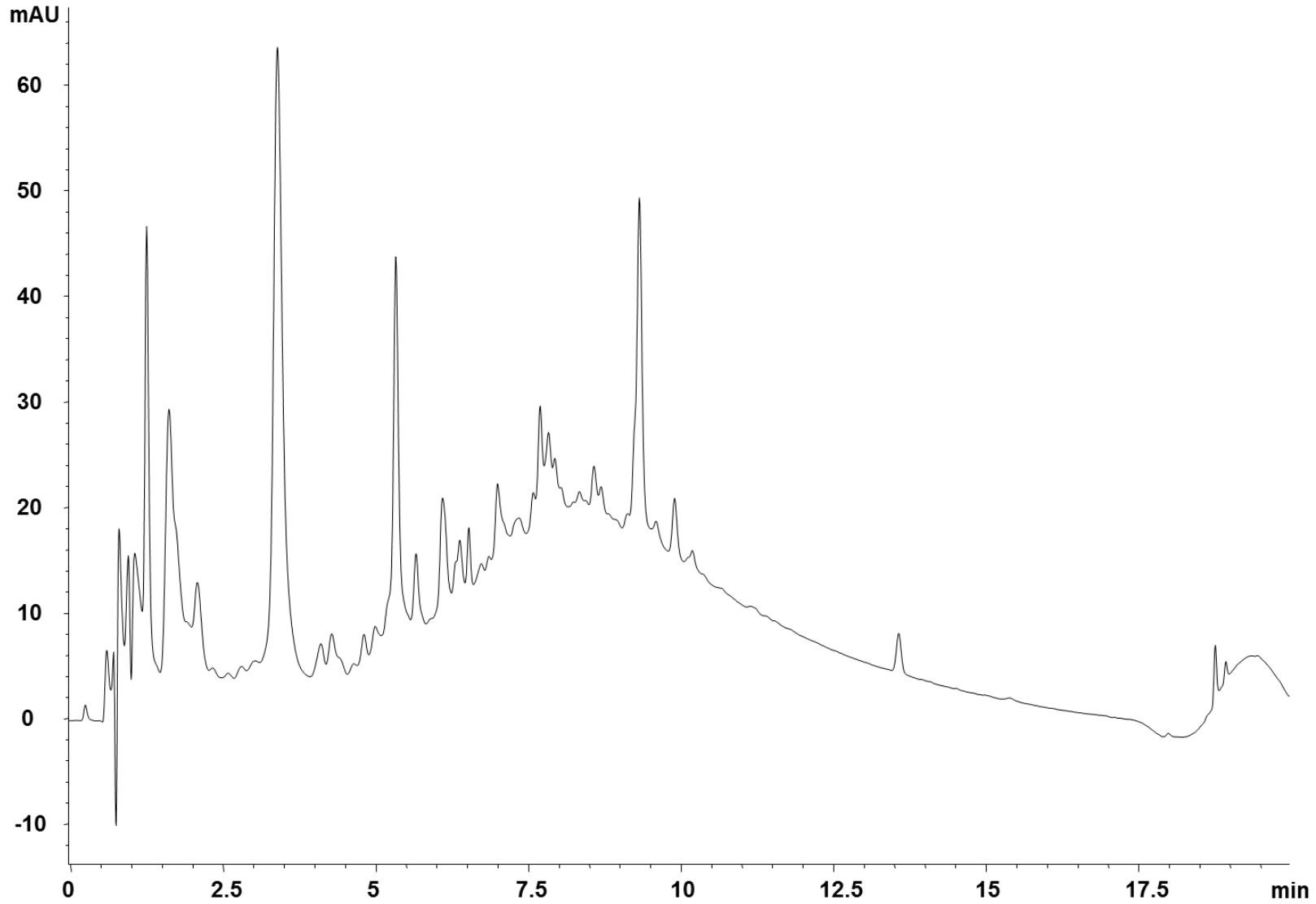


Figure 12 HPLC–DAD chromatogram of *Blakea cuneata* secondary fraction BC–II–Q–1 at 280 nm.

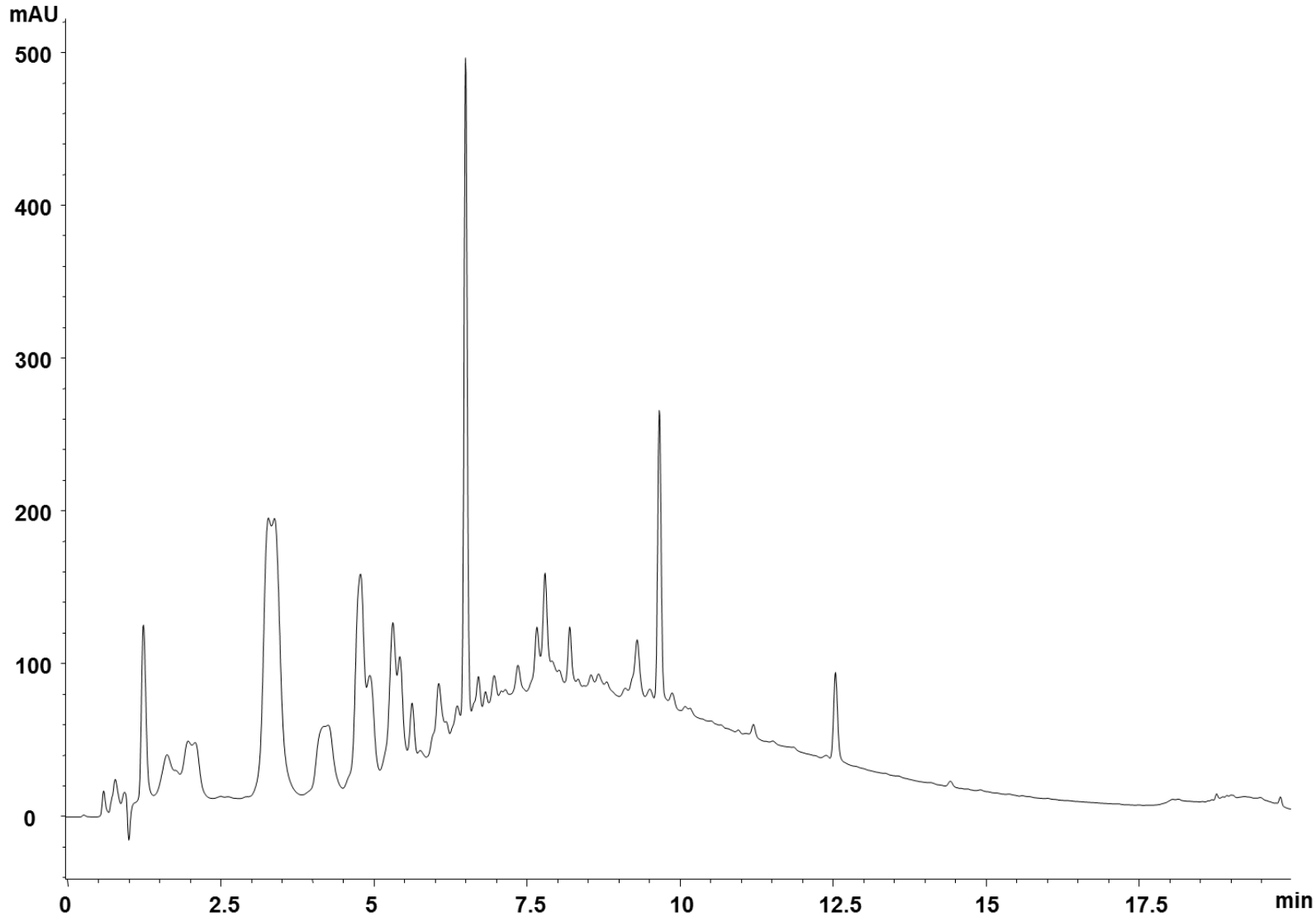


Figure 13 HPLC–DAD chromatogram of *Blakea cuneata* secondary fraction BC–II–Q–2 at 280 nm.

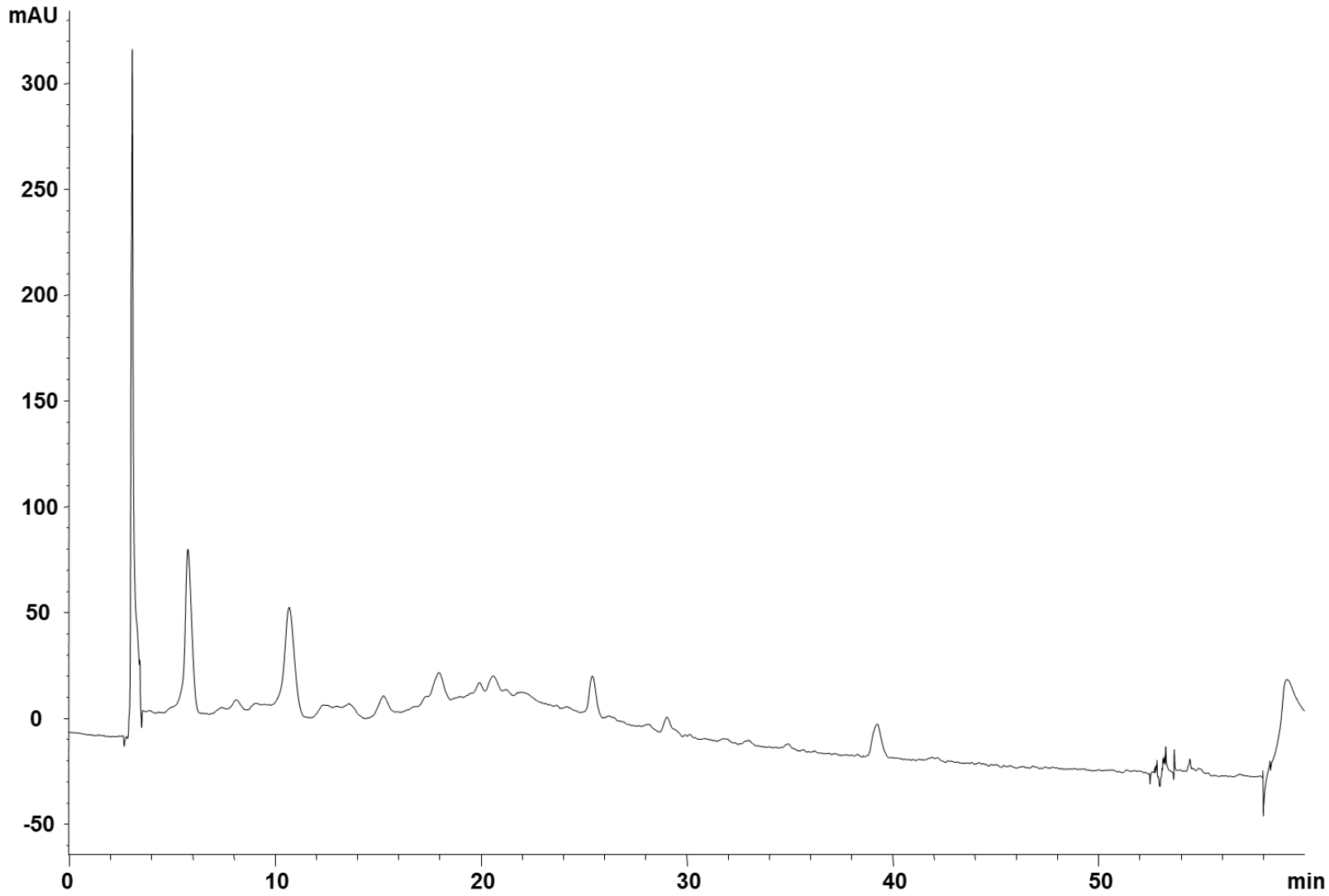


Figure 14 HPLC–DAD chromatogram of *Blakea cuneata* secondary fraction BC–III–H 0 to 24 min time collection at 280 nm.

