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Investigation of the Antimicrobial Activity and Secondary Metabolites of Leaf Extracts from Vachellia Rigidula, Vachellia Farnesiana, Senegalia Berlandiery, and Senegalia Gregii

Hector Benjamin Lozano

INVESTIGATION OF THE ANTIMICROBIAL ACTIVITY AND SECONDARY METABOLITES OF LEAF EXTRACTS FROM *VACHELLIA RIGIDULA, VACHELLIA FARNESIANA, SENEGALIA BERLANDIERI,* AND *SENEGALIA GREGGII*

A Thesis

by

HECTOR BENJAMIN LOZANO

Submitted to Texas A&M International University
in partial fulfillment of the requirements
for the degree of

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Major Subject: Biology

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Approved as to style and content by:

Chair of Committee, Dr. Ruby A. Ynalvez
Committee Members, Dr. Mónica O. Mendez
Dr. Fernando G. Quintana
Dr. Alfred K. Addo-Mensah
Head of Department, Dr. Michael R. Kidd

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ABSTRACT

Investigation of the Antimicrobial Activity and Secondary Metabolites of Leaf Extracts from *Vachellia rigidula, Vachellia farnesiana, Senegalia berlandieri,* and *Senegalia greggii*

(December 2019)

Hector Benjamin Lozano, B.S., Texas A&M International University;

Chair of Committee: Dr. Ruby A. Ynalvez

The rise of antibiotic resistance is pressuring scientists to investigate natural products for novel antimicrobial agents. Plants are widely used as an ethnomedicine and are thought to have been used therapeutically since the middle paleolithic age. These medicinal plants contain secondary metabolites, such as phenols, that may confer antimicrobial properties. Studies have reported antimicrobial activity of plant species within the *Vachellia* and *Senegalia* genera. The objective of this study was to investigate the antimicrobial activity and secondary metabolites of the following South Texas plant species: *Vachellia rigidula, Vachellia farnesiana, Senegalia berlandieri*, and *Senegalia greggii*.

The disc diffusion test was performed to assess antimicrobial activity against selected bacteria and qualitative phytochemical tests were done to screen for secondary metabolites. The *V. rigidula* 70% ethanol extract inhibited the growth of *Providencia alcalifaciens* (7.85±1.19 mm). The *V. farnesiana* ethyl acetate extract showed inhibition against *M. roseus* (13.54±5.44). The *V. rigidula* 70% ethanol extract tested positive for phenols, tannins, diterpenes, sterols, triterpenes, and saponins. The *V. farnesiana* ethyl acetate extract tested positive for phenols, tannins, diterpenes, sterols, and triterpenes. The *V. rigidula* 70% ethanol extract may be a novel

treatment for diarrhea-inducing *P. alcalifaciens* infections. The *V. farnesiana* ethyl acetate extract may be able to combat *M. roseus* infections in immunocompromised patients.

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INTRODUCTION

Background of the study

The golden era (1940s-1960s) of antibiotic development and discovery has long since ended. We have transitioned into an era of unprecedented antibiotic resistance and the number of multi-drug resisting bacteria are increasing. Previous infections easily treated with modern antibiotics are now presenting a greater challenge due to increased antibiotic resistance (Gelband *et al.*, 2015). There are several reasons for the decline of antibiotic discovery and increased antibiotic resistance, involving individual misuse of antibiotics, bacterial mechanisms of antibiotic resistance, and sensitivity of the antibiotic market.

There are few incentives for pharmaceutical or biotechnology firms to invest into antibiotic research and development. The number of antibacterial new drug application approvals has decreased dramatically over the past three decades (Centers for Disease Control and Prevention [CDC], 2013). Relative to other drug categories, antibiotics are less profitable due to national conservation programs and the temporal nature of antibiotic prescriptions (Renwick *et al.*, 2015). Additionally, because of the inevitable onset of antibiotic resistance, there are diminishing returns in profitability even when a newly developed antibiotic becomes marketable. Regardless of the issues in antibiotic research and development, there is undeniable need for novel antibiotic drug discovery. The CDC (2013) reports 23,000 annual deaths in the United States and 25,000 in the European Union as a direct result of antibiotic resistance. The economic impact of antibiotic resistance is significant. In the United States alone, antibiotic resistance accounts for $55 billion dollars annually (Smith and Coast, 2013). Antibiotic resistant *Staphylococcus aureus* and *Enterococcus* species, both gram-positive bacteria, have proven to be

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a worldwide epidemic. Methicillin-resistant *S. aureus* (MRSA), for instance, claims more American lives each year relative to those claimed by HIV/AIDS, Parkinson's disease, emphysema, and homicide combined (Ventola, 2015).

The use of plants as an ethnomedicine is recorded in the fossil records 60,000 years ago and dated to the Middle Paleolithic age (Fabricant and Farnsworth, 2001). Since then, the exploitation of plants for their healing properties has continued, primarily propagated by indigenous peoples. The advent of increasing antibiotic resistance has pressured pharmaceutical and biotechnological communities to investigate these ethnomedicinal plants. These plants are an untapped source of natural products for the development of novel antibiotic drugs. It is estimated that angiosperm and gymnosperm plant species number from 215,000 to 500,000. However, only about 6% of species have been evaluated for potential biological activity and only 15% have been investigated using phytochemical analysis (Fabricant and Farnsworth, 2001). There is still a generous wealth of knowledge to be discovered from species yet to be investigated, particularly regarding secondary metabolite production.