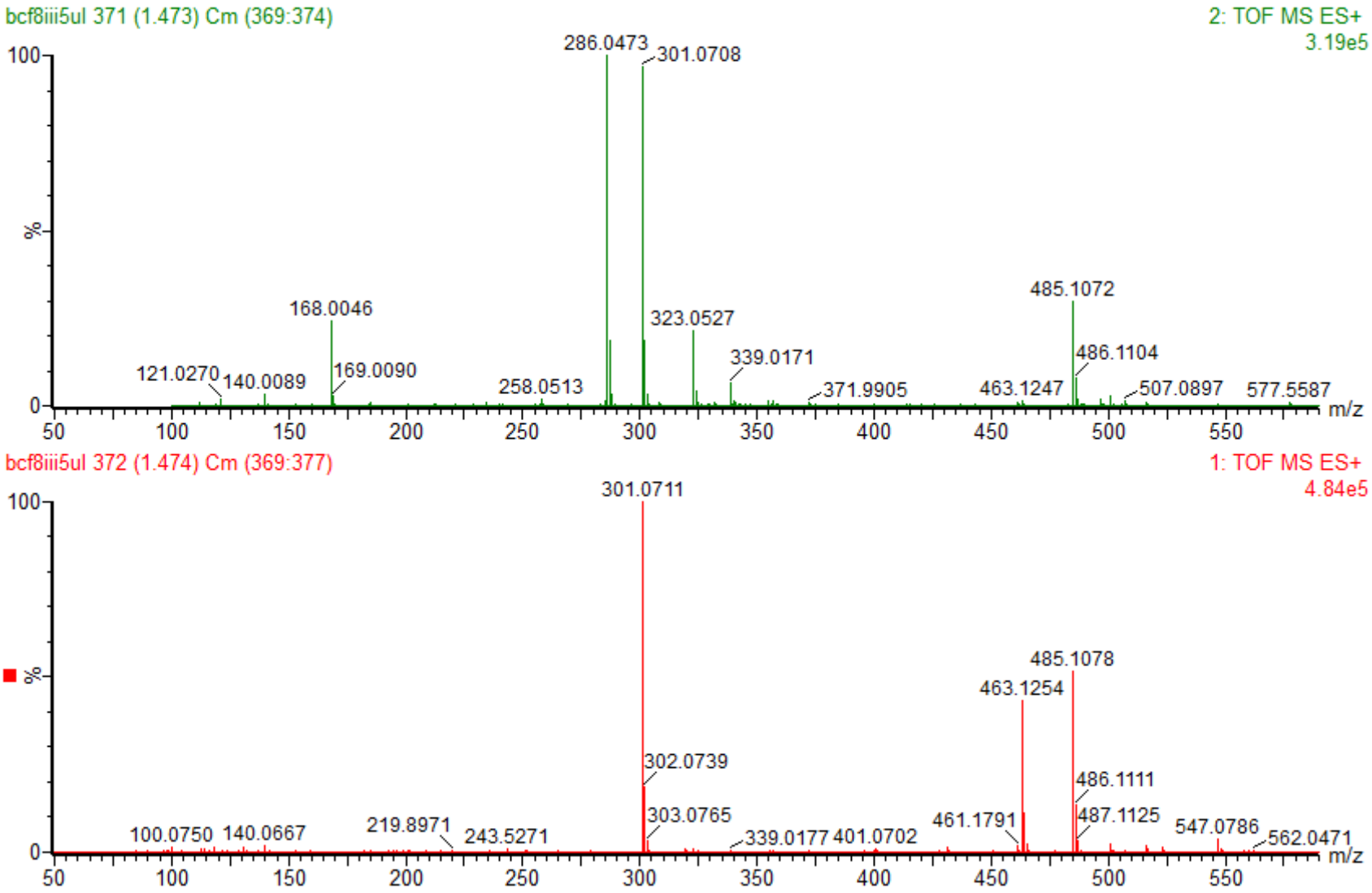
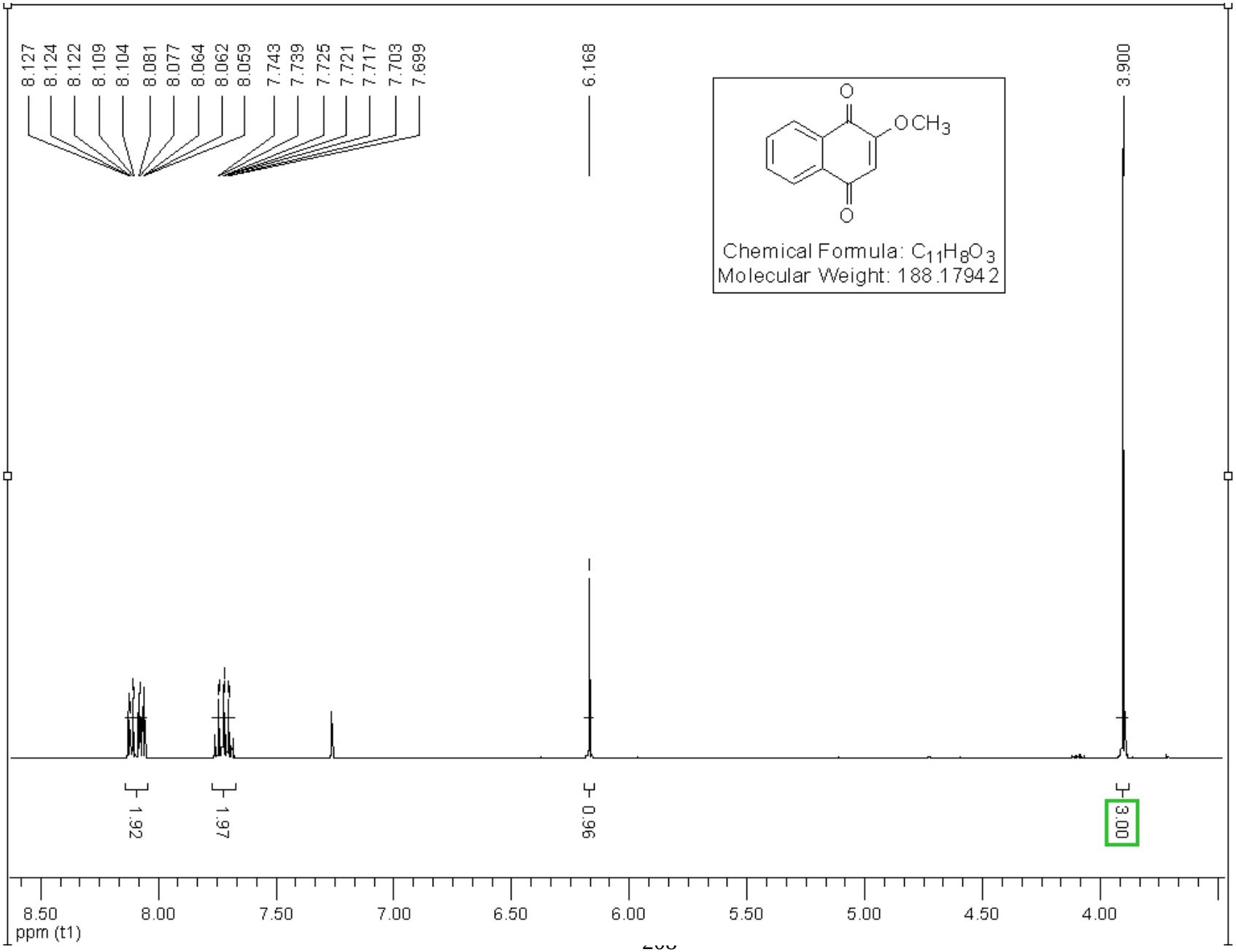
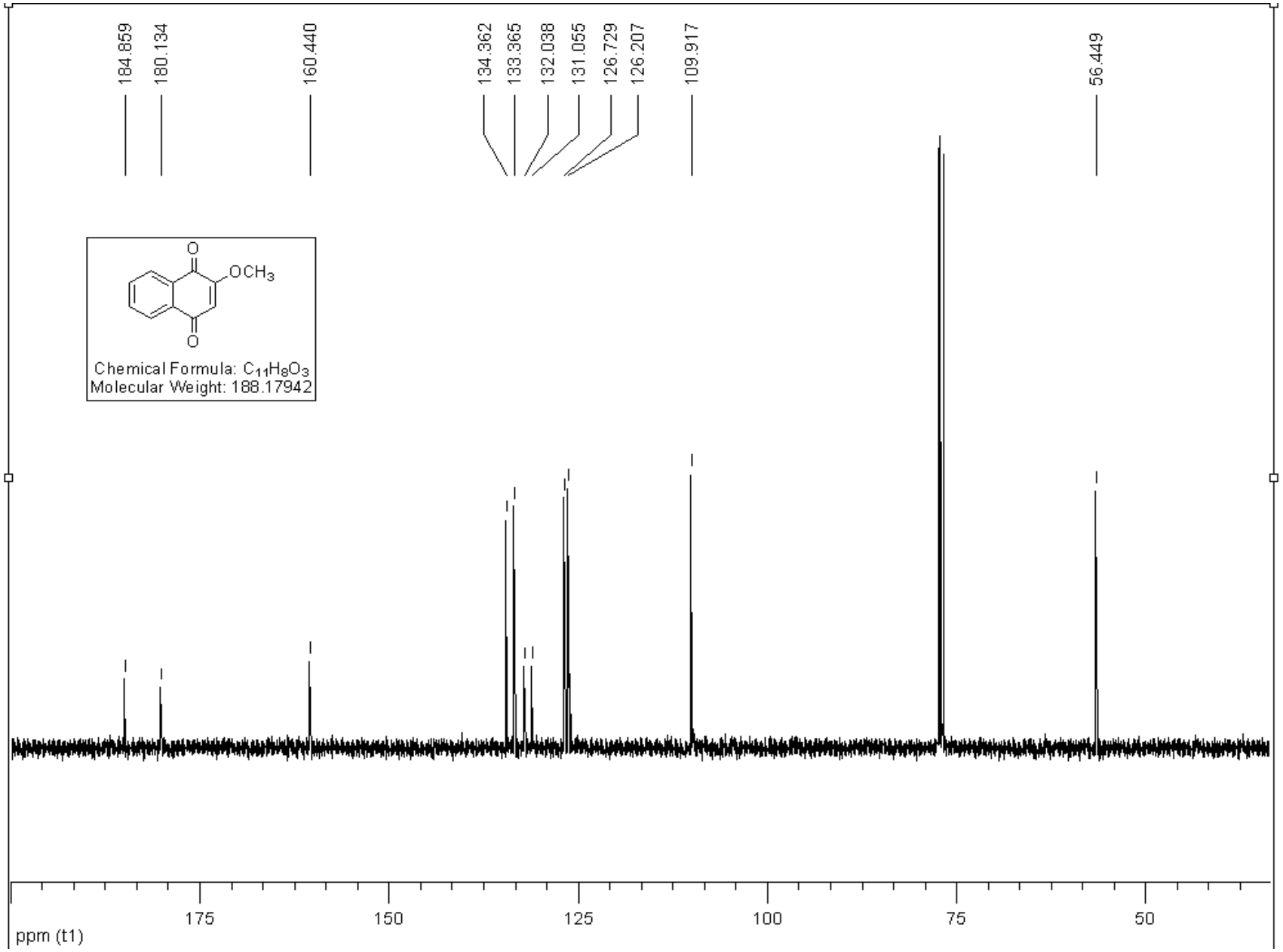


Figure 15 HR–ESI–MS/MS spectrum of compound **1** (positive mode).

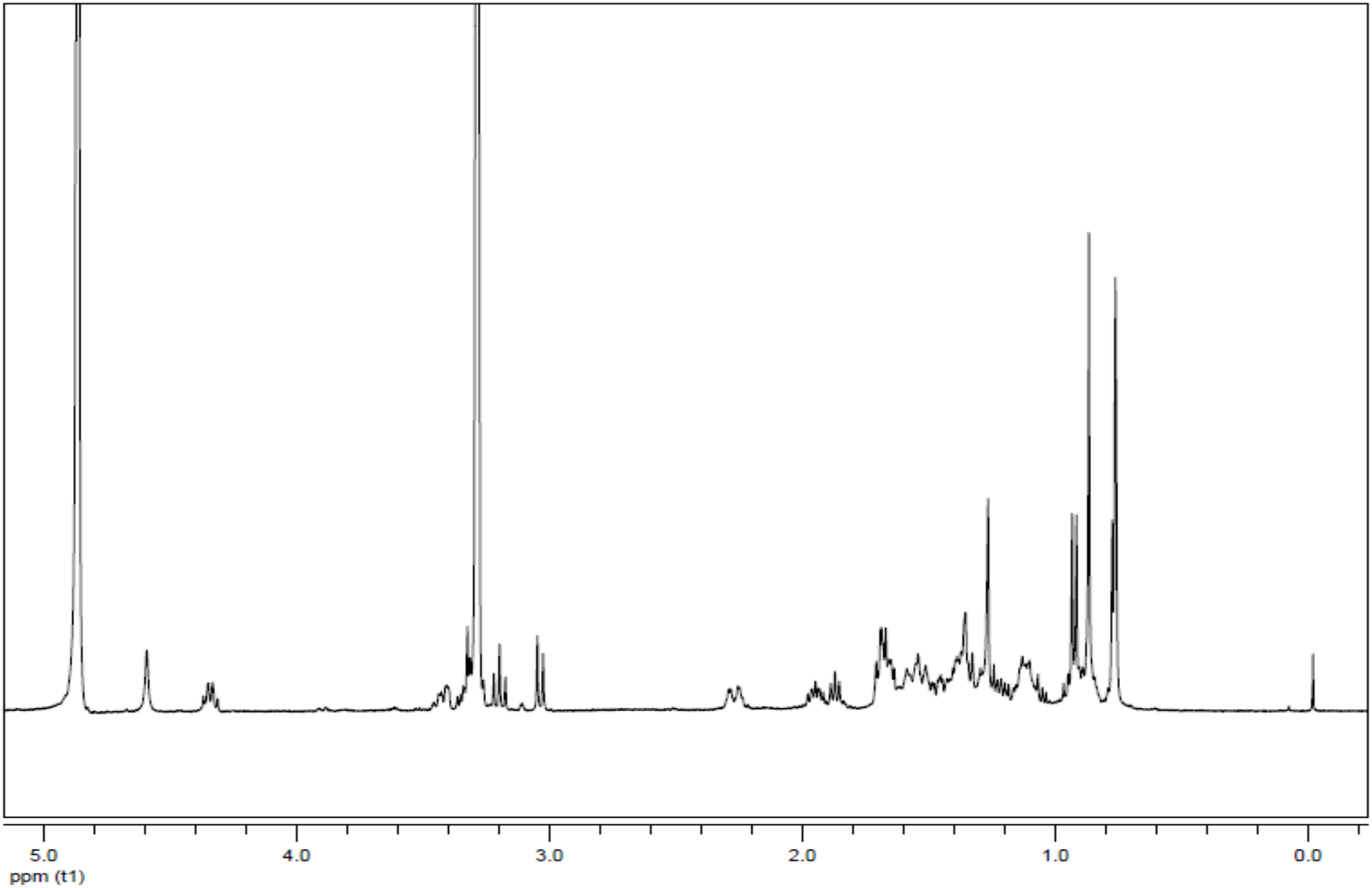
Appendix IIa – ^{1}H NMR spectrum of 2–methoxy–1,4–naphthoquinone isolated from *Marcgravia nervosa* (400 MHz, $CDCl_3$)



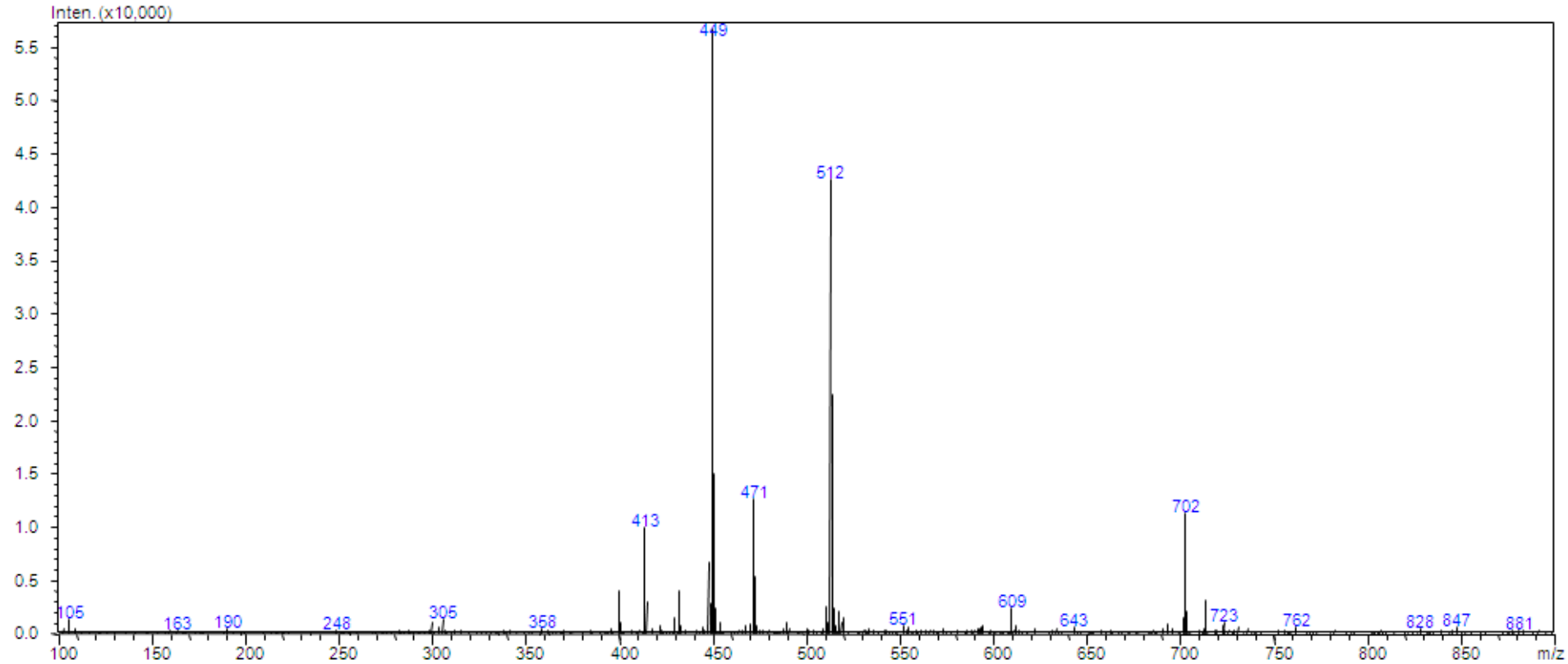
Appendix IIb – ^{13}C NMR spectrum of 2–methoxy–1,4–naphthoquinone isolated from *Marcgravia nervosa* (400 MHz, $CDCl_3$)



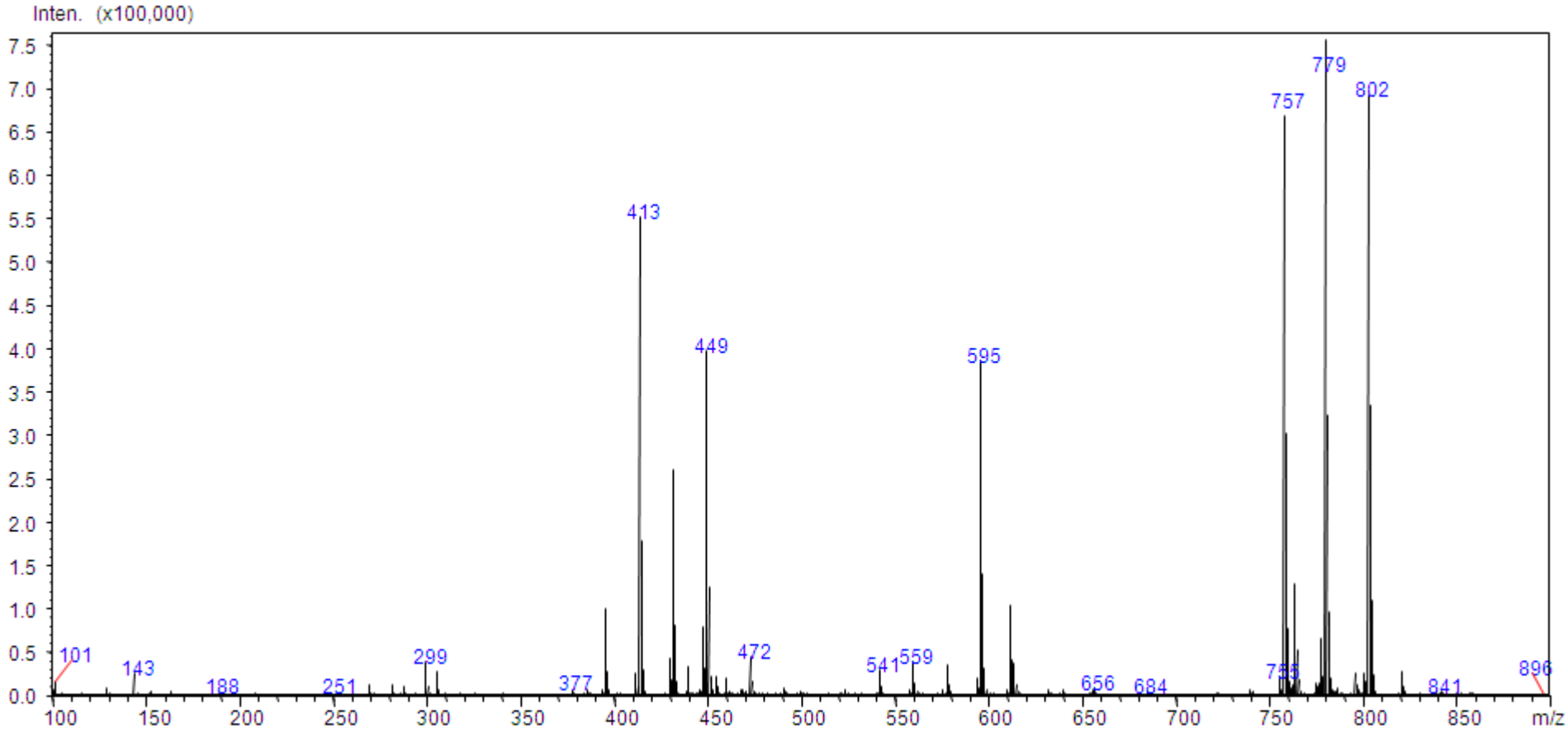
Appendix IIIa – ^{1}H NMR spectrum of sapogenin 1A (500 MHz, CD_3OD)