In the past, secondary metabolites were disregarded as a waste product of primary metabolism (Bennett and Wallsgrove, 1994). Secondary metabolites are organic compounds that are not directly responsible for the growth and development of a plant, whereas primary metabolites are. However, secondary metabolites contribute greatly to a plant's defense mechanisms. Secondary metabolites play a role in a plant's defense against herbivory, parasites, and pathogens (Bennett and Wallsgrove, 1994). Furthermore, secondary metabolites are specific to the species of a plant. Over the years, many scientists have performed research on different plant families in aims to identify secondary metabolites and antimicrobial activities. These antibacterial properties are important for future pharmacological uses.

There is a lack of scientific literature regarding antimicrobial studies on *Vachellia* and *Senegalia* species native to South Texas. *Vachellia rigidula* (Blackbrush Acacia), *V. farnesiana* (Huisache), *S. berlandieri* (Guajillo), and *S. greggii* (Cat's claw) are indigenous to Laredo, Texas (USDA, n.d.). There are several factors involved in deciding to select these species for the experiment. First, plant collection is convenient given the proximity of the habitat and location of species. Potential costs that may be associated with outsourcing or collaboration are avoided. Although there is lack of research on these species, there are substantial reports on antimicrobial activity exhibited by other species of the *Vachellia* and *Senegalia* genera (Arias *et al.*, 2004; Amoussa *et al.*, 2016; Mutai *et al.*, 2009) Therefore, since the local species are taxonomically categorized under the same genera, the species selected for this study may exhibit similar antimicrobial properties. Specifically, there will be at least one plant extract that exhibits antimicrobial activity against the bacterial pathogens (*Bacillus cereus*, *B. subtilis*, *B. thuringienisis*, *Citrobacter freundii*, *Enterobacter aerogenes*, *Enterococcus faecalis*, *Erwinia sp*, *Escherichia coli* β, *Hafnia sp*, *Micrococcus luteus*, *M. roseus*, *Providencia alcalifaciens*, *Staphylococcus epidermidis*, and *S. saprophyticus*) used in this study

This study aimed to investigate the antimicrobial activities of secondary metabolites from various leaf extracts of *V. rigidula*, *V. farnesiana*, *S. berlandieri*, *and S. greggii*. Once antimicrobial activity was revealed, the secondary metabolites of the respective leaf extract was screened. The screening of secondary metabolites in the most promising extracts are an exciting and impacting research endeavor, since there are few to no literature reports on the classes of secondary metabolites present in the plant species of interest.

Mechanisms and contributions to bacterial antibiotic resistance

The mechanisms by which antibiotic resistance in microorganisms develops are well-documented. Investigation of these mechanisms have led to further understanding of the fundamental knowledge regarding cell structure and function (Davies and Davies, 2010). Some microorganisms are intrinsically resistant to a class or classes of antibiotics. Intrinsic resistance may be present simply due to relative structure and resulting function of the microorganism. Triclosan, for instance, has a broad spectrum of antimicrobial activity against Gram-positive and Gram-negative bacteria. However, triclosan is unable to effectively inhibit the growth of the *Pseudomonas* genus because of its intrinsic FabV homologue, a triclosan-resistant enoyl-acyl carrier protein reductase (Zhu *et al.*, 2010). One mechanism by which resistance is developed in general is through increased efflux. The microorganism actively transports antibiotics out of the cell, contributing to intrinsic resistance (Blair *et al.*, 2015). Increased expression of efflux pumps may grant additional resistance by allowing the ability to individually pump out several classes of antibiotics, thereby becoming MDR efflux pumps.

Mutations in the antibiotic target also increases the resistance of a microorganism. Antibiotics have specificity, which can be compromised when mutations occur. Infections usually involve a variety of pathogens. One mutation to an antibiotic target gene renders the antibiotic ineffective, allowing the microorganism to survive and proliferate (Blair *et al.*, 2015). Microorganisms may also acquire resistance through the external environment through transformation. In transformation, the microorganism uptakes DNA from the environment. Modification to the antibiotic target protein because of transformation reduces the efficacy of the antibiotic. Furthermore, modification of the antibiotic target may occur through protection, where chemical groups may be added to the binding site. Mutations of the target are not necessary involved in protection modification, since the chemical groups bind to the target site,

reducing the efficacy of the antibiotic. Aside from mutation and modification of the target, microorganisms may directly modify the antibiotic. Inactivation of antibiotics can occur through hydrolysis, where enzymes from the microorganisms are able to catalyze the modification of the antibiotic. Additionally, bacterial enzymes inactivate antibiotics through chemical group transfer, where vulnerable sites on the antibiotic are targeted. Chemical group transfer causes steric hindrance and prevents the ability of the antibiotic to bind the target protein (Blair *et al.*, 2015).

Anthropogenic activity significantly contributes to antibiotic resistance. Since the golden era of antibiotic discovery, the amount of antibiotics designed for human applications has risen dramatically. In turn, they have been spread and released into the environment on an enormous scale. Over the last half century, it is estimated that millions of metric tons of antibiotic remnants or compounds have been disseminated into the biosphere (Davies and Davies, 2010). Anthropogenic activities that have contributed to antibiotic resistance are largely commercial. These activities include prophylactic use in animals, humans, aquaculture, and household pets. Antimicrobial agents are used for pest control in plants and agriculture, which can result in runoff of excess agents into the environment. Irresponsible use of antimicrobial agents in the household and personal antibiotic drugs also contribute to antibiotic resistance (Davies and Davies, 2010).