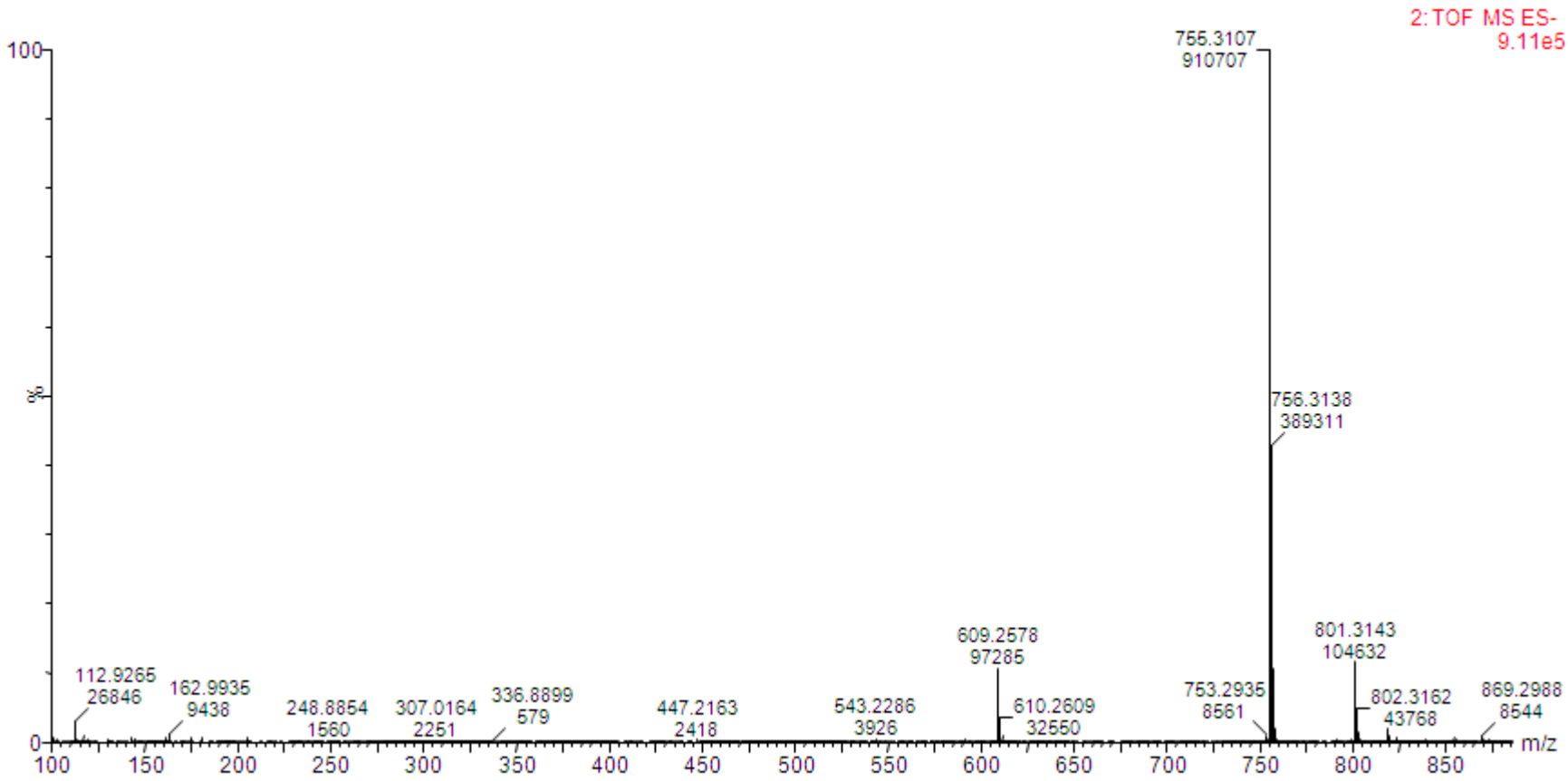
Appendix IIIb – ESI–MS spectrum of sapogenin 1A (positive mode)



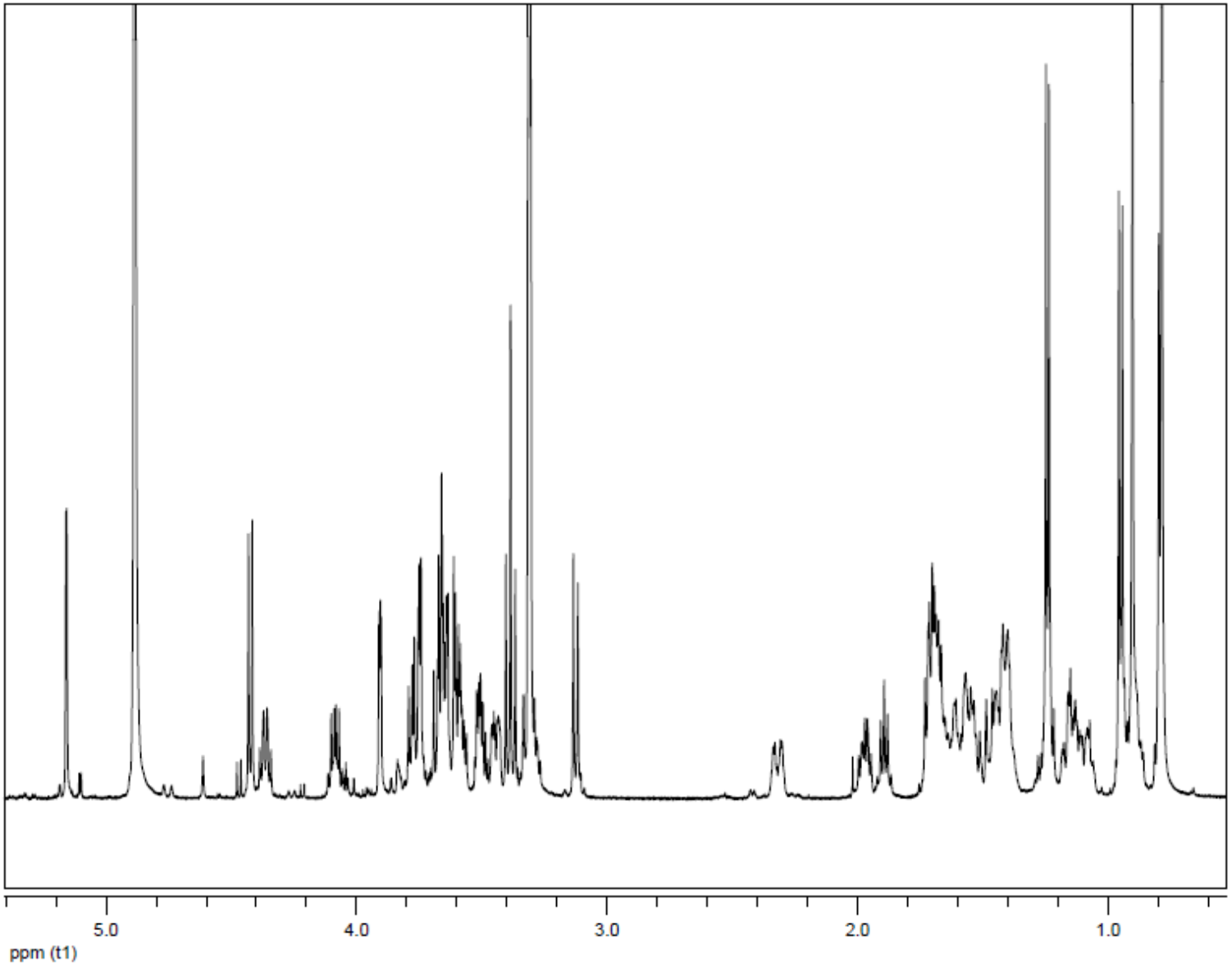
Appendix IIIc – ESI–MS spectrum of the major saponin isolated from *Cestrum schlechtendahlii* (positive mode)



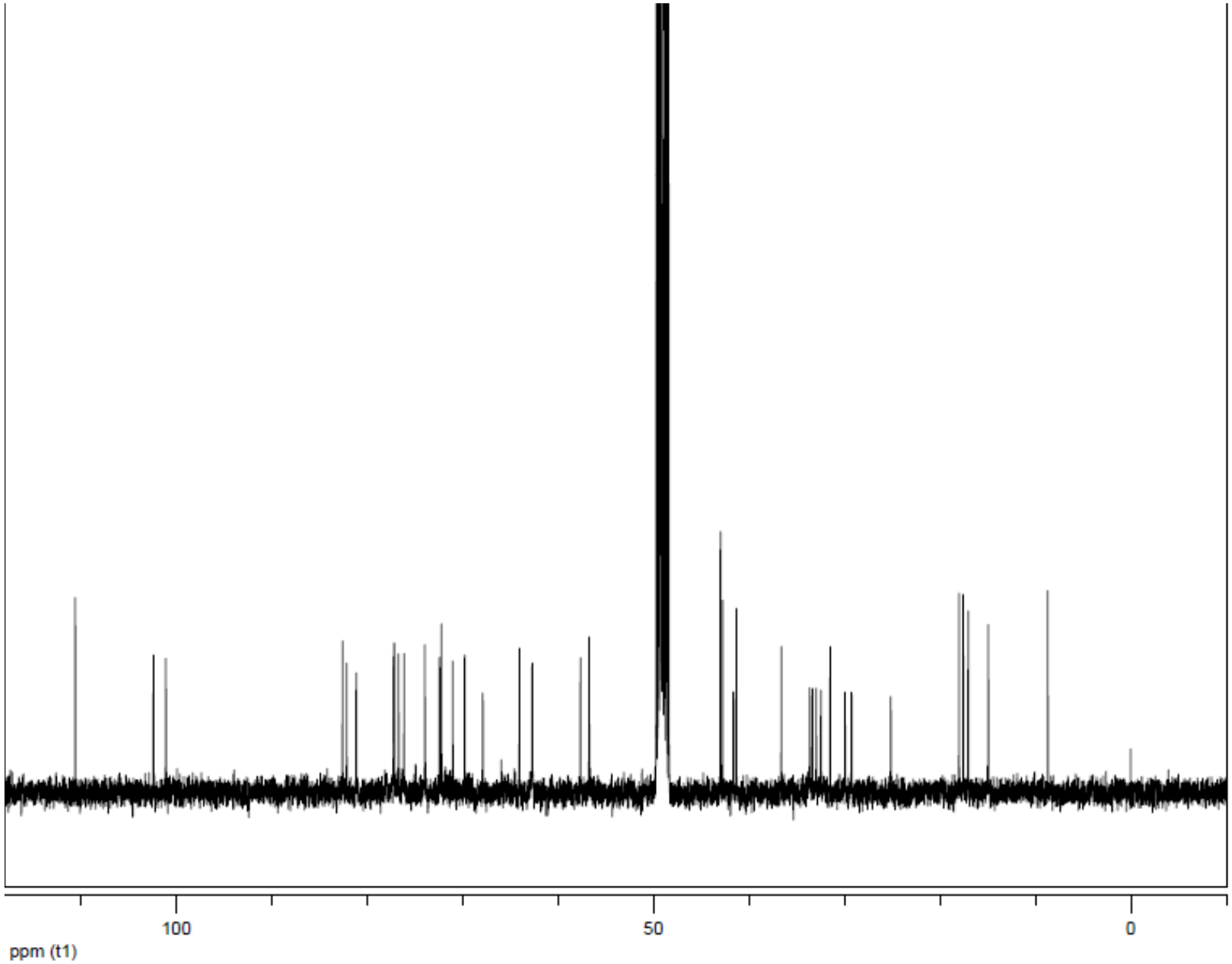
Appendix IIId – HRESI–MS spectrum of the major saponin isolated from *Cestrum schlechtendahlii* (negative mode)



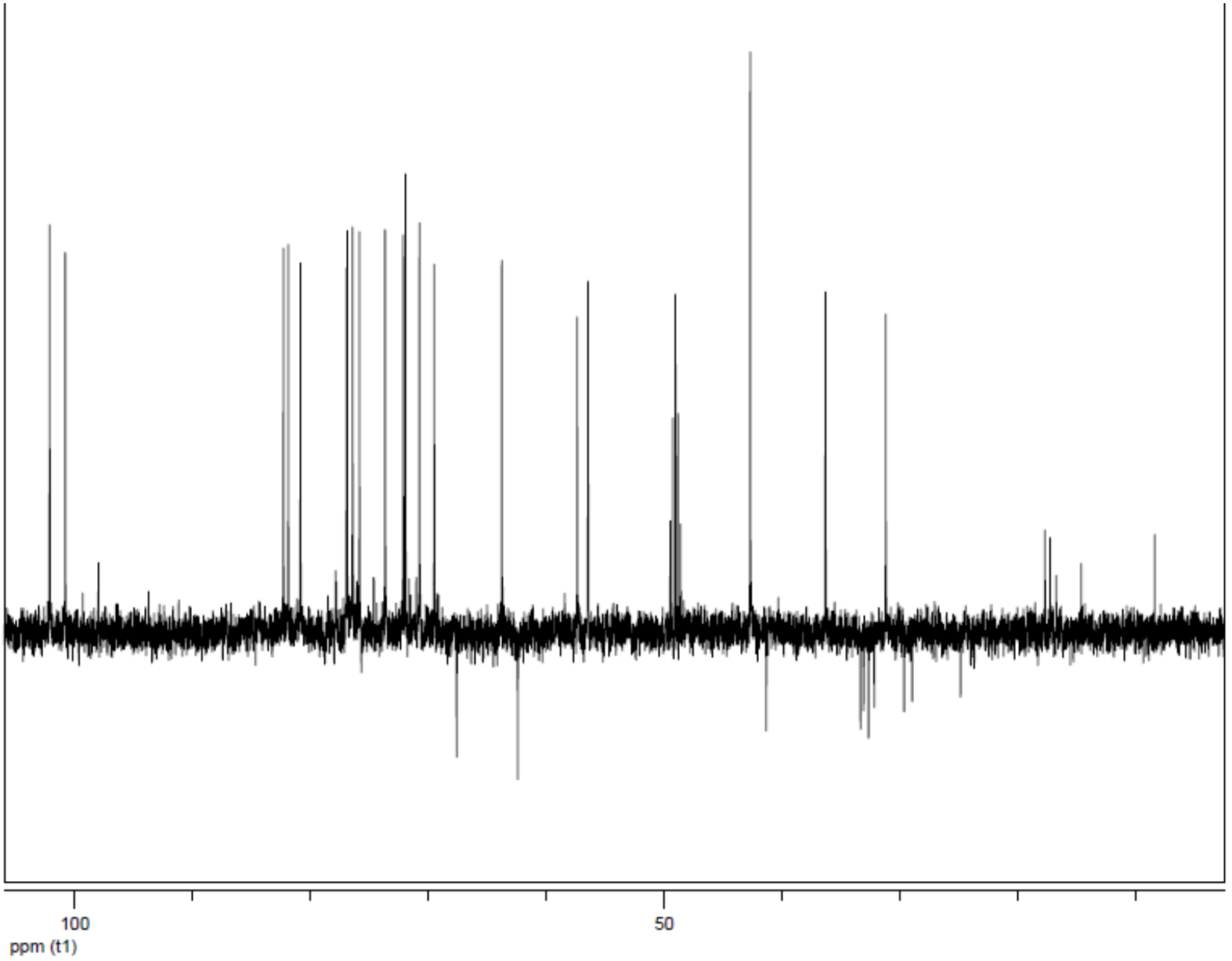
Appendix IIIe – ^{1}H NMR spectrum of the major saponin isolated from *Cestrum schlechtendahlii* (500 MHz, CD_3OD)



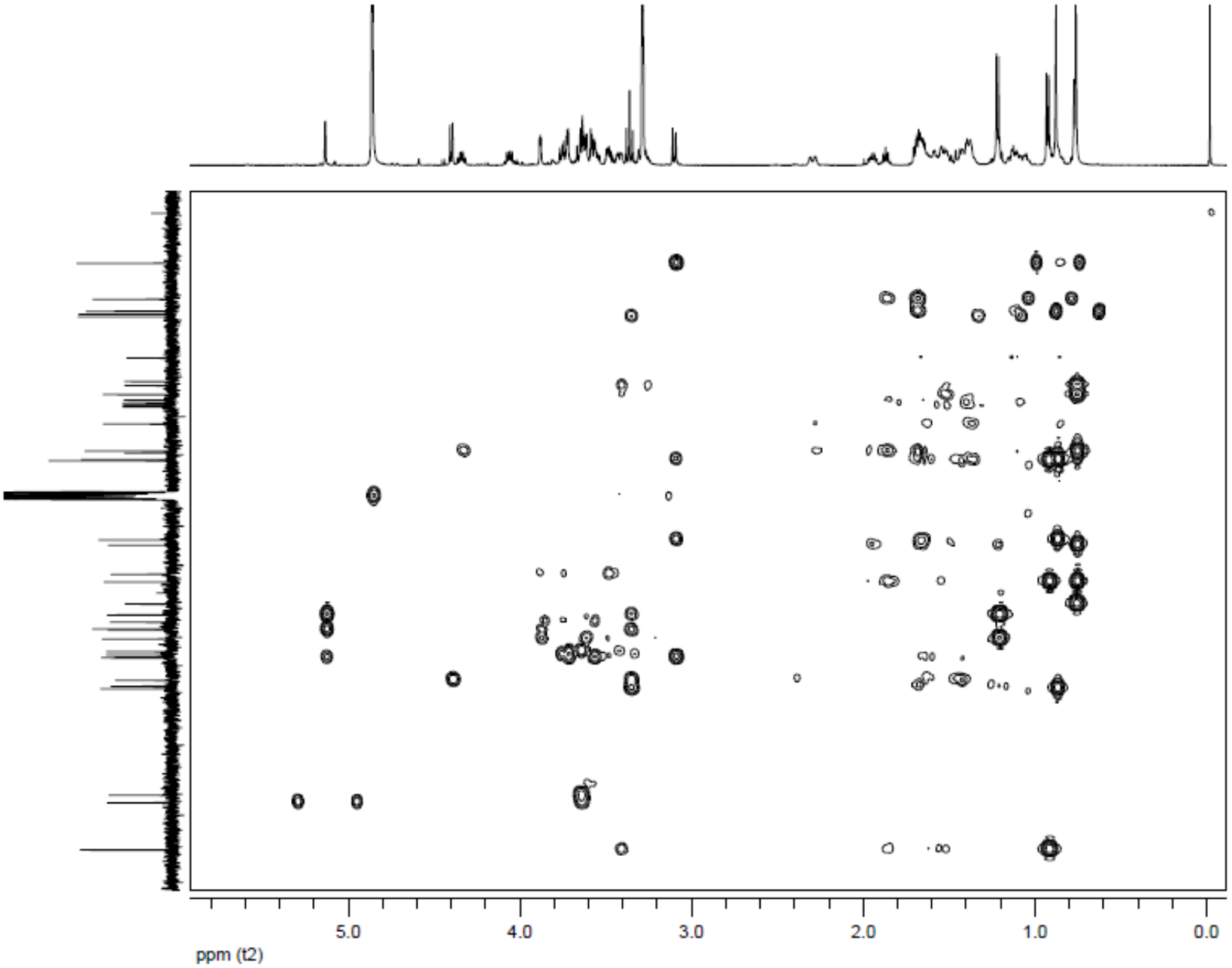
Appendix IIIf – ^{13}C NMR spectrum of the major saponin isolated from *Cestrum schlechtendahlii* (125 MHz, CD_3OD)



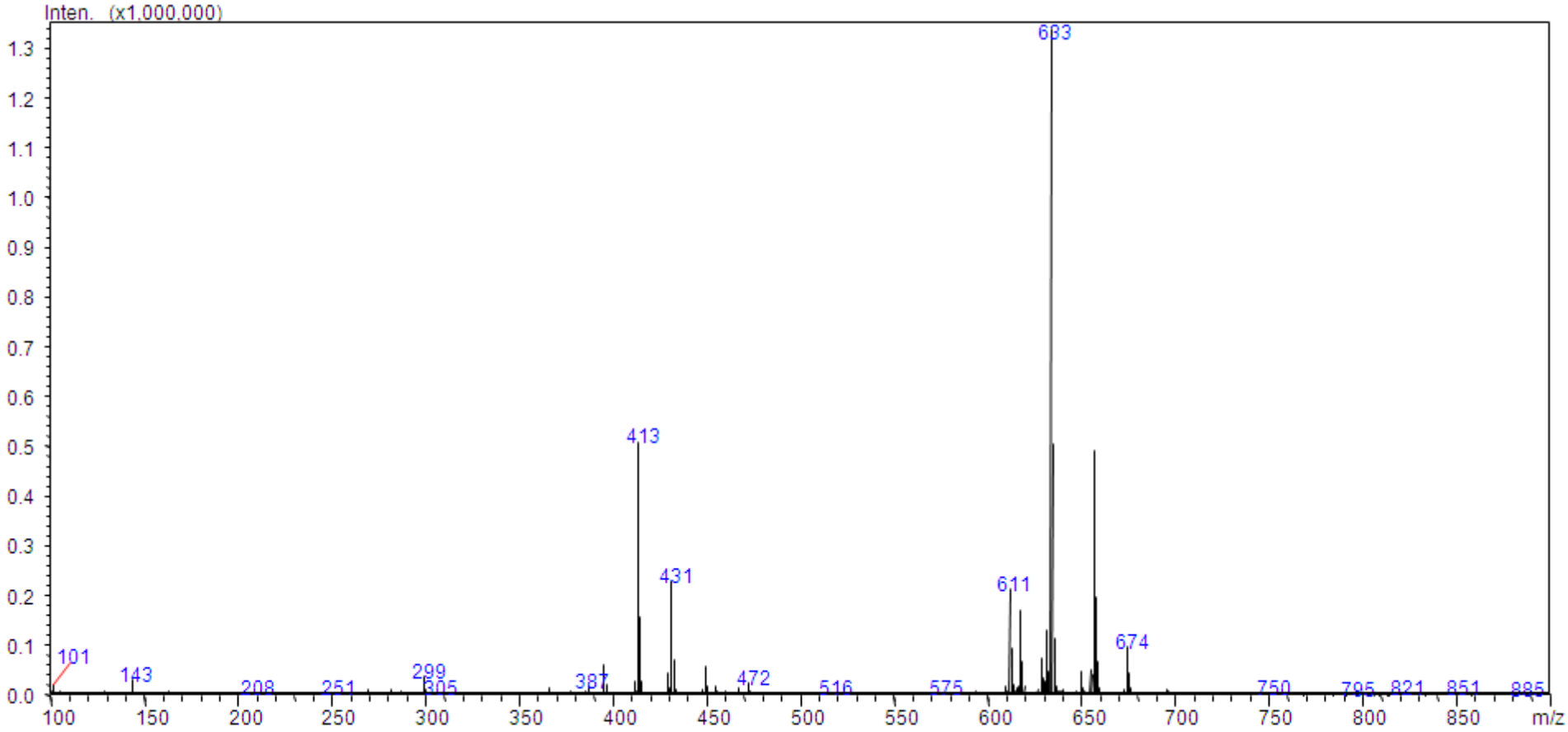
Appendix IIIg – DEPT NMR spectrum of the major saponin isolated from *Cestrum schlechtendahlii* (125 MHz, CD_3OD)



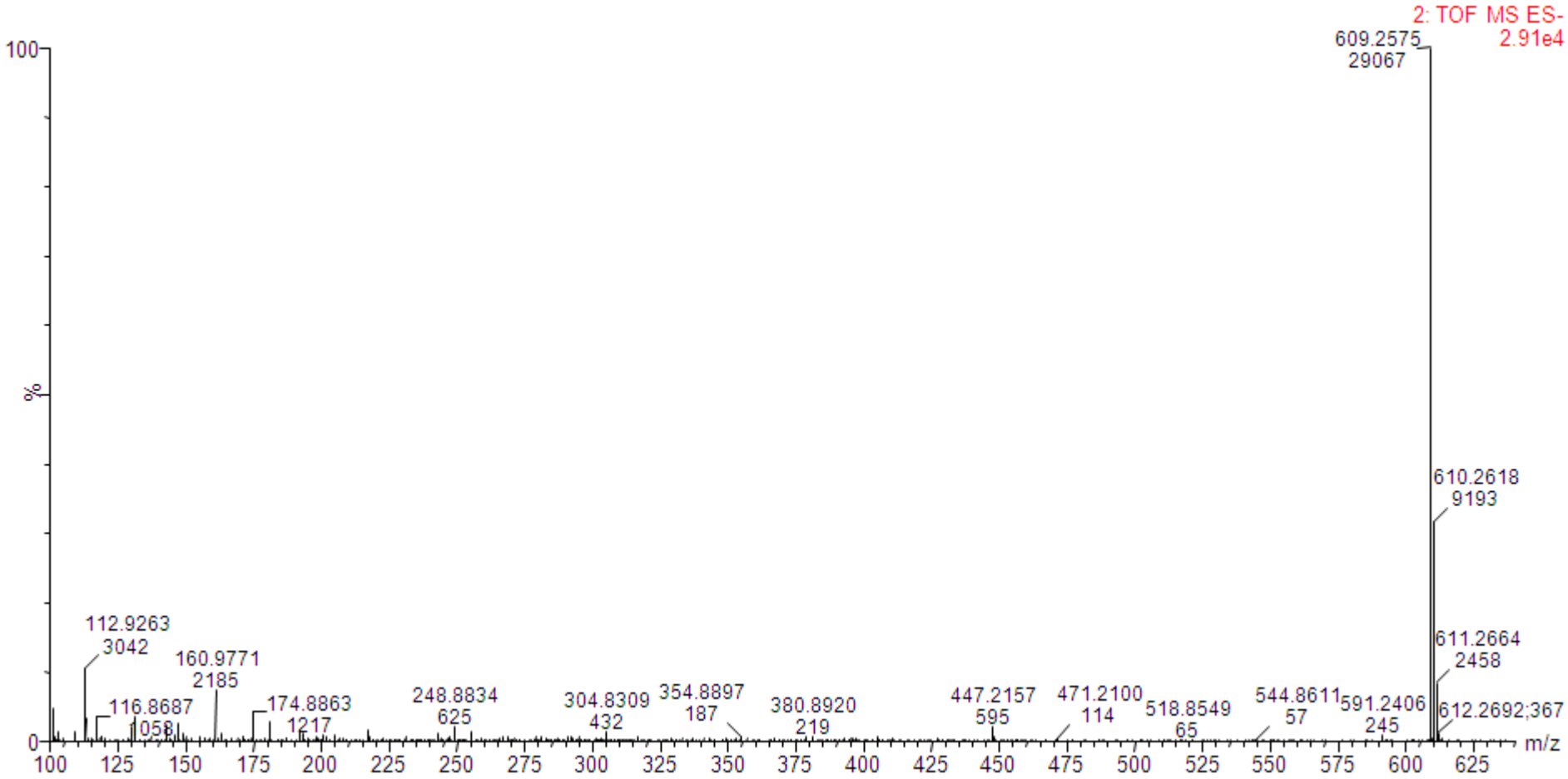
Appendix IIIh – HMBC NMR spectrum of the major saponin isolated from *Cestrum schlechtendahlii* (500 MHz, CD_3OD)



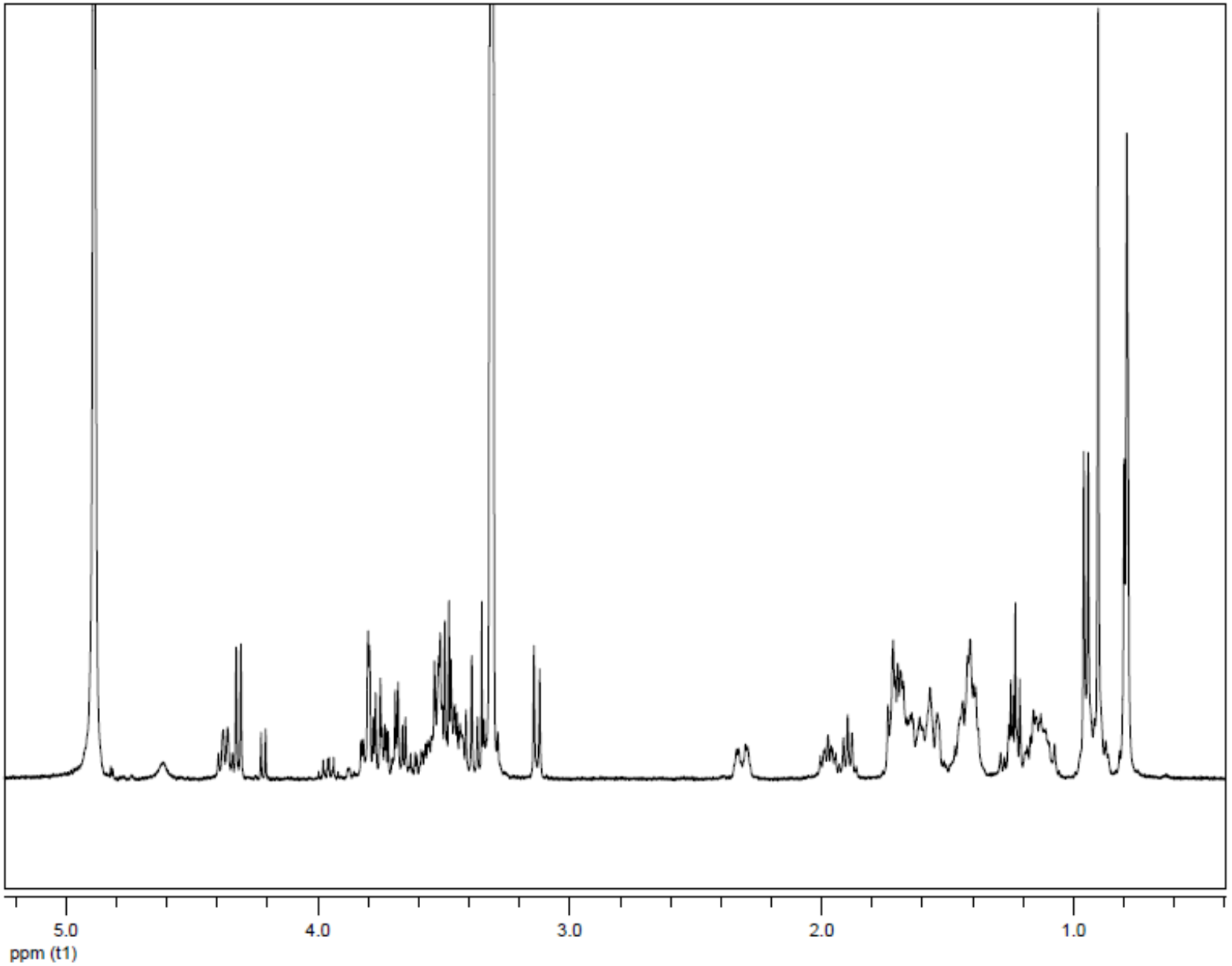
Appendix IIIi – HRESI–MS spectrum of the minor saponin isolated from *Cestrum schlechtendahlii* (positive mode)



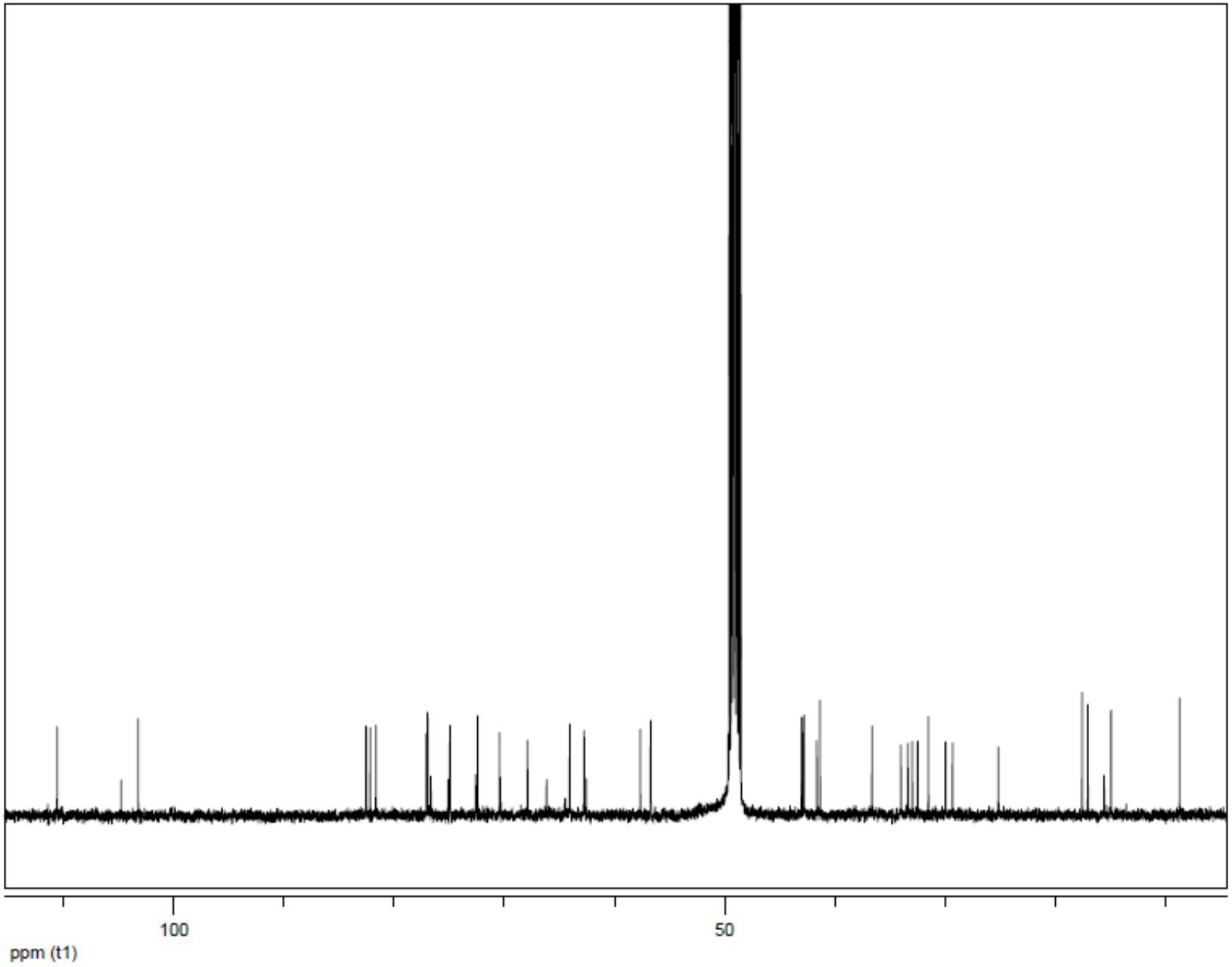
Appendix IIIj – HRESI–MS spectrum of the minor saponin isolated from *Cestrum schlechtendahlii* (negative mode)