Use of plant extracts for pharmaceutical purposes

Plants have been used as an ethnomedicine for over a millennium. Investigations of ethnomedicinal plants have led to the development of significantly beneficial drugs. In modern times, these plant-derived drugs are still commercially available. Morphine, a pain medication, was discovered in the 19th century by German apothecary assistant Friedrich Sertürner. Alkaloids, a nitrogen-containing secondary metabolite, were extracted from the opium poppy,

identified, and named. Morphine is still being used today and has given rise to several other pain medications as a precursor, such as hydrocodone and oxycodone.

Artemisinin is a recently discovered plant-derived drug that combats the life-threatening malaria disease. It has been reported that artemisinin has significantly reduced the mortality rates in individuals afflicted with malaria (Shen, 2015). Artemisinin was discovered by Youyou Tu from *Artemisia annua*, a Chinese ethnomedicinal plant. Tu used diethyl ether to extract artemisinin from *A.* annua, most of which was found in the leaves (Shen, 2015). Tu was awarded with half of the 2015 Nobel Prize in Medicine for this discovery.

Alzheimer's disease (AD) is a progressive neurodegenerative disease where cognitive function is severely impaired. It is the most common form of dementia and memory loss (Ng *et al.*, 2015). The inhibition of acetylcholinesterase (AChe) is the main pharmaceutical strategy used to combat AD and two alkaloid AChe inhibitors have been approved by the FDA for mild to moderate treatment of AD (Konrath *et al.*, 2013; Ng *et al.*, 2015). Galantamine, one of the two FDA approved alkaloids, was initially extracted from *Galanthus nivalis* L, but is now extracted by the pharmaceutical industry through daffodil bulbs (Konrath *et al.*, 2013). Rivastigmine is a synthetic derivative of the alkaloid physostigmine and inhibits both AChe and butyrylocholinesterase (BuChE) (Dall'Acqua, 2013).

Plant secondary metabolites

Natural products are chemical compounds derived from any living organism, including plants. Raw materials from a plant are collected and further analyzed for the bioactive constituents exhibiting the biological activity, with the goal of eventually being used therapeutically. Natural products include secondary metabolites, which are products of primary metabolism and are known for plant defense mechanisms (Ramawat, 2007). These secondary

metabolites are vital defense mechanisms against microorganisms, insects, and herbivores (Vaghasiya *et al.*, 2011). Over the years, many scientists have performed research on different plant families aiming to identify antimicrobial activities and secondary metabolites. These antibacterial properties are important for future pharmacological uses. Phytochemical screening of different plants has revealed numerous bioactive compounds including alkaloids, tannins, flavonoids, glycosides, and saponins (Compean and Ynalvez, 2014).

The common structure of all phenolic compounds contains one or more aromatic rings and one or more hydroxyl groups (Dai and Mumper, 2010). They are one of the most abundant classes of secondary metabolites, with upwards of 8,000 structures already known and can vary significantly in their structural complexity. Perhaps the most recognized property of plant phenolics is their contributions to a plant's pigmentation. This is incredibly useful for the plant's ability to become conspicuous to parasites or to signal potential pollinators. However, plant phenolics have also been reported to play a major role in a plant's defense against ultraviolet radiation, herbivory, and pathogens (Dai and Mumper, 2010). Flavonoids are an example of a bioactive compound classified under the phenolic class. Flavonoids function as pigmentation for the plant, but also protect its cells from UV-B radiation by accumulating in the epidermal layers of leaves and stems (Mazid *et al.*, 2011). Tannins on the other hand, primarily function as defense against herbivores. Tannins are toxins that repel predators by reducing survivorship of prey, while also acting as an astringent for mammalian herbivores (Mazid *et al.*, 2011).

Terpenoids are the most abundant class of secondary metabolites. Terpenes, in addition to most secondary metabolites, consist of isoprene. Terpenoids refer to terpenes in which a hydrocarbon molecule (isoprene) has been modified, such as by oxidation (Basu and Zwenger, 2008). Like phenolics, terpenoids contribute to a plant's defense mechanisms by conferring anti-

herbivorous and anti-venin properties. Terpenoids may also stimulate pollinators by producing pheromones. Terpenoids are more commonly known for their significant contributions in industrial commerce. They are used to produce perfumes, flavors, spices, and other cosmetic products (Singh and Sharma, 2015). Diterpenes are terpenoids that may be present within the canals of tree trunks as resin. Insects that pierce the canals may be intercepted by the resin and be chemically deterred. Furthermore, diterpenes deter mammalian herbivores by functioning as a skin irritant and/or internal toxin (Mazid *et al.*, 2011). Triterpenes, an important bioactive compound found in plant cell membranes, serve as regulatory channels and facilitate permeability to small molecules. Triterpenes may act as a bittering agent that deters herbivorous predators and may even disrupt developmental processes of insects (Mazid *et al.*, 2011).