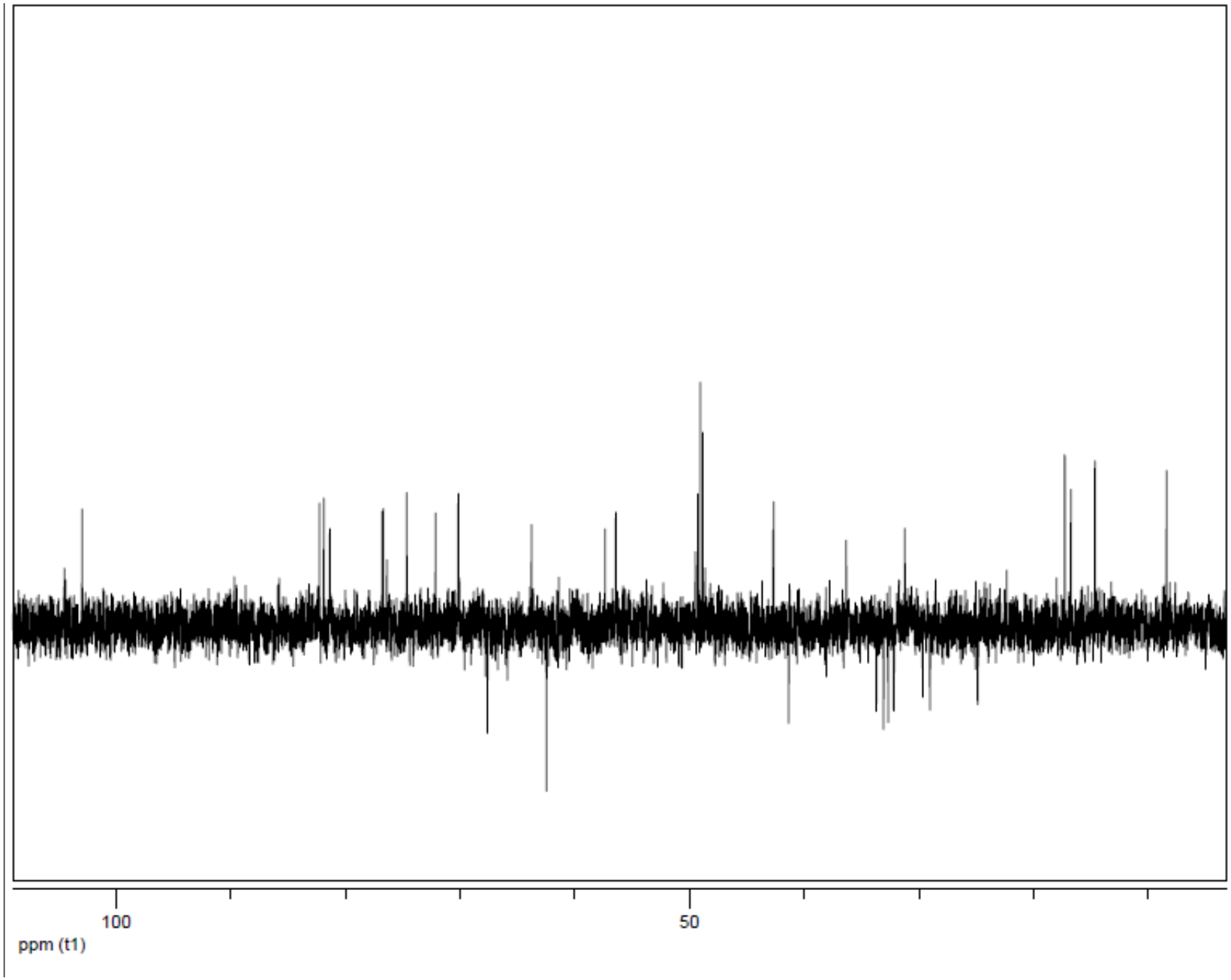
Appendix IIIk – ^{1}H NMR spectrum of the minor saponin isolated from *Cestrum schlechtendahlii* (500 MHz, CD_3OD)



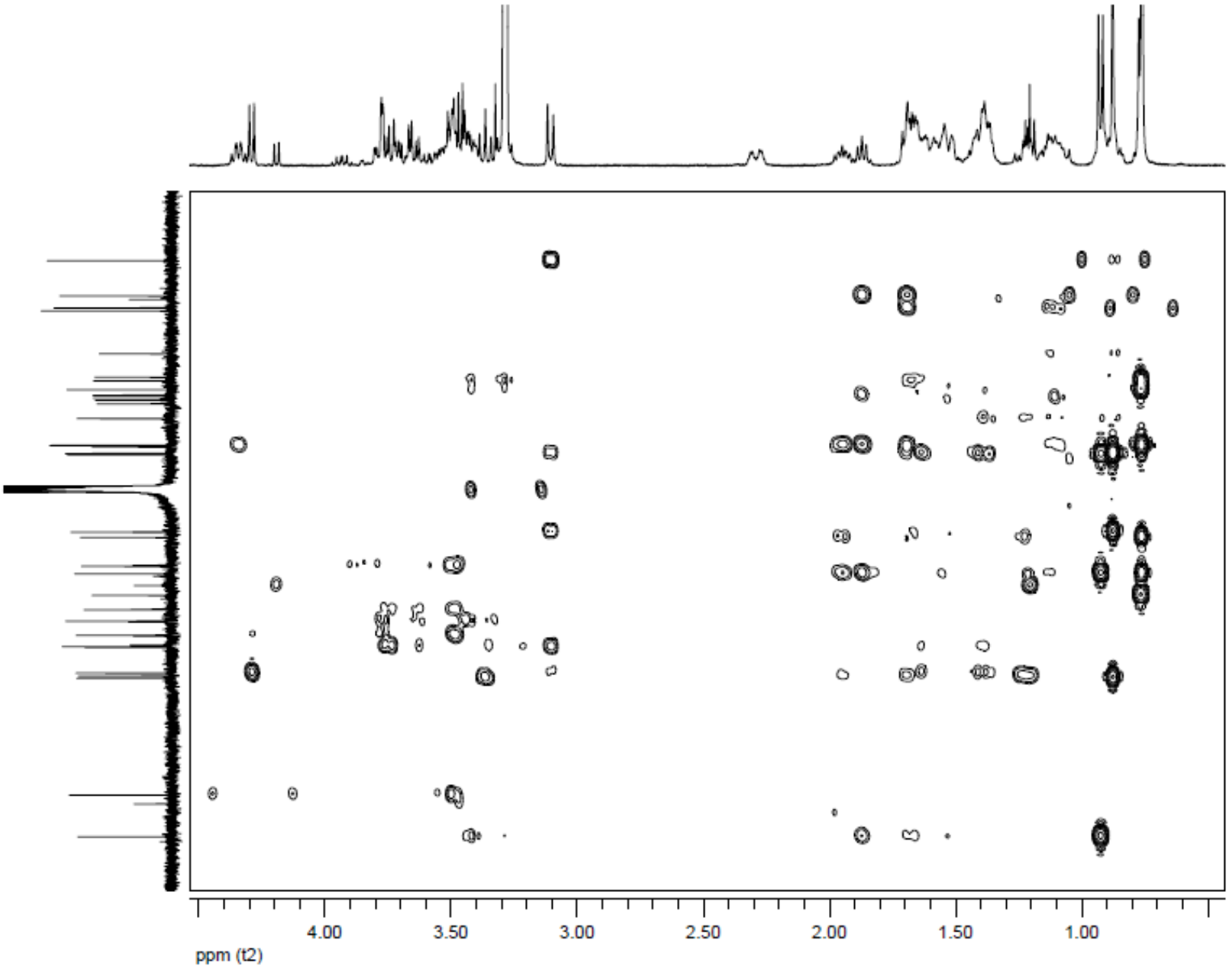
Appendix IIIl – ^{13}C NMR spectrum of the minor saponin isolated from *Cestrum schlechtendahlii* (125 MHz, CD_3OD)



Appendix IIIm – DEPT NMR spectrum of the minor saponin isolated from *Cestrum schlechtendahlii* (125 MHz, CD_3OD)



Appendix IIIn – HMBC NMR spectrum of the minor saponin isolated from *Cestrum schlechtendahlii* (500 MHz, CD_3OD)



Appendix IV – Diploid heterozygous *Saccharomyces cerevisiae* mutants affected by saponin at 15 μg/mL in solid media.

ORF[a]	Gene[b]	Gene function[c]
YAL035W	*FUN12*	GTPase involved in general translation
YAL049C	*AIM2*	Cytoplasmic protein involved in mitochondrial function or organization
YAL060W	*BDH1*	NAD-dependent (R,R)-butanediol dehydrogenase
YAL061W	*BDH2*	Putative medium-chain alcohol dehydrogenase1
YAL066W	N/A[d]	Dubious open reading frame unlikely to encode a protein
YAL067C	*SEO1*	Putative permease, member of the allantoate transporter subfamily
YBL001C	*ECM15*	Non-essential protein of unknown function, may be involved in cell-wall biogenesis
YBL005W	*PDR3*	Transcriptional activator of the pleiotropic drug resistance network which regulates the expression of ATP-binding cassette (ABC) transporters
YBL008W	*HIR1*	Subunit of the HIR complex involved in nucleosome and kinetochores as well as heterochromatic gene silencing
YBL008W-A	N/A	Putative protein of unknown function
YBL011W	*SCT1*	Glycerol 3-phosphate/dihydroxyacetone phosphate sn-1 acyltransferase from the glycerolipid biosynthesis pathway
YBL013W	*FMT1*	Methionyl-tRNA formyltransferase which catalyzes the formylation of initiator Met-tRNA in mitochondria
YBL014C	*RRN6*	Component of the core factor (CF) rDNA transcription factor complex
YBL032W	*HEK2*	RNA binding protein involved in the asymmetric localization of ASH1 mRNA
YBL047C	*EDE1*	Key endocytic protein involved in many interactions with other endocytic proteins and binds membranes in a ubiquitin-dependent manner
YBL048W	*RRT1*	Dubious open reading frame unlikely to encode a protein
YBL050W	*SEC17*	Peripheral membrane protein required for vesicular transport between ER and Golgi
YBL055C	N/A	3'-->5' exonuclease and endonuclease with a possible role in apoptosis
YBL060W	*YEL1*	Guanine nucleotide exchange factor specific for Arf3p required for localization to the bud neck and tip
YBL069W	*AST1*	Peripheral membrane protein that interacts with the plasma membrane ATPase Pma1p and has a role in its targeting to the plasma membrane, possibly by influencing its incorporation into lipid rafts"
YBL071C	N/A	Dubious open reading frame, predicted protein contains a peroxisomal targeting signal"
YBL075C	*SSA3*	ATPase involved in protein folding and the response to stress; member of the heat shock protein 70 (HSP70) family
YBL084C	*CDC27*	Subunit of the Anaphase-Promoting Complex/Cyclosome (APC/C) required for degradation of anaphase inhibitors during metaphase/anaphase transition
YBL094C	N/A	Dubious open reading frame unlikely to encode a protein
YBR090C-A	N/A	Putative protein of unknown function; green fluorescent protein (GFP)-fusion protein localizes to the cytoplasm and nucleus
YBR097W	*VPS15*	Myristoylated serine/threonine protein kinase involved in vacuolar protein sorting
YBR175W	*SWD3*	Essential subunit of the COMPASS (Set1C) complex required in transcriptional silencing near telomeres
YBR176W	*ECM31*	Ketopantoate hydroxymethyltransferase required for pantothenic acid biosynthesis
YBR188C	*NTC20*	Member of the NineTeen Complex (NTC) that binds to snRNAs
YBR260C	*RGD1*	GTPase-activating protein (RhoGAP) for Rho3p and Rho4p, possibly involved in control of actin cytoskeleton organization
YCL007C	N/A	Dubious open reading frame unlikely to encode a protein

Appendix IV – Continued

ORF	Gene	Gene function
YCL021W-A	YCL021W-A	Putative protein of unknown function
YCL026C-B	YCL026C-B	Putative protein of unknown function similar to bacterial nitroreductases
YCL051W	*LRE1*	Protein involved in control of cell wall structure and stress response
YCR002C	*CDC10*	Component of the septin ring of the mother-bud neck that is required for cytokinesis
YCR006C	N/A	Dubious open reading frame unlikely to encode a protein
YCR073W-A	*SOL2*	Protein possibly involved in tRNA export
YCR085W	N/A	Dubious open reading frame unlikely to encode a protein
YDL038C	N/A	Merged open reading frame; does not encode discrete protein
YDL043C	*PRP11*	Subunit of the SF3a splicing factor complex required for spliceosome assembly
YDL053C	*PBP4*	Pbp1p binding protein
YDL096C	*OPI6*	Dubious open reading frame unlikely to encode a protein
YDL115C	*IWR1*	RNA polymerase II transport factor
YDL120W	*YFH1*	Mitochondrial matrix iron chaperone
YDL159W-A	N/A	Putative protein of unknown function
YDL160C-A	N/A	Protein of unknown function
YDR003W	*RCR2*	Vacuolar protein potentially involved in the endosomal-vacuolar trafficking pathway
YDR114C	N/A	Putative protein of unknown function
YDR121W	*DPB4*	Shared subunit of DNA polymerase (II) epsilon and of ISW2/yCHRAC chromatin accessibility complex
YDR246W	*TRS23*	Subunit of the transport protein particle (TRAPP) complex of the cis-Golgi responsible for vesicle docking and fusion
YDR249C	N/A	Putative protein of unknown function
YDR250C	N/A	Dubious open reading frame unlikely to encode a protein
YDR251W	*PAM1*	Essential protein of unknown function
YDR279W	*RNH202*	Ribonuclease H2 subunit
YDR288W	*NSE3*	Essential subunit of the Mms21-Smc5-Smc6 complex; protein of unknown function; required for DNA repair and growth
YDR292C	*SRP101*	Signal recognition particle (SRP) receptor alpha subunit; involved in SRP-dependent protein targeting
YDR299W	*BFR2*	Essential component of 90S pre-ribosome possibly involved in rRNA processing
YDR304C	*CPR5*	Peptidyl-prolyl cis-trans isomerase (cyclophilin) of the endoplasmic reticulum
YDR315C	*IPK1*	Inositol 1,3,4,5,6-pentakisphosphate 2-kinase
YDR317W	*HIM1*	Protein of unknown function involved in DNA repair
YDR318W	*MCM21*	Protein involved in minichromosome maintenance
YDR328C	*SKP1*	Conserved kinetochore protein that is part of multiple protein complexes
YDR329C	*PEX3*	Peroxisomal membrane protein (PMP) required for proper localization and stability of PMPs
YDR330W	*UBX5*	UBX (ubiquitin regulatory X) domain-containing protein that interacts with Cdc48p
YDR424C	*DYN2*	Cytoplasmic light chain dynein; possibly involved in nuclear pore complex assembly
YDR433W	N/A	Dubious open reading frame unlikely to encode a functional protein
YDR507C	*GIN4*	Protein kinase involved in bud growth and assembly of the septin ring
YEL041W	*YEF1*	ATP-NADH kinase
YEL066W	*HPA3*	D-Amino acid N-acetyltransferase

Appendix IV – Continued

ORF	Gene	Gene function
YEL071W	*DLD3*	D-lactate dehydrogenase
YER004W	*FMP52*	Protein of unknown function, localized to the mitochondrial outer membrane
YER121W	N/A	Putative protein of unknown function
YER130C	N/A	Protein of unknown function
YFL014W	*HSP12*	Plasma membrane protein involved in maintaining membrane organization in stress conditions
YFL028C	*CAF16*	Part of conserved CCR4-NOT regulatory complex
YFL030W	*AGX1*	Alanine:glyoxylate aminotransferase (AGT); part of glycine biosynthesis
YFL031W	*HAC1*	Basic leucine zipper (bZIP) transcription factor (ATF/CREB1 homolog) that regulates the unfolded protein response
YFL041W	*FET5*	Multicopper oxidase; integral membrane protein possibly involved in iron transport
YFL042C	N/A	Putative protein of unknown function; not an essential gene
YFL048C	*EMP47*	Integral membrane component of endoplasmic reticulum-derived COPII-coated vesicles involved in ER to Golgi transport
YFL051C	N/A	Putative protein of unknown function; not an essential gene
YFL054C	N/A	Putative channel-like protein which mediates passive diffusion of glycerol in the presence of ethanol
YFL063W	N/A	Dubious open reading frame
YFR007W	*YFH7*	Putative kinase with similarity to the phosphoribulokinase/uridine kinase/bacterial pantothenate kinase (PRK/URK/PANK) subfamily of P-loop kinases
YFR011C	*AIM13*	Putative protein of unknown function
YFR049W	*YMR31*	Mitochondrial ribosomal protein of the small subunit
YGL006W	*PMC1*	Vacuolar Ca2+ ATPase involved in sequestering Ca2+ ions in cytosol
YGL007W	*BRP1*	Dubious ORF located in the upstream region of PMA1
YGL012W	*ERG4*	C-24(28) sterol reductase; catalyzes the final step in ergosterol biosynthesis
YGL017W	*ATE1*	Arginyl-tRNA-protein transferase
YGL020C	*GET1*	Subunit of the GET complex involved in insertion of proteins into the ER membrane
YGL031C	*RPL24A*	Ribosomal protein L30 of the large (60S) ribosomal subunit
YGL033W	*HOP2*	Meiosis-specific protein that prevents synapsis between nonhomologous chromosomes and ensures synapsis between homologs
YGL044C	*RNA15*	Cleavage and polyadenylation factor I (CF I) component involved in cleavage and polyadenylation of mRNA 3' ends
YGL046W	N/A	Unknown
YGL101W	N/A	Putative protein of unknown function; non-essential gene
YGL134W	*PCL10*	Pho85p cyclin involved in phosphorylation
YGR029W	*ERV1*	Flavin-linked sulfhydryl oxidase of the mitochondrial intermembrane space (IMS)
YGR108W	*CLB1*	B-type cyclin involved in cell cycle progression from G2 to M phase
YGR139W	N/A	Dubious ORF unlikely to encode a functional protein
YGR142W	*BTN2*	v-SNARE binding protein that facilitates specific protein retrieval from a late endosome to the Golgi; modulates arginine uptake
YGR199W	*PMT6*	Protein O-mannosyltransferase; essential for cell wall rigidity
YGR223C	*HSV2*	Phosphatidylinositol 3,5-bisphosphate-binding protein involved in micronucleophagy

Appendix IV – Continued

ORF	Gene	Gene function
YGR224W	*AZR1*	Plasma membrane transporter of the major facilitator superfamily, involved in resistance to azole
YGR250C	N/A	Putative RNA binding protein; localizes to stress granules induced by glucose deprivation
YGR264C	*MES1*	Methionyl-tRNA synthetase
YGR275W	*RTT102*	Component of both SWI/SNF and RSC chromatin remodeling complexes
YGR280C	*PXR1*	Essential protein involved in rRNA and snoRNA maturation
YHL023C	*NPR3*	Subunit conserved Npr2/3 complex involved in downregulation of TORC1 activity upon amino acid limitation
YHR007C	*ERG11*	Lanosterol 14-alpha-demethylase, part of ergosterol biosynthesis pathway
YHR146W	*CRP1*	Protein that binds to cruciform DNA structures
YHR150W	*PEX28*	Peroxisomal integral membrane peroxin, involved in the regulation of peroxisomal size, number and distribution
YHR151C	*MTC6*	Protein of unknown function
YIL004C	*BET1*	Type II membrane protein required for vesicular transport between the endoplasmic reticulum and Golgi complex
YIL009C-A	*EST3*	Component of the telomerase holoenzyme involved in telomere replication
YIL020C	*HIS6*	Phosphoribosyl-5-amino-1-phosphoribosyl-4-imidazolecarboxiamide isomerase, part the fourth step in histidine biosynthesis
YIL021W	*RPB3*	NA polymerase II third largest subunit B44, part of central core
YIL039W	*TED1*	Conserved phosphoesterase domain-containing protein that acts together with Emp24p/Erv25p in cargo exit from the ER
YIL064W	*SEE1*	Probable lysine methyltransferase involved in the dimethylation of eEF1A (Tef1p/Tef2p); involved in vesicular transport
YIL076W	*SEC28*	ε-COP subunit of the coatomer; regulates retrograde Golgi-to-ER protein traffic
YIL077C	N/A	Putative protein of unknown function
YIL078W	*THS1*	Threonyl-tRNA synthetase, essential cytoplasmic protein
YIL086C	N/A	Dubious open reading frame unlikely to encode a functional protein
YIL123W	*SIM1*	Protein of SUN family (Sim1p, Uth1p, Nca3p, Sun4p) with possible role in DNA replication
YIR017C	*MET28*	Basic leucine zipper (bZIP) transcriptional activator in the Cbf1p-Met4p-Met28p complex; participates in the regulation of sulfur metabolism
YIR020W-B	N/A	Unknown
YIR021W	*MRS1*	Protein required for the splicing of two mitochondrial group I introns (BI3 in COB and AI5beta in COX1
YIR022W	*SEC11*	18kDa catalytic subunit of the Signal Peptidase Complex (SPC; Spc1p, Spc2p, Spc3p, and Sec11p); cleaves the signal sequence of proteins targeted to the endoplasmic reticulum
YIR024C	N/A	Protein of unknown function
YIR025W	*MND2*	Subunit of the anaphase-promoting complex (APC); required to maintain sister chromatid cohesion in prophase I of meiosis
YIR031C	*DAL7*	Malate synthase, unknown role in allantoin degradation
YIR033W	*MGA2*	ER membrane protein involved in regulation of OLE1 transcription
YIR039C	*YPS6*	Putative GPI-anchored aspartic protease, member of the yapsin family of proteases involved in cell wall growth and maintenance
YJL059W	*YHC3*	Vacuolar membrane protein involved in the ATP-dependent transport of arginine into the vacuole and possibly in balancing ion homeostasis

Appendix IV – Continued

ORF	Gene	Gene function
YJL090C	*DPB11*	Replication initiation protein that loads DNA pol epsilon onto pre-replication complexes at origins
YJL091C	*GWT1*	Protein involved in the inositol acylation of glucosaminyl phosphatidylinositol (GlcN-PI) to form glucosaminyl(acyl)phosphatidylinositol (GlcN(acyl)PI), an intermediate in the biosynthesis of glycosylphosphatidylinositol (GPI) anchors
YJL111W	*CCT7*	Subunit of the cytosolic chaperonin Cct ring complex required for the assembly of actin and tubulins in vivo
YJL136W-A	N/A	Dubious open reading frame unlikely to encode a protein
YJR062C	*NTA1*	Amidase, removes the amide group from N-terminal asparagine and glutamine residues
YJR063W	*RPA12*	RNA polymerase I subunit A12.2
YJR118C	*ILM1*	Protein of unknown function; may be involved in mt-DNA maintenance
YJR135W-A	*TIM8*	Mitochondrial intermembrane space protein, forms a complex with Tim13p that delivers a subset of hydrophobic proteins to the TIM22 complex for inner membrane insertion
YKL054C	*DEF1*	RNAPII degradation factor, forms a complex with Rad26p in chromatin
YKL056C	*TMA19*	Protein that associates with ribosomes
YKL059C	*MPE1*	Essential conserved subunit of CPF (cleavage and polyadenylation factor); involvedin 3' end formation of mRNA
YKL060C	*FBA1*	Fructose 1,6-bisphosphate aldolase, required for glycolysis and gluconeogenesis
YKL064W	*MNR2*	Vacuolar membrane protein required for magnesium homeostasis; putative magnesium transporter
YKL068W-A	N/A	Putative protein of unknown function
YKL096C-B	N/A	Putative protein of unknown function
YKL154W	*SRP102*	Signal recognition particle (SRP) receptor beta subunit; involved in SRP-dependent protein targeting
YKL155C	*RSM22*	Mitochondrial ribosomal protein of the small subunit; predicted to be an S-adenosylmethionine-dependent methyltransferase
YKL156W	*RPS27A*	Protein component of the small (40S) ribosomal subunit
YKL157W	*APE2*	Aminopeptidase yscII; may have a role in obtaining leucine from dipeptide substrates
YKL205W	*LOS1*	Nuclear pore protein involved in nuclear export of pre-tRNA and in re-export of mature tRNAs after their retrograde import from the cytoplasm
YKL208W	*CBT1*	Protein involved in 5' end processing of mitochondrial COB, 15S_rRNA, and RPM1 transcripts; may also have a role in 3' end processing of the COB pre-mRNA
YKL213C	*DOA1*	WD repeat protein required for ubiquitin-mediated protein degradation
YKR004C-A	*YKR004C*	Merged open reading frame, does not encode a discreet protein
YKR009C	*FOX2*	Multifunctional enzyme of the peroxisomal fatty acid beta-oxidation pathway
YKR042W	*UTH1*	Mitochondrial outer membrane and cell wall localized SUN family member involved in cell wall biogenesis and mitochondrial autophagy
YKR044W	*UIP5*	Protein of unknown function that interacts with Ulp1p, a Ubl (ubiquitin-like protein)-specific protease
YKR045C	N/A	Putative protein of unknown function
YKR049C	*FMP46*	Putative redox protein containing a thioredoxin fold
YKR051W	N/A	Putative protein of unknown function
YKR055W	*RHO4*	Non-essential small GTPase of the Rho/Rac subfamily of Ras-like proteins; likely to be involved in the establishment of cell polarity

Appendix IV – Continued

ORF	Gene	Gene function
YKR067W	*GPT2*	Glycerol-3-phosphate/dihydroxyacetone phosphate dual substrate-specific sn-1 acyltransferase located in lipid particles and the ER; involved in lipid biosynthesis
YKR069W	*MET1*	S-adenosyl-L-methionine uroporphyrinogen III transmethylase, involved in the biosynthesis of siroheme and methionine biosynthesis
YKR081C	*RPF2*	Essential protein involved in the processing of pre-rRNA and the assembly of the 60S ribosomal subunit
YLL017W	N/A	Non-essential Ras guanine nucleotide exchange factor (GEF)
YLL018C	*DPS1*	Aspartyl-tRNA synthetase
YLR067C	*PET309*	Specific translational activator for the COX1 mRNA
YLR081W	*GAL2*	Galactose permease, required for utilization of galactose; also able to transport glucose
YLR082C	*SRL2*	Protein of unknown function
YLR182W	*SWI6*	Transcription cofactor, forms complexes with Swi4p and Mbp1p to regulate transcription at the G1/S transition
YLR188W	*MDL1*	Mitochondrial inner membrane half-type ATP-binding cassette (ABC) transporter; mediates export of peptides generated upon proteolysis of mitochondrial proteins; involved in regulation of resistance to oxidative stress
YLR208W	*SEC13*	Component of the Nup84 nuclear pore sub-complex, the Sec13p-Sec31p complex of the COPII vesicle coat, and the SEA (Seh1-associated) complex; required for vesicle formation in ER to Golgi transport and nuclear pore complex organization
YLR211C	N/A	Putative protein of unknown function; not an essential gene
YLR212C	*TUB4*	Gamma-tubulin, involved in nucleating microtubules from both the cytoplasmic and nuclear faces of the spindle pole body
YLR213C	*CRR1*	Putative glycoside hydrolase of the spore wall envelope; required for normal spore wall assembly, possibly for cross-linking between the glucan and chitosan layers; expressed during sporulation
YLR332W	*MID2*	O-glycosylated plasma membrane protein that acts as a sensor for cell wall integrity signaling and activates the pathway
YLR460C	N/A	Member of the quinone oxidoreductase family
YLR461W	*PAU4*	Member of the seripauperin multigene family encoded mainly in subtelomeric regions; active during alcoholic fermentation
YML009C	*MRPL39*	Mitochondrial ribosomal protein of the large subunit
YML107C	*PML39*	Protein required for nuclear retention of unspliced pre-mRNAs along with Mlp1p and Pml1p
YML108W	N/A	Putative protein of unknown function
YMR078C	*CTF18*	Subunit of a complex with Ctf8p that shares some subunits with Replication Factor C and is required for sister chromatid cohesion
YMR083W	*ADH3*	Mitochondrial alcohol dehydrogenase isozyme III; involved in the shuttling of mitochondrial NADH to the cytosol under anaerobic conditions and ethanol production
YMR089C	*YTA12*	Component of mitochondrial inner membrane m-AAA protease that mediates degradation of misfolded or unassembled proteins; required for correct assembly of mitochondrial enzyme complexes
YMR125W	*STO1*	Large subunit of the nuclear mRNA cap-binding protein complex; interacts with Npl3p to carry nuclear poly(A)+ mRNA to cytoplasm; also involved in nuclear mRNA degradation and telomere maintenance

Appendix IV – Continued

ORF	Gene	Gene function
YMR130W	N/A	Putative protein of unknown function; not an essential gene
YMR173W-A	N/A	Dubious open reading frame unlikely to encode a protein
YMR174C	*PAI3*	Cytoplasmic proteinase A (Pep4p) inhibitor
YMR175W	*SIP18*	Phospholipid-binding hydrophilin with a role in dessication resistance; expression is induced by osmotic stress
YMR194C-B	*CMC4*	Protein localized to mitochondrial intermembrane space via the Mia40p-Erv1p system; contains twin cysteine-x(9)-cysteine motifs
YMR303C	*ADH2*	Glucose-repressible alcohol dehydrogenase II
YNL011C_1	N/A	Unknown
YNL031C	*HHT2*	Histone H3, core histone protein required for chromatin assembly
YNL032W	*SIW14*	Tyrosine phosphatase that plays a role in actin filament organization and endocytosis; localized to the cytoplasm
YLL019C	*KNS1*	Nonessential putative protein kinase of unknown function
YLL027W	*ISA1*	Mitochondrial matrix protein involved in biogenesis of the iron-sulfur (Fe/S) cluster of Fe/S proteins
YNL034W	N/A	Putative protein of unknown function; not an essential gene
YNL038W	*GPI15*	Protein involved in the synthesis of N-acetylglucosaminyl phosphatidylinositol (GlcNAc-PI), first intermediate in the synthesis of glycosylphosphatidylinositol (GPI) anchors
YNL064C	*YDJ1*	Type I HSP40 co-chaperone involved in regulation of the HSP90 and HSP70 functions; involved in protein translocation across membranes; member of the DnaJ family
YNL081C	*SWS2*	Putative mitochondrial ribosomal protein of the small subunit; involved in controlling sporulation efficiency
YNL084C	*END3*	EH domain-containing protein involved in endocytosis, actin cytoskeletal organization and cell wall morphogenesis
YNL086W	*SNN1*	Putative protein of unknown function; likely member of BLOC complex involved in endosomal cargo sorting
YNL162W	*RPL42A*	Protein component of the large (60S) ribosomal subunit
YNL183C	*NPR1*	Protein kinase that stabilizes several plasma membrane amino acid transporters by antagonizing their ubiquitin-mediated degradation
YNL237W	*YTP1*	Probable type-III integral membrane protein of unknown function, has regions of similarity to mitochondrial electron transport proteins
YNL274C	*GOR1*	Glyoxylate reductase
YNR023W	*SNF12*	73 kDa subunit of the SWI/SNF chromatin remodeling complex involved in transcriptional regulation
YNR072W	*HXT17*	Hexose transporter
YOL004W	*SIN3*	Component of the Sin3p-Rpd3p histone deacetylase complex, involved in transcriptional repression and activation of diverse processes, including mating-type switching and meiosis; involved in the maintenance of chromosomal integrity
YOL013W-A	N/A	Putative protein of unknown function
YOL091W	*SPO21*	Component of the meiotic outer plaque of the spindle pole body, involved in modifying the meiotic outer plaque that is required prior to prospore membrane formation
YOL093W	*TRM10*	tRNA methyltransferase, methylates the N-1 position of guanosine in tRNAs

Appendix IV – Continued

ORF	Gene	Gene function
YOL096C	*COQ3*	O-methyltransferase, catalyzes two different O-methylation steps in ubiquinone (Coenzyme Q) biosynthesis; component of a mitochondrial ubiquinone-synthesizing complex
YOL098C	N/A	Putative metalloprotease
YOL099C	N/A	Dubious open reading frame unlikely to encode a protein
YOL100W	*PKH2*	Serine/threonine protein kinase involved in sphingolipid-mediated signaling pathway that controls endocytosis; activates Ypk1p and Ykr2p, components of signaling cascade required for maintenance of cell wall integrity
YOL122C	*SMF1*	Divalent metal ion transporter with a broad specificity for di-valent and tri-valent metals
YOL131W	N/A	Putative protein of unknown function
YOL132W	*GAS4*	1,3-beta-glucanosyltransferase, involved with Gas2p in spore wall assembly
YOL133W	*HRT1*	RING finger containing subunit of Skp1-Cullin-F-box ubiquitin protein ligases (SCF)
YOL134C	N/A	Dubious open reading frame unlikely to encode a protein
YOL141W	*PPM2*	AdoMet-dependent tRNA methyltransferase also involved in methoxycarbonylation
YOR013W	*IRC11*	Dubious opening reading frame unlikely to encode a protein
YOR077W	*RTS2*	Basic zinc-finger protein involved in UV response and DNA replication"
YOR098C	*NUP1*	Nuclear pore complex (NPC) subunit, involved in protein import/export and in export of RNAs
YOR149C	*SMP3*	Alpha 1,2-mannosyltransferase involved in glycosyl phosphatidyl inositol (GPI) biosynthesis; required for addition of the fourth, side branching mannose to the GPI core structure
YOR223W	N/A	Protein of unknown function found in the ER and vacuole lumen
YOR239W	*ABP140*	AdoMet-dependent tRNA methyltransferase and actin binding protein
YOR289W	N/A	Putative protein of unknown function
YOR290C	*SNF2*	Catalytic subunit of the SWI/SNF chromatin remodeling complex involved in transcriptional regulation; contains DNA-stimulated ATPase activity
YOR293C-A	N/A	Putative protein of unknown function
YOR316C-A	N/A	Putative protein of unknown function
YOR386W	*PHR1*	DNA photolyase involved in photoreactivation, repairs pyrimidine dimers in the presence of visible light; induced by DNA damage
YPL096C-A	*ERI1*	Endoplasmic reticulum membrane protein that binds to and inhibits GTP-bound Ras2p at the ER; component of the GPI-GnT complex which catalyzes the first step in GPI-anchor biosynthesis
YPR018W	*RLF2*	Largest subunit (p90) of the Chromatin Assembly Complex (CAF-1)
YPR084W	N/A	Putative protein of unknown function
YPR091C	N/A	Putative protein of unknown function; may interact with ribosomes
YPR173C	*VPS4*	AAA-ATPase involved in multivesicular body (MVB) protein sorting; regulates cellular sterol metabolism

[a] code of ORF (open reading frame) which has been deleted from the mutant
[b] gene name associated with a particular ORF annotated from *Saccharomyces* Genome Database (SGD)
[c] function of the associated gene
[d] no known gene associated with this ORF

Appendix V – Phytochemicals affecting microbial quorum sensing (QS) and/or biofilm formation published as of December 2014.