Sulfur-containing secondary metabolites are critical for the plant's ability to respond to stress in an abiotic or biotic environment (Rausch and Wachter, 2005). Glutathione (GSH), glucosinolates (GSL), and phytoalexins are included within the sulfur-containing secondary metabolism class. GSH is reported to be responsible for its antioxidant activity during periods of plant stress and detoxification of heavy metals (Mazid *et al.*, 2011). GSL is responsible for anti-herbivorous activities by producing toxins that repel predators. On the other hand, phytoalexins are secondary metabolites with antimicrobial activities. Phytoalexins are produced by a plant during the invasion of foreign bacterial or fungal pathogens (Mazid *et al.*, 2011). Nitrogen-containing secondary metabolites include alkaloids, cyanogenic glycosides, and non-protein amino acids. Like the sulfur-containing class, nitrogen-containing secondary metabolites are important for anti-herbivorous defense and antimicrobial activity. However, nitrogen-containing secondary metabolites are synthesized from common amino acids. Alkaloids are considered toxic to a certain degree and serve primarily to deter pathogens and herbivorous predators. (Mazid *et*

al., 2011). Cyanogenic glycosides allow for the release of hydrogen cyanide (HDC), an incredibly potent toxin and disrupts cellular respiration. Non-protein amino acids are incorporated into other proteins within the plant but act as defensive substances by blocking the synthesis or acquisition of amino acids upon being digested by a predator (Mazid *et al.*, 2011).

Antimicrobial activity in *Vachellia* (*Acacia*)

Vachellia karroo is a medicinally resourceful tree native to India, Sri Lanka, and the United States. Its leaves, bark, and latex are used as a traditional medicine for treatment of several diseases. (Priyanka *et al.*, 2014). It has been used to treat conjunctivitis (pink eye), hemorrhages, and is used as an astringent agent for the common cold. Leaf and root extracts from *V. karroo* have been reported to have broad spectrum antimicrobial activity against clinical isolates of bacteria (Priyanka *et al.*, 2014). Antimicrobial activity has been exhibited when tested against the following bacteria: *Staphylococcus aureus*, *Escherichia coli*, *Salmonella typhi*, *Pseudomonas aeruginosa*, *Klebsiella pneumoniae*, *Proteus vulgaris*, and *Bacillus subtilis*. Through the Kirby-Bauer disc-diffusion method, it was determined that the ethyl acetate root extract of *V. karroo* was the most effective antimicrobial agent. Priyanka *et al.* (2014) performed phytochemical analysis via gas chromatography-mass spectrometry (GC-MS), a method of identifying compounds within a sample. The following six biologically active compounds thought to be phytoprotectants were identified: Hexamethylcyclotrisiloxane, Methyl-2,4-bis(4'-trimethylsilyloxyphenyl) pentene-, p-Menth-8-ene, trans-, Furan, 2-hexyl-, Trans-decalin, 2-methyl-, and Cyclotrisiloxane, hexamethyl (Priyanka *et al.*, 2014).

Vachellia nilotica is another species native to India and utilized for its medicinal properties. The powdered bark mixed with salt is traditionally used as an anti-diarrheal drug. The bark is also heavily used in the treatment of colds and bronchitis (Deshpande and Kadam, 2013).

Deshpande and Kadam (2013) report antimicrobial activity of ethanolic and petroleum ether bark extracts when tested against the following bacteria: *S. aureus*, *P. vulgaris*, *P. mirabilis*, *K. pneumoniae*, *E. coli*, and *S. paratyphi B*. Ethanolic and petroleum ether bark extracts were obtained using Soxhlet extraction and the tube dilution method was used to assess antimicrobial activity. It was determined that the ethanolic bark extract was more effective relative to the petroleum ether extract. *Escherichia coli* was found to be most susceptible to the ethanolic extract (Deshpande and Kadam, 2013).

Vachellia aroma is highly valued not only for its ecological functions, but its medicinal properties as well. The tree itself plays a role in reforestation and wasteland reclamation (Arias *et al.*, 2004). As an Argentine ethnomedicine, it is used for its cicatrizing properties, while the leaf and bark extracts are also known for their cicatrizing and anti-inflammatory properties (Arias *et al.* 2004). The ethanol and aqueous extracts have been reported to have antimicrobial properties. Arias *et al.* (2004) tested the ethanol and aqueous extracts against several Gram-positive and Gram-negative bacteria, reporting that antimicrobial activity was highest against Gram-positive bacteria. *Vachellia aroma* has also been reported to have antimicrobial activity against a medicinally significant pathogen, Methicillin-resistant *Staphylococcus aureus* (MRSA). The ethanolic and ethyl acetate extracts have been shown to exhibit higher antimicrobial activity relative to petroleum ether, dichloromethane, and methanolic extracts (Mattana *et al.*, 2010).

Vachellia farnesiana is well known for its flowers, which are cultivated to produce cassie perfume, and the glycosidal fraction has been reported to act as an anti-inflammatory agent (Ramli *et al.*, 2011). Additionally, the root of *V. farnesiana* has previously been reported to have antivenin activity against snake bites (Trivedi *et al.*, 1986). Ramli *et al.* (2011) evaluated the antimicrobial potential of the leaf extract through successive Soxhlet extraction using petroleum

ether, dichloromethane, and ethanol. It was determined that antimicrobial activity was exhibited against *B. subtilis* with a minimum inhibitory concentration (MIC) value of 0.8 mg/ml (Ramli *et al.*, 2011).

Acacia saligna has been widely used as a traditional medicine in Saudi Arabia. The bark has been used for treatment of colds and bronchitis, while also having anti-diarrheal properties (Gumgumjee and Hajar, 2015). It has been reported that the leaf ethanolic extract exhibit antimicrobial activity when tested against *K. pneumoniae* and *Micrococcus luteus* (Gumgumjee and Hajar (2015). Additionally, Gumgumjee and Hajar (2015) report the leaf ethanol extract as a substantial antifungal agent. The leaf extract exhibited antifungal activity against *Aspergillus flavus, A. fumigatus, A. niger,* and *Candida albicans,* with *A. flavus* being most susceptible.