Active constituents	Source plant(s)	Biological activities	Reference
Sulfur-containing compounds			
allicin	*Allium sativum* L. (Amaryllidaceae)	• Inhibited biofilm formation by >90 % in *S. epidermidis* strains at 4 mg/mL • Reduced adhesion of GFP-transformed *P. aeruginosa* PAO1, EPS production (70%), biofilm thickness (50%), and expression of virulence factors at 128 μg/mL	Pérez-Giraldo et al. 2003 Lin et al. 2013
ajoene	*Allium sativum* L. (Amaryllidaceae)	• Inhibited QS in *P. aeruginosa lasB-gfp* (IC_{50} = 15 μM) , *rhlA-gfp* (IC_{50} = 50 μM), *E. coli luxI-gfp* (IC_{50} = 100 μM) reporter strains • Downregulated QS-regulated virulence factors (5-fold at 80 μg.mL) and improved bacteria clearance in mouse infection model (subcutaneous treatment of 25 mg/kg body weight)	Jakobsen et al. 2012
sulforaphane	Brassiceae species	• Inhibited *lasR*-mediated QS in *E. coli* DH5 (pJN105L) (pSC11) completely at 100 μM • Inhibited biofilm formation (60% at 37 μM) and pyocyanin production in *P. aeruginosa* PAO1 (70% at 100 μM)	Ganin et al. 2013
erucin	Brassiceae species	• Inhibited *lasR*-mediated QS in *E. coli* DH5 (pJN105L) (pSC11) completely at 100 μM • Inhibited pyocyanin production in *P. aeruginosa* PAO1 (70% at 100 μM)	Ganin et al. 2013
allyl isothiocyanate	Brassiceae species	• Inhibited biofilm formation in *L. monocytogenes* (61%), *P. aeruginosa* (90%), *E. coli* (100%) at 1 mg/mL • Inhibited violacein production in *C. violaceum* ATCC 12472 (70% at 5 μg/mL)	Borges et al. 2013 Borges et al. 2014
phenylethyl isothiocyanate	Brassiceae species	• Inhibited biofilm formation in *S. aureus* (75% at 1 mg/mL) • Inhibited violacein production in *C. violaceum* ATCC 12472 (47% at 5 μg/mL)	Borges et al. 2013 Borges et al. 2014
benzyl isothiocyanate	Brassicaeae species	• Inhibited violacein production in *C. violaceum* ATCC 12472 (81% at 5 μg/mL)	Borges et al. 2014
Terpenoids			
acanthospermolides	*Acanthospermum hispidum* DC. (Asteraceae)	• Inhibited biofilm formation in *P. aeruginosa* (70% at 2.5 μg/mL)	Cartagena et al. 2007
4-*epi*-pimaric acid	*Aralia cachemirica* Decne. (Araliaceae)	• Inhibited formation of new biofilm (77% at 4 μg/mL) and reduced growth of existing biofilm (59% at 64 μg/mL) in *S. mutans*	Ali et al. 2012
thymol	*Thymus vulgaris* L. (Lamiaceae)	• Inhibited formation of new and inactivated preformed biofilms in *L. monocytogenes* (0.5 mM and 5 mM, respectively) • Downregulated genes critical to *L. monocytogenes* biofilm formation (0.5 mM) • Downregulated expression of enterotoxin genes in *S. aureus* at 64μg/mL (>5-fold) • Inhibited formation of biofilm in *P. aeruginosa* ATCC 27853, CIP A22, and IL5 at 0.1% by 86%, 54%, and 70%, respectively	Upadyay et al. 2013 Qiu et al. 2010a Soumya et al. 2011
carvacrol	*Thymus vulgaris* L. and other Lamiaceae species	• Inhibited formation of new and inactivated preformed biofilms in *L. monocytogenes* (0.65 mM and 10 mM, respectively) • Downregulated genes critical to *L. monocytogenes* biofilm formation (0.65 mM) • Inhibited *P. aeruginosa* ATCC 27853, CIP A22, and IL5 biofilms at 0.04% by >90%	Upadyay et al. 2013 Soumya et al. 2011

Appendix V – Continued

Active constituents	Source plant(s)	Biological activities	Reference
salvipisone	*Salvia sclarea* L. (Lamiaceae)	• Reduced preformed biofilm of *S. epidermidis* RP12 (>90%), 6756/99 (85%) and *S. aureus* 1474/01(85%) at 37.5 μg/mL	Kuźma et al. 2007 Walencka et al. 2007
aethiopinone	*Salvia sclarea* L. (Lamiaceae)	• Reduced preformed biofilm of *S. epidermidis* RP12 (83% at 18.75 μg/mL), 6756/99 (67% at 9.375 μg/mL) and *S. aureus* 1474/01(67% at 37.5 μg/mL)	Walencka et al. 2007
isolimonic acid	*Citrus* × *auratium* L. (Rutaceae)	• Inhibited AI-induced bioluminescence in *V. harveyi* BB170 (60% at 6.25 μg/mL) and biofilm formation in *V. harveyi* BB120 (40% at 100 μg/mL)	Vikram et al. 2011
ichangin	*Citrus* × *auratium* L. (Rutaceae)	• Inhibited AI-induced bioluminescence in *V. harveyi* BB170 (90%) and biofilm formation in *V. harveyi* BB120 (40%) at 100 μg/mL	Vikram et al. 2011
deacetylnomilinic acid glucoside	*Citrus* × *auratium* L. (Rutaceae)	• Inhibited AI-induced bioluminescence in *V. harveyi* BB170 by 40% at 6.25 μg/mL	Vikram et al. 2011
isoobacunoic acid	*Citrus* × *auratium* L. (Rutaceae)	• Inhibited AI-induced bioluminescence in *V. harveyi* BB170 by 90% at 6.25 μg/mL	Vikram et al. 2011
isoobacunoic acid glucoside	*Citrus* × *auratium* L. (Rutaceae)	• Inhibited AI-induced bioluminescence in *V. harveyi* BB170 by 70% at 6.25 μg/mL	Vikram et al. 2011
1,4-dihydroxy-2E,6E-12E-triene-5-one-casbane	*Croton nepetifolius* Baill. (Euphorbiaceae)	• Inhibited formation of new and existing single and mixed-species biofilms of *S. aureus* JKD 6008, *P. aeruginosa* ATCC 10145, *C. albicans* ATCC 90028, and *C. glabrata* ATCC 2001 (40 to 70% at 31.25 to 500 μg/mL	Vasconcelos et al. 2014
betulinic acid	various species	• Enhanced biofilm formation in *P. aeruginosa* PA14 at 100 μg/mL	Cho HS et al. 2013
3*α*-*O*-*cis*-*p*-Coumaroyl-20*α*-hydroxy-12-ursen-28-oic acid	*Diospyros dendo* Welw. ex Hiern (Ebenaceae)	• Inhibited biofilm formation in *P. aeruginosa* PAO1 by 62% at 10 μg/mL	Hu et al. 2006
3*β*-*O*-*trans*-*p*-coumaroyl-2*α*-hydroxy-12-ursen-28-oic acid	various species	• Inhibited biofilm formation in *P. aeruginosa* PAO1 by 35% at 10 μg/mL	Hu et al. 2006
***3β*-*O*-*cis*-*p*-coumaroyl-2*α*-hydroxy-12-ursen-28-oic acid**	various species	• Inhibited biofilm formation in *P. aeruginosa* PAO1 by 32% at 10 μg/mL	Hu et al. 2006
3*β* -*O*-*trans*-feruloyl-2 *α* -hydroxy-12-ursen-28-oic acid	various species	• Inhibited biofilm formation in *P. aeruginosa* PAO1 by 48% at 10 μg/mL	Hu et al. 2006
ursolic acid	various species	• Inhibited biofilm formation in *P. aeruginosa* PAO1 (>87%), *E. coli* JM109 (50%), and *V. harveyi* BB120 (57%) at 10 μg/mL • Induced expression of chemotaxic and motility genes and repressed sulphur metabolism in *E. coli* K-12 at 10 μg/mL • Inhibited biofilm formation in *P.aeruginosa* PAO1 by 35% at 10 μg/mL	Ren et al. 2005 Hu et al. 2006

Appendix V – Continued

Active constituents	Source plant(s)	Biological activities	Reference
gymnemic acids	*Gymnema sylvestre* (Retz.) R.Br. ex Sm. (Apocynaceae) and Asclepiadaceae species	• Mixture of 4 acids at 40 μg/mL inhibited yeast-to-hypha transition in *C. albicans* SC5314 and induced conversion of hyphae back to yeast form (100% after 11 hours) • Inhibited conidial germination and hyphal growth in *Aspergillus fumigates* by 74% at 40 μg/mL	Vediyappan et al. 2013
Flavonoids and stilbenoids			
***trans*-stilbene**	various species	• Enhanced biofilm formation in *P. aeruginosa* PA14 at 100 μg/mL	Cho HS et al. 2013
resveratrol	Vitaceae, Ericaceae species, *Carex* species (Cyperaceae)	• Inhibited *S. aureus* biofilm formation (30%) and enhanced *S. epidermidis* biofilm formation (1.5-fold) at 100 μg/mL • Inhibited *P. acnes* biofilm formation by 80% at 0.32% • Inhibited biofilm formation in *P. aeruginosa* PA14 and *E. coli* O157:H7 at 50 μg/mL	Moran et al. 2014 Coenye et al. 2012 Cho HS et al. 2013
pterostilbene	*Vitis* and Ericaceae species	• Inhibited formation of new and mature *C. albicans* SC5314, Y0109, 0304103, and 01010 biofilms at 16 μg/mL • Inhibited hyphal formation in *C. albicans* at 4 μg/mL • Treatment of 16 μg/mL altered expression of genes involved in morphological transition, ergosterol biosynthesis, filamentation, and cell surface proteins; also effective in rat central venous catheter infection model	Li DD et al. 2014
ε-viniferin	*Carex* species (Cyperaceae)	• Inhibited biofilm formation in *P. aeruginosa* PA14 and *E. coli* O157:H7 at 50 μg/mL	Cho HS et al. 2013
vitisin A	*Vitis* species (Vitaceae)	• Inhibited biofilm formation in *E. coli* O157:H7 (>70% at 10 μg/mL)	Lee J et al. 2014
vitisin B	*Vitis* species (Vitaceae)	• Inhibited biofilm formation in *E. coli* O157:H7 (>70% at 10 μg/mL)	Lee J et al. 2014
naringenin	*Citrus × paradisi* Macfad. and other Rutaceae species	• Inhibited AI-induced bioluminescence in *V. harveyi* BB886, MM32 (80% at 6.25 μg/mL) and biofilm formation in *E. coli* O157:H7, *V. harveyi* BB120 (50% at 6.25 μg/mL)	Vikram et al. 2010
naringin	*Citrus × paradisi* Macfad. and other Rutaceae species	• Inhibited biofilm formation in *E. coli* O157:H7 (50% at 100 μg/mL) and *V. harveyi* BB120 (50%)	Vikram et al. 2010
neohesperidin	*Citrus* spp. (Rutaceae)	• Inhibited AI-induced bioluminescence in *V. harveyi* MM32 by 50% at 12.5 μg/mL	Vikram et al. 2010
kaempferol	various species	• Inhibited AI-induced bioluminescence in *V. harveyi* BB886, MM32 (100% at 6.25 μg/mL) and biofilm formation in *E. coli* O157:H7, *V. harveyi* BB120 (100% at 6.25 μg/mL)	Vikram et al. 2010
quercetin	various species	• Inhibited AI-induced bioluminescence in *V. harveyi* BB886, MM32 (75% at 6.25 μg/mL) and biofilm formation in *E. coli* O157:H7, *V. harveyi* BB120 (60% at 6.25 μg/mL) • Inhibited biofilm formation in MRSA (>80%) and MSSA (>50%) strains at 1 μg/mL • Reduced expression of genes involved in QS and virulence in *S. aureus* at 10 μg/mL	Vikram et al. 2010 Lee et al. 2013

Appendix V – Continued

Active constituents	Source plant(s)	Biological activities	Reference
apigenin	various species	• Inhibited AI-induced bioluminescence in *V. harveyi* BB886, MM32 (60% at 6.25 μg/mL) and biofilm formation in *E. coli* O157:H7 (60% at 12.5 μg/mL) • Reduced biofilm mass (30%) and toxic end products production in *S. mutans* UA159 at 1.33 mM	Vikram et al. 2010 Koo et al. 2003
isoorientin	various species	• Inhibited violacein production in *C. violaceum* ATCC 31532 (40 μg/mL) and bioluminescence in *E. coli* PSB403 (45 μg/mL)	Brango-Vanegas et al. 2014
orientin	various species	• Inhibited violacein production in *C. violaceum* ATCC 31532 at 50 μg/mL	Brango-Vanegas et al. 2014
isovitexin	various species	• Inhibited bioluminescence in *E. coli* PSB403 at 45 μg/mL	Brango-Vanegas et al. 2014
rutin	various species	• Inhibited violacein production in *C. violaceum* ATCC 31532 (15 μg/mL) and bioluminescence in *E. coli* PSB403 (30 μg/mL)	Brango-Vanegas et al. 2014
sinensetin	*Citrus sinensis* (L.) Histoire (Rutaceae) and others	• Inhibited biofilm formation in *E. coli* O157:H7, *V. harveyi* BB120 by 60% at 12.5 μg/mL	Vikram et al. 2010
fisetin	various species	• Inhibited biofilm formation in *S. aureus* (90%) and *S. dysgalactiae* (70%) at 16 μg/mL	Dürig et al. 2010
genistein	Many species	• Inhibited biofilm formation in *S. aureus* (45%) and enhanced *S. epidermidis* biofilms (>10-fold) at 500 μg/mL	Moran et al. 2014
icariin	Berberidaceae species	• Inhibited biofilm formation in *P. acnes* by 70% at 0.08%	Coenye et al. 2012
baicalin	*Scutellaria* species (Lamiaceae)	• Inhibited QS in *E. coli* JB523 and biofilm formation in *B. multivorans* LMG13010, LMG17588, *B. cenocepacia* LMG18828 (40 to 60% at 100 μM)	Brackman et al. 2009
phloretin	*Malus* species (Rosaceae)	• Inhibited biofilm formation in *E. coli* O157:H7 (89% at 25 μg/mL)	Lee et al. 2011
licochalcone A	*Glycyrrhiza* species (Fabaceae)	• Downregulated expression of enterotoxin genes in *S. aureus* ATCC 29213 and MRSA 2985 at 2 μg/mL after 4 hour growth • Treatment of 64 μg/mL to mature *S. aureus* ATCC 29213 biofilms downregulated expression of pathogenic factors, cell wall and biofilm-related proteins	Qiu et al. 2010b Shen et al. 2014
(+)-catechin	various species	• Enhanced biofilm formation in *S. epidermidis* by 4-fold at 2 mg/mL • Increased biofilm formation in *E. corrodens* at 1 mM	Moran et al. 2014 Matsunaga et al. 2010
(–)-catechin	*Camellia sinensis* (L.) Kuntze (Theaceae) and others	• Inhibited violacein production in *C. violaceum* CV026 (50% at 2 mM), pyocyanin (50% at 0.25 mM) and elastase production (30% at 4 mM) in *P. aeruginosa* PAO1 • Reduced biofilm formation and downregulated QS genes expression in *P. aeruginosa* PAO1 by at 4 mM	Vandeputte et al. 2010