Acacia auriculiformis is typically grown as an ornamental plant and desired for the many benefits of its wood. It is also known to possess anti-helminthic activity (Ghosh *et al.*, 1996). Mandal *et al.* (2005) reports antibacterial and antifungal activity from funicles of *A. auriculiformis*. Using a mixture of already extracted saponins (a secondary metabolite), specifically, Acaciaside A and B, antimicrobial and antifungal activity was investigated. The saponin mixture exhibited antifungal activity against *A. ochraceous* and *Curvularia lunata*. However, the concentration required to inhibit the fungi was much less (300µg/ml) than the concentration required to inhibit the bacterial strains. With a concentration of 700 µg/ml or higher, antimicrobial activity was exhibited against *B. megaterium, S. typhimurium,* and *P. aeruginosa* (Mandal *et al.*, 2005).

Acacia caesia is traditionally used as a medicine in Southeast Asia and Northeast India. The bark of *A. caesia* has been utilized for treatment of gastrointestinal infections and lice infestations. Additionally, *A. caesia* is used as a soap and possesses anti-helminthic activity.

(Smitha *et al.*, 2012). Through Soxhlet extraction, Smitha *et al.* (2012) obtained the methanolic bark extract from *A. caesia* and tested it against the following bacteria: *B. subtilis*, *B. cereus*, *S. aureus*, *P. aeruginosa*, *Proteus vulgaris*, *E. coli*, and *Salmonella sp.* The Kirby-Bauer disc diffusion method was utilized for the assessment of antimicrobial activity. Although no antimicrobial activity was exhibited against *E. coli* and *Salmonella*, the remaining bacterial strains were susceptible to the methanolic bark extract. Among the effected bacteria, *B. cereus* and *P. aeruginosa* were most susceptible to the extract (Smitha *et al.*, 2012).

Acacia mearnsii is an invasive tree native to Australia, which has not been traditionally used as a medicine, but whose tannins are extensively used for industrial applications (Santos *et al.*, 2017). As a sol-gel, a solid material derived from small molecules, *A. mearnsii* has been reported to exhibit antimicrobial activity against *S. aureus*, *E. coli*, *A. niger*, and *Candida* sp. Santos *et al.* (2017) encapsulated the tannins from *A. mearnsii* using four different methods of sol-gel development: acid, basic, silicate, and non-hydrolytic. The acid, basic, and non-hydrolytic sol-gels exhibited ineffective to selectively moderate antimicrobial activity. However, the silica sol-gel showed much more promise and demonstrated the most broadly effective antimicrobial activity. It moderately prevented the growth of *S. aureus, A. niger*, and *C.* sp., while only having weak antimicrobial activity against *E. coli*. The silica sol-gel exhibited the most effective antimicrobial activity due to preservation of tannins and appropriate texture that facilitates the tannin release (Santos *et al.*, 2017).

Acacia ataxacantha, a shrub native to sub-Saharan Africa, has been traditionally used as a medicine in the Benin republic (Amoussa *et al.*, 2016). It is traditionally used for the treatment of tooth decay and dysentery, while also being used as an analgesic. Amoussa *et al.* (2016) extracted the powdered bark of *A. ataxacantha* through the maceration method and successive

extraction with hexane, dichloromethane, ethyl acetate, and methanol. The resulting extract was tested against *S. epidermidis*, *E. faecalis*, MRSA, and *P. aeruginosa*. Although lupeol, betulinic acid, and betulinic acid-3-trans-caffeate were isolated and identified as the bioactive compounds, betulinic acid-3-trans-caffeate was determined to be the most effective bioactive compound. This was done through several antimicrobial tests, including the disc-diffusion assay, wherein betulinic acid-3-trans-caffeate was the only isolated compound to show antimicrobial activity against all the test microorganisms (Amoussa *et al.*, 2016).

Acacia mollissima and *A. cyclops* are native to Tunisia in Africa and have recently assessed for potential antimicrobial activity (Jelassi at al., 2017). The essential oils from the flower extract have been tested against *Salmonella enteric*a, *E. coli*, *S. aureus*, *B. subtilis*, *P. aeruginosa*, and *Candida albicans*. Jelassi *et al.* (2017) reports that the essential oil of *A. mollissima* exhibited greater antimicrobial activity relative to *A. cyclops* against all the test microorganism. Furthermore, *A. mollissima* was four times more effective in inhibiting the growth of *C. albicans*. The antimicrobial activity of *A. cyclops* may be attributed to the high number of hydrocarbons in the essential oil compared to *A. mollissima*, which has been linked to lower antifungal activity (Jelassi *et al.*, 2017).

Antimicrobial activity in *Senegalia*

Senegalia mellifera is a highly valued ethnomedicinal plant commonly used in Kenyan's folk medicine, whose bark is used to treat syphilis, pneumonia, and even malaria (Mutai *et al.*, 2009). The chloroform and methanol bark extracts have been reported to have antimicrobial activity. Additionally, antimicrobial activity is also reported when the bark is extracted with mixtures of chloroform and methanol. Mutai *et al.* (2009) tested these bark extracts against several Gram-positive and Gram-negative bacteria, where the methanol extract was shown to be

most effective against *S. aureus*. However, the chloroform and methanol mixture extracts were also shown to have strong antimicrobial activity against *S. aureus* (Mutai *et al.*, 2009). The extracts were also assessed for antifungal activity, with the chloroform and 75% methanol extract exhibiting the most antifungal activity against *Trichophyton mentagrophytes* (Mutai *et al.*, 2009).

Senegalia modesta twigs are traditionally used as a miswak (teeth cleaning twig), treating gastric problems, and for hemostasis (Ashgar *et al.*, 2003). Previous research by Ashgar *et al.* (2003) determined that the twig extract exhibits antimicrobial activity against dental pathogens, particularly *Lactobacillus*. Additionally, the root extract has been shown to possess bacteriostatic activity against two Gram-negative and Gram-positive strains of bacteria (Rashid and Hashmi, 1999). Using water and methanol, Khalid *et al.* (2011) produced leaf and stem extracts. Both extracts were tested against the following bacteria: *B. subtilis*, *Enterococcus faecalis*, *P. aeruginosa*, *S. aureus*, and *S. typhi*. It was determined that both leaf and bark aqueous/methanol extracts exhibited competent antimicrobial activity against all test bacterial strains.

Senegalia catechu has an incredible number of medicinal uses. The concentrated extract possesses antidiarrheal, hemostatic, and anti-hemorrhoid properties. It is traditionally used to treat stomatitis and irritable bowel syndrome. Furthermore, it is a power astringent, and is used as a leprostatic agent (Khare, 2008). Negi and Dave (2010) report antimicrobial activity from the leaf extract of *S. catechu*. Methanol, hexane, acetone, and aqueous leaf extracts were obtained and tested against the following bacteria: *S. aureus*, *B. subtilis*, *E. coli*, *S. typhimurium*, and *P. aeruginosa*. Through the Kirby-Bauer disc diffusion method, it was determined that the methanolic leaf extract was most effective in inhibiting microbial growth across all test bacterial strains (Negi and Dave, 2010).

Other biological activities in *Vachellia* and *Senegalia*

Although the focus of this experiment is on antimicrobial activity, it is important to acknowledge other properties that the *Vachellia* and *Senegalia* genera may have to highlight their significance. *Acacia concinna* is native to Thailand and Southeast Asia, renowned for its medicinal properties. The plant's pod (case withholding seeds) is traditionally used as an emetic, laxative, and for general hygiene. Wuthi-udomlert and Vallisuta (2011) report antifungal activity from the extracts of the plant's dried fruits. Using Soxhlet extraction and aqueous lyophilization, ethanol, chloroform, and aqueous extracts were obtained. Antifungal activity was observed when tested against three species of fungi: *Candida albicans*, *Cryptococcus neoformans*, and *Penicillium marneffi*. Furthermore, 35 clinical strains of dermatophytes were assessed for antifungal susceptibility using the same extracts. The ethanol and chloroform Soxhlet extracts exhibited the highest levels of antifungal activity against the dermatophytes and *P. merneffi,* while the aqueous lyophilized extract was most effective against *C. neoformans* (Wuthi-udomlert and Vallisuta, 2011).

The human immunodeficiency virus (HIV), perhaps the most significant pathogen of the twentieth century, has caused a worldwide epidemic. Nutan *et al.* (2003) reports that the aqueous and ethanol stem bark extracts of *S. catechu* can suppress HIV-1 replication. It was suggested that this suppression may be possible due to the extract's inhibitory effect on the HIV-1 protease and interaction interference of the viral Tat protein.

Significance of selected pathogens

The bacteria selected in this study were chosen due to their developing antibiotic resistance. For instance, the *Enterobacter* genera may become increasingly resistant to antibiotic classes such as aminoglycosides, β-lactams, and fluoroquinolones. These are typically used for treatment of Gram-negative infections. The resistance is in part due to the newly reported NDM-

1 carbapenem resistance gene (Kumarasamy *et al.*, 2010). The NDM-1 gene has also been reported to be produced by *Citrobacter freundii*, which was found to be significantly resistant to all β-lactams (Poirel *et al.*, 2010). Although *Providencia stuartii* is not being tested in this study, *P. alcalifaciens*, a member of the same genus, is under investigation. *P. stuartii* has been reported to have porins that contribute to antibiotic resistance. *P. stuartii* already shows intrinsic resistance to many antibiotic classes through the AmpC gene, but the OmpPst1 and OmpPst2 outer membrane porins have been correlated with antibiotic resistance (Tran *et al.*, 2010). The *Enterococcus* genera has been thought to be medically insignificant and non-pathogenic (Fisher and Phillips, 2009). However, it has become one of the most notorious pathogens that causes nosocomial infections, with a startling mortality rate of up to 61% (Lopes *et al.*, 2006).

Several species of the *Bacillus* genus have been considered safe and beneficial to humans as a probiotic. However, antibiotic resistance is not necessarily considered a concern when considered for approval as a probiotic in food (Gueimonde *et al.*, 2013). Several genera of probiotic bacteria, including *Lactobacillus, Bifidobacterium,* and *Bacillus* have been reported as having intrinsic resistance to several antibiotics. The *Bacillus* genus have several genes which encode for mechanisms of antibiotic resistance, those of which are already effective against respective antibiotics. The *cat*(Bcl) gene encodes for antibiotic acetylation, which confers antibiotic resistance towards chloramphenicol. The BCL-1 gene targets β-lactams, a broad-spectrum antibiotic, through antibiotic hydrolysis. (Gueimonde *et al.*, 2013).

Staphylococcus epidermidis was once disregarded as a harmless commensal microorganism part of the human skin flora. However, it is now being recognized as an opportunistic pathogen and is the leading cause of nosocomial infections (Otto, 2009). *Staphylococcus epidermidis* produces a biofilm on several medical devices, particularly those

that are implanted into patients. This biofilm accounts for a dramatic decrease in antibiotic efficacy from several antibiotics (Uçkay *et al.*, 2009). *Staphylococcus saprophyticus* is a major pathogen involved in female lower urinary tract infections (UTI), with *E. coli* being the only other microorganism to be found more abundant in UTI microorganism isolations (Widerström *et al.*, 2012). Reports indicate that *Escherichia coli* mutations in the *marO* gene and amino acid changes in the MarR gene may be responsible for its antibiotic resistance to ampicillin (*Sáens et al*., 2004).

MATERIALS AND METHODS

Plant collection

This study was done on the surrounding shrubbery of the Texas A&M International University (TAMIU) (27°35'N, 99°26'W) campus in Laredo, Texas. Low precipitation and high average temperatures characterize the habitat of the test species (McReynolds, 2008). Nine individual trees were sampled for each the test species: *V. rigidula*, *V. farnesiana*, *S. berlandieri*, and *S. greggii*. Tree age was not a considered factor when sampling. Stems were collected and defoliated. Leaves from the samples were stored in a Ziploc® bag and refrigerated at -40℃ until used for preparation and subsequent extraction. Species identification and authentication were carried out by Dr. Neal McReynolds from the Department of Biology and Chemistry, TAMIU. Voucher specimens were deposited at the Department of Biology and Chemistry TAMIU in a -40℃ refrigerator within LBV 289.

Preparation and extraction of plant material

The plant leaves were thoroughly washed using distilled water. After washing, the leaves were placed in a Ziploc® bags and refrigerated at -40℃. The leaves were later lyophilized until dry. The dried leaves were then ground into a fine powder using an electronic coffee bean

grinder. This was done to increase the surface area of the exposed plant material during Soxhlet extraction. Then, the leaves were subject to a Soxhlet extractor apparatus (Chemglass® CG-1368-03). Thirty grams of ground leaves were used to fill a glass Soxhlet thimble, while 210 mL of 70% ethanol (VWR® Cat# 97065-058), chloroform (Sigma-Aldrich® Cat# 288306-1L), or acetyl acetate (VWR® Cat# BDH1123-4LG) was added to the round bottom flask. The solvent was heated and allowed to evaporate, eventually arriving at the Soxhlet condenser. The condenser section of the apparatus then releases water as droplets into the reservoir containing the thimble. As the solvent reaches the siphon, the cycle will begin again as it pours back into the round bottom flask (Redfern *et al.*, 2014). The Soxhlet extraction was run for at least 24-48 hours, depending on the solvent used.

Figure 1. Photo of the Soxhlet extractor and setup used in this study. Ground leaves (30g) are being extracted with 210 mL of solvent.

Plant extract solvent removal

Two methods were employed to remove the excess solvent from the Soxhlet extract, rotary evaporation and air-drying. Rotary evaporation (Heidolph® Laborota 4000) was used to remove most of the excess solvent from the Soxhlet extract. After most of the solvent was removed from the Soxhlet extracts, lyophilized using the Labconco® FreeZone 2.5 Liter Benchtop Freeze Dry System (Catalog# 7670520) or air-drying. The 70% ethanol extracts were frozen at -80℃ prior to freeze-drying. Chloroform and ethyl acetate extracts were air-dried under a fume hood for approximately 24-48 hours. On the other hand, the 70% ethanol extracts were ground into a fine powder using a pestle and mortar after freeze-drying. After air-drying, the chloroform and ethyl acetate extracts had a paste-like consistency. These extracts were then used for the disc diffusion assay.

Bacterial culturing and turbidity adjustment

The bacterial strains were obtained from Dr. Sebastian Schmidl, Department of Biology and Chemistry, TAMIU. The bacteria were purchased from Presque Isle. The bacterial strains used in this study were: *Bacillus cereus* (617), *B. subtilis* (620), *B. thuringenisis* (619a), *Citrobacter freundii* (239), *Enterobacter aerogenes* (341), *Enterococcus faecalis* (522A), *Erwinia sp* (351), *Escherichia coli* B (337), *Hafnia sp* (342A), *Micrococcus luteus* (456), *M. roseus* (461), *Providencia alcalifaciens* (368), *Staphylococcus epidermidis* (4653), and *S. saprophyticus* (4654). Glycerol stocks were prepared and stored in a -80℃ freezer for later use. Overnight cultures were prepared by incubation at 37℃ prior to an assay. Using a sterile toothpick, each bacterium from the glycerol stock were placed into two individual 25 mL tubes with 2 mL of Lurteria-Burteni (LB) Broth (Teknova® Cat# L9105) for turbidity adjustment. The turbidity of an overnight broth culture was adjusted to a level of A= 0.132 ± 0.005 at 625 nm. This level is optically comparable to the 0.5 McFarland standards. A spectrophotometer was

used to measure the absorbance of the suspension. This yielded a bacterial suspension of approximately 0.5-1.0 x 10^8 CFU/mL.

Antimicrobial assay (Disc diffusion method)

Nutrient agar plates were prepared using Meuller-Hinton (MH) agar (Himedia® Cat# M1084-2.5KG) and VWR® 100x15 mm petri dishes (Cat# 25384-342) for cultivation of bacteria. Each petri dish was prepared with 25 mL of MH agar. The disk diffusion method of Pirbalouti *et al.* (2010) was used to determine the zone of inhibition values for the plant extracts, with some modifications. The disc-diffusion method is a standard for antimicrobial susceptibility testing due to its cost and practicality relative to alternative tests (Reller *et al.*, 2009). MH agar plates were inoculated with 100 µL of respective bacterial suspension. A petri dish turntable and sterile, glass L-shaped rods were used to spread the inoculum. Sterile, blank antibiotic discs (Carolina® Cat# 806491, BD BBL® Cat# 470111-928, Whatman® Cat# WHA2017006) were impregnated with 20 µl of the plant extracts at a concentration of 100 mg/mL or respective control solvents. The 100 mg/mL concentration for plant extracts was selected for homogenous mixture with the extraction solvent. Smitha *et al.* has previously used a concentration of 100 mg/mL for antimicrobial assessment in *A. caesia* (2012).Solvents (70% ethanol, chloroform, or ethyl acetate) were used as a negative control in the experiment. Gentamicin (10 µg) (Carolina® Cat# 806152) was used as the positive control for each bacterium tested. After all the impregnated antibiotic and gentamicin discs were placed on the inoculated MH agar plates, the bacteria were placed in an incubator (Fisher Scientific® Isotemp 650D Incubator Oven) at 37℃ for approximately 24 hours. Measurements of any ZoI were then taken after the incubation period. Quantitative readings were recorded by zones of inhibition (ZoI) in millimeters using a

digital Vernier caliper. The longest and shortest diameters of any ZoI were measured and averaged. If no ZoI was observed, it was recorded as 6 mm. The diameter of the disc is 6 mm.

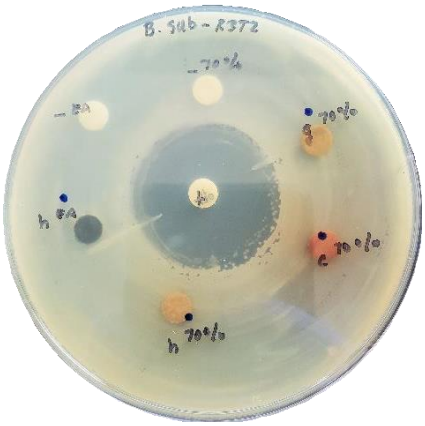

Figure 2. Example of the disc diffusion setup used in this study. Impregnated discs (control and test discs) placed radially, with gentamicin placed in the middle.

Secondary metabolite screening tests

Several standard phytochemical screening tests were performed according to Lu *et al.* (2014) and Panti *et al.* (2014), with some modifications. This was done to identify the class of secondary metabolites present in the plant extracts.

For the alkaloids test (Dragendorf test), 0.06 g of lyophilized extract was dissolved in 1.5 mL of ethanol. Then, 1.5 mL HCl was added. The solution was then heated in a boiling water bath for five minutes. After cooling to room temperature, the solution was filtered by centrifugation (Heraeus® Multifuge® X3 Centrifuge Series). The filtrate was then treated with 60 µL of Dragendorf reagent. The solution was then observed for turbidity or precipitation, which indicates presence of alkaloids. Scores were given to the respective plant extracts based on turbidity. Scores of "(+)" provided slight opaqueness, "(++)" for definite turbidity without flocculation, and "(+++)" for definite heavy turbidity and/or flocculation.

For the phenols test (Ferric chloride test), 0.02 g/2 mL extract was prepared and treated with 10% ferric chloride solution. The formation of a bluish-black solution indicated the presence of phenols.

For the cardiac glycosides test (Legal's Test), 0.012 g of solid plant extract was dissolved in 600 µL of ethanol, followed by centrifugation. The filtrate was then treated with 300 µL of glacial acetic acid, 60 µL of 10% ferric chloride, and 60 µL of 18 *M* concentrated sulfuric acid. The formation of a green-blue color in the solution indicated the presence of cardiac glycosides. For the saponins test (Froth test), 0.02 g/2 mL extract was prepared and diluted with 18 mL of distilled water. The solution was then mixed and observed for a persistent foam layer. A 1 cm or greater foam layer indicated the presence of saponins.

For the diterpenes test (Copper acetate test), 0.02 g/2 mL extract was prepared and treated with three drops of copper acetate solution. The formation of an emerald green color in the solution indicated the presence of diterpenes.

For the sterols/triterpenes test, 0.012 g of solid plant extract was dissolved in 600 µL of chloroform, followed by centrifugation. The filtrate was then treated with 300 µL of 18 *M* concentrated sulfuric acid. A two-phase formation with red coloration in the chloroform phase indicated the presence of sterols/triterpenes.

For the tannins test (Gelatin test), 0.06 g of solid plant extract was dissolved in 3 mL of hot distilled water, followed by centrifugation. The filtrate was then equally portioned into three test tubes containing 3 mL of: (1) 0.9% sodium chloride (2) 0.9% sodium chloride (1.5 mL) and gelatin solution (1.5 mL) and (3) 60 µL 1% ferric chloride solution. The formation of precipitate in the second treatment and blue/green/black coloration in the third indicated the presence of tannins.

For the resins test (precipitate test), 0.06 g of solid plant extract was dissolved in 4.5 mL of 96% ethanol. The solution was then decanted into a test tube containing 6 mL of distilled water. The formation of a precipitate indicated the presence of resins.

RESULTS

Determination for presence of antimicrobial activity

The mean zone of inhibition (ZoI) from the antimicrobial disc diffusion assay is shown in Table 1. The *V. rigidula* 70% ethanol extract was shown to inhibit microbial growth of *P. alcalifaciens* with a ZoI of 7.85±1.19 mm. This zone of inhibition was higher compared