Appendix V – Continued

Active constituents	Source plant(s)	Biological activities	Reference
(–)-epicatechin	*Camellia sinensis* (L.) Kuntze (Theaceae) and others	• Inhibited elastase activity in *P. aeruginosa* PAO1 by 40% at 4 mM • Enhanced biofilm formation in *P. aeruginosa* PAO1 (2-fold at 200 μg/mL) and *A. tumefaciens* C58 (2-fold at 400 μg/mL) • Increased AHL production in *E. coli* JDL271/pAL105 at 40 to 200 μg//mL • Inhibited violacein production in *C. violaceum* ATCC 12472 (33% at 1 mg/mL) • Inhibited biofilm formation in *E. coli* JM109 by 40% at 1 mg/mL	Vandeputte et al. 2010 Plyuta et al. 2013 Borges et al. 2014
(–)-gallocatechin	*Camellia sinensis* (L.) Kuntze (Theaceae)	• Inhibited biofilm formation in *E. corrodens* by >60% at 1 mM (MIC = 0.5 mM)	Matsunaga et al. 2010
(–)-epigallocatechin	*Camellia sinensis* (L.) Kuntze (Theaceae) and others	• Inhibited biofilm formation in *E. corrodens* by >60% at 1 mM (MIC = 0.25 mM)	Matsunaga et al. 2010
(–)-catechin gallate	*Camellia sinensis* (L.) Kuntze (Theaceae)	• Inhibited biofilm formation in *E. corrodens* by >60% at 1 mM (MIC = 0.1 mM)	Matsunaga et al. 2010
(–)-epicatechin gallate	*Camellia sinensis* (L.) Kuntze (Theaceae) and others	• Inhibited biofilm formation in *E. corrodens* by >60% at 1 mM (MIC = 0.1 mM)	Matsunaga et al. 2010
(–)-gallocatechin gallate	*Camellia sinensis* (L.) Kuntze (Theaceae)	• Inhibited biofilm formation in *E. corrodens* by >60% at 1 mM (MIC = 0.1 mM)	Matsunaga et al. 2010
(–)-epigallocatechin gallate	*Camellia sinensis* (L.) Kuntze (Theaceae)	• Inhibited QS in *E. coli* MT102 (pSB403) and *P. putida* (pKR-C12) (>50% and 40% at 40 μg/mL, respectively) • Reduced biofilm formation (30%) and swarming motility in *B. cepacia* (100%) at 40 μg/mL • Inhibited biofilm formation in *E. corrodens* by >60% at 1 mM (MIC = 0.1 mM)	Huber et al. 2003 Matsunaga et al. 2010
phloridzin	*Malus* species (Rosaceae)	• Inhibited violacein production in *C. violaceum* ATCC 12472 (48% at 1 mg/mL)	Borges et al. 2014
oleuropein glucoside	*Olea europaea* L. (Oleaceae)	• Inhibited violacein production in *C. violaceum* ATCC 12472 (51% at 1 mg/mL)	Borges et al. 2014
cyanidin 3-*O*-galactoside	various species	• Enhanced biofilm formation in *B. cereus* 407 (3-fold) and *E. coli* JM109 (40%) at 1 mg/mL	Bräunlich et al. 2013
cyanidin 3-*O*-glucoside	various species	• Enhanced biofilm formation in *B. cereus* 407 (3-fold) and *E. coli* JM109 (40%) at 1 mg/mL	Bräunlich et al. 2013

Appendix V – Continued

Active constituents	Source plant(s)	Biological activities	Reference
cyanidin 3-*O*-arabinoside	various species	• Enhanced biofilm formation in *B. cereus* 407 (2-fold) and *E. coli* JM109 (30%) at 1 mg/mL	Bräunlich et al. 2013
cyanidin 3- *O*-xyloside	various species	• Inhibited biofilm formation in *B. cereus* 407 (50%) and *E. coli* JM109 (40%) at 1 mg/mL	Bräunlich et al. 2013
procyanidin B5	various species	• Inhibited biofilm formation in *B. cereus* 407 by 50% by 1 mg/mL	Bräunlich et al. 2013
procyanidin C1	various species	• Inhibited biofilm formation in *B. cereus* 407 by 50% by 1 mg/mL	Bräunlich et al. 2013
proanthocyanidin A2	various species	• Inhibited biofilm formation in *S. aureus* 6538P (50% at 7 μM) and *S. epidermidis* RP62A (50% at 8 μM)	Artini et al. 2012
Other phenolics and acid derivatives			
pyrogallol	*Phyllanthus emblica* L. (Phyllanthaceae) and others	• Inhibited AI-2 QS in *V. harveyi* with IC_{50} of 1 to 3 μM • Inhibited biofilm formation (50%) and surface adhesion (58%) in *C. albicans* at 50 μg/mL	Ni et al. 2008 Shahzad et al. 2014
eugenol	various species	• Inhibited *las* QS-mediated elastase production (32% at 200 μM) and *pqs* QS-mediated pyocanin production (56% at 50 μM) in *P. aeruginosa* , QS-mediated violacein production in *C. violaceum* (48% at 150 μM) • Decreased biofilm formation in *P. aeruginosa* PAO1 (43% at 400 μM) • Inhibited formation of new and inactivated preformed biofilms in *L. monocytogenes* (2.5 mM and 25 mM, respectively) • Downregulated genes critical to *L. monocytogenes* biofilm formation (2.5 mM) • Inhibited biofilm formation in *K. pneumoniae* clinical isolates (MIC = 63.5 μg/mL)	Zhou et al. 2013 Upadhyay et al. 2013 Magesh et al. 2013
methyleugenol	various species	• Inhibited violacein production in *C. violaceum* CV026 by 50% at 5 μg/mL • Inhibited bioluminescence in *V. harveyi* MTCC 3438 by 80% at 7.5 μg/mL • Inhibited biofilm formation in *P. aeruginosa* PAO1 by 60% at 7.5 μg/mL	Packiavathy et al. 2012
cinnamaldehyde	*Cinnamomum* species (Lauraceae)	• Inhibited AI-2-mediated QS in *Vibrio* spp. (65% at 100 μM) Inhibited formation of new and inactivated preformed biofilms in *L. monocytogenes* (0.75 mM and 10 mM, respectively) • Downregulated genes involved in *L. monocytogenes* biofilm formation (0.75 mM) • Inhibited biofilm formation in *S. epidermidis* (MIC = 125 μg/mL) • Inhibited formation of new and inactivated mature biofilms in *C. sakazakii* (750 μM and 38 mM, respectively) and downregulated expression biofilm-related genes	Brackman et al. 2008 Upadhyay et al. 2013 Sharma et al. 2014 Amalaradjou and Kumar 2011
zingerone	*Zingiber officinale* Roscoe (Zingiberaceae)	• Inhibited swimming, swarming, and twitching motility (50%) and biofilm formation (25 to 50% over 7 days) in *P. aeruginosa* PAO1 at 10 mg/mL	Kumar et al. 2013
malabaricone C	*Myristica cinnamomea* King (Myristicaceae)	• Inhibited violacein production in *C. violaceum* CV026 (40% at 1 mg/mL) • Reduced by 50% pyocyanin production and biofilm formation in *P. aeruginosa* PAO1 (1 mg/mL)	Chong et al. 2011

Appendix V – Continued

Active constituents	Source plant(s)	Biological activities	Reference
4-hydroxybenzoic acid	various species	• Enhanced biofilm formation in *P. aeruginosa* PAO1 (2-fold at 25 μg/mL) and *A. tumefaciens* C58 (2-fold at 50 μg/mL) • Increased AHL production in *E. coli* JDL271/pAL105 at 40 to 200 μg//mL	Plyuta et al. 2013
protocatechuic acid	various species	• Increased biofilm formation in *S. epidermidis* by 4-fold at 1.5 mg/mL	Moran et al. 2014
ginkgolic acid	*Ginkgo biloba* L. (Ginkgoaceae)	• Inhibited biofilm formation in *E. coli* O157:H7 (>70% at 10 μg/mL)	Lee J et al. 2014
cinnamic acid	various species	• Enhanced biofilm formation in *P. aeruginosa* PAO1 (2-fold at 100 μg/mL) and *A. tumefaciens* C58 (2-fold at 12 μg/mL)	Plyuta et al. 2013
salicylic acid	various species	• Inhibited biofilm formation in *P. carotovorum* 29 (90%), swimming motility in *P. carotovorum* 29, *P. corrugata* 3'M, *P. syringae* pv *syringae* 13 (25 mM) • Reduced twitching & swimming motility, production of protease IV (37%), elastase (46%), and AHLs (89%) in *P. aeruginosa* 6294 (30 mM) • Downregulated QS genes expression in *P. aeruginosa* PAO1 at 4 mM • Inhibited swimming motility in *P. fluorescens* ATCC 13525 and *B. cereus* at 100 μg/mL	Lagonenko et al. 2013 Bandara et al. 2006 Vandeputte et al. 2010 Lemos et al. 2014
gallic acid	various species	• Increased biofilm formation in *S. epidermidis* by 3-fold at 188 μg/mL • Inhibited biofilm formation in *E. corrodens* by 80% at 1 mM • Enhanced biofilm formation in *P. aeruginosa* PAO1 (2-fold at 25 μg/mL) and *A. tumefaciens* C58 (2-fold at 100 μg/mL) • Reduced biofilm formation in *P.aeruginosa* PAO1 by 30% at 200 μg/mL • Inhibited violacein production in *C. violaceum* ATCC 12472 (59% at 1 mg/mL)	Moran et al. 2014 Matsunaga et al. 2014 Plyuta et al. 2013 Borges et al. 2014
ellagic acid	various species	• Inhibited QS in *E. coli* MT102 (pSB403) by 40% at 40 μg/mL and *P. putida* (pKR-C12) by 40% at 30 μg/mL • Inhibited biofilm formation (50% at 40 μg/mL) and swarming motility (100% at 20 μg/mL) in *B. cepacia* • Reduced biofilm formation in *S. dysgalactiae* strains by 70% at 4 μg/mL • Inhibited biofilm formation in *S. aureus* ATCC 11632 (60% at 15 μg/mL), MRSA ATCC 33591 (70% at 20 μg/mL), *E. coli* ATCC 10536 (60% at 15 μg/mL), and *C. albicans* ATCC 90028 (50% at 20 μg/mL)	Huber et al. 2003 Dürig et al. 2010 Biakkiyaraj et al. 2013
ferulic acid	various species	• Enhanced biofilm formation in *P. aeruginosa* PAO1 and *A. tumefaciens* C58 by 2-fold at 200 μg/mL • Inhibited violacein production in *C. violaceum* ATCC 12472 (72% at 1 mg/mL) • Inhibited swimming motility in *P. fluorescens* ATCC 13525 and *B. cereus* at 100 μg/mL	Plyuta et al. 2013 Borges et al. 2014 Lemos et al. 2014
sinapic acid	various species	• Enhanced biofilm formation in *P. aeruginosa* PAO1 (2-fold at 200 μg/mL) and *A. tumefaciens* C58 (2-fold at 100 μg/mL)	Plyuta et al. 2013
caffeic acid	various species	• Inhibited violacein production in *C. violaceum* ATCC 12472 (75% at 1 mg/mL)	Borges et al. 2014

Appendix V – Continued

Active constituents	Source plant(s)	Biological activities	Reference
chlorogenic acid	various species	• Inhibited biofilm formation in *P. aeruginosa* PA14 (45% at 128 μg/mL) • Enhanced biofilm formation in in *P. aeruginosa* PAO1 (2-fold at 200 μg/mL) and *A. tumefaciens* C58 (2-fold at 3 μg/mL)	D'Abrosca et al. 2013 Plyuta et al. 2013
anacardic acids	Anacardiaceae species	• Mixture of 4 acids showed inhibition of violacein production in *C. violaceum* ATCC 12472 (94% at 166 μg/mL) and virulence factors production in *P. aeruginosa* PA14 (75 to 91% at 200 to 500 μg/mL)	Castillo-Juárez et al. 2013
1,2,3,4,6-penta-*O*-galloyl-ß-D-glucopyranose	various species	• Inhibited biofilm formation in *S. aureus* (IC_{50} = 3.6 μM) • Inhibited biofilm formation in *S. aureus* by 60% at 4 μM	Lin et al. 2011 Payne et al. 2013
tannic acid	various species	• Enhanced biofilm formation in *P. aeruginosa* PA14 at 100 μg/mL • Inhibited QS in *P. putida* (pKR-C12) by 40% at 30 μg/mL and *E. coli* MT102 (pSB403) by 20% at 60 μg/mL • Inhibited biofilm formation in *S. aureus* by >50% at 20 μg/mL • Reduced expression of genes responsible for QS and virulence in *S. aureus* at 20 μg/mL • Inhibited *S. aureus* biofilm formation (60% at 2 μM) and increased *isaA* expression (a transglycosylase)	Huber et al. 2003 Cho HS et al. 2013 Payne et al. 2013
Coumarins and derivatives			
dihydroxybergamottin	*Citrus × paradisi* Macfad. (Rutaceae)	• Inhibited AI-1 and AI-2 QS in *Vibrio harveyi* BB886 (>95%) and biofilm formation in *E. coli* O157:H7 (72%) at 10 μg/mL	Girennavar et al. 2008
bergamottin	*Citrus × paradisi* Macfad. (Rutaceae)	• Inhibited AI-1 and AI-2 QS in *Vibrio harveyi* BB886 (>95%) and biofilm formation in *E. coli* O157:H7 (58%), *S. typhimurium* (46%) at 10 μg/mL	Girennavar et al. 2008
aesculetin	various species	• Inhibited QS in *C. violaceum* CV026, *P. aeruginosa*, *E. coli* JB523 (30 to 60% at 100 μM) • Inhibited biofilm formation in *S. aureus* strains (53 to 77% at 128 μg/mL) • Reduced expression of biofilm-related genes (motility, adhesion, virulence) at 50 μg/mL	Brackman et al. 2009 Dürig et al. 2010 Lee J et al. 2014
aesculin	various species	• Inhibited QS in *P. aeruginosa* (30% at 500 μM)	Brackman et al. 2009
coladonin	*Ferula loscosii* (Lange) Willk. (Apiaceae)	• Inhibited biofilm formation in *E. coli* O157:H7 (>70% at 10 μg/mL)	Lee J et al. 2014
coumarin	various species	• Inhibited biofilm formation in *E. coli* O157:H7 (80% inhibition at 50 μg/mL • Reduced expression of biofilm-related genes (motility and adhesion) at 50 μg/mL	Lee J et al. 2014
umbelliferone	Apiaceae species	• Inhibited biofilm formation in *E. coli* O157:H7 (90% inhibition at 50 μg/mL • Reduced expression of biofilm-related genes (motility and adhesion) at 50 μg/mL • Inhibited formation of *S. aureus* CECT 976 biofilm by 50% at 800 μg/mL	Lee J et al. 2014 Monte et al. 2014

Appendix V – Continued

Active constituents	Source plant(s)	Biological activities	Reference
nodakenetin	*Angelica* sp. (Apiaceae)	• Inhibited biofilm formation in *P. aeruginosa* PAO1 (50%) and *S. maltophilia* (30%) at 200 μM	Ding et al. 2011
fraxin	various species	• Inhibited biofilm formation in *P. aeruginosa* PAO1 by 31% at 200 μM	Ding et al. 2011
Quinones			
chrysophanol	various species	• Inhibited biofilm formation in *P. aeruginosa* PAO1 (44%) and *S. maltophilia* (38%) at 200 μM	Ding et al. 2011
emodin	various species	• Inhibited biofilm formation in *P. aeruginosa* PAO1 (75%) and *S. maltophilia* (43%) at 20 μM	Ding et al. 2011
purpurin	*Rubia tinctorum* L. (Rubiaceae)	• Inhibited yeast-to-hypha transition in *C. albicans* SC5314 at 3 μg/mL • Inhibited formation of new and preformed *C. albicans* biofilms (50% at 5 μg/mL and 30% at 10 μg/mL, respectively) • Downregulated expression of hypha-specific genes in *C. albicans* at 5 to10 μg/mL	Tsang et al. 2012
rhein	various species	• Inhibited biofilm formation in *P. aeruginosa* PAO1 by 37% at 200 μM	Ding et al. 2011
shikonin	Boraginaceae species	• Inhibited biofilm formation in *P. aeruginosa* PAO1 (44%) and *S. maltophilia* (54%) at 200 μM	Ding et al. 2011
Polyketides			
motrilin	*Annon cherimola* Mill. (Annonaceae)	• Inhibited biofilm formation in *P. aeruginosa* (70% at 2.5 μg/mL)	Cartagena et al. 2007
Alkaloids			
berberine	Berberidaceae species and others	• Inhibited biofilm formation in *S. epidermidis* ATCC 35984 (50% at 30 μg/mL) and SE243 (50% at 45 μg/mL) • Inhibited biofilm formation in *K. pneumoniae* clinical isolates (MIC = 63.5 μg/mL)	Wang et al. 2009 Magesh et al. 2013
chelerythrine	Papaveraceae species	• Inhibited biofilm formation in *S. aureus* 6538P (50% at 15μM) and *S. epidermidis* RP62A (50% at 9 μM)	Artini et al. 2012
sanguinarine	Papaveraceae species	• Inhibited biofilm formation in *S. aureus* 6538P (50% at 25μM) and *S. epidermidis* RP62A (50% at 5 μM)	Artini et al. 2012
reserpine	*Rauwolfia* sp (Apocynaceae)	• Inhibited biofilm formation in *K. pneumoniae* clinical isolates (MIC = 15.6 μg/mL	Magesh et al. 2013
tomatidine	*Lycopersicon esculentum* Mill. (Solanaceae)	• Inhibited quorum sensing and expression of virulence factors in *S. aureus* ATCC 29213, NewbouldΔ*sigB*, and 8325-4 strains at 12.8 mg/mL	Mitchell et al. 2012
Fatty acids			
oleic acid	various species	• Inhibited adhesion of *S. aureus* to polysterene substrate (IC_{50} = 0.016%)	Stenz et al. 2008

Appendix V – Continued

Active constituents	Source plant(s)	Biological activities	Reference
linolenic acid	various species	• Inhibited biofilm formation in *K. pneumonia* clinical isolates (MIC = 31.2 μg/mL)	Magesh et al. 2013
Amino acid derivatives			
L-canavanine	*Medicago* sp and other Fabaceae species	• Moderate inhibition of *B. cenocepacia* LMG16656 biofilm formation (20% at 20 μM)	Brackman et al. 2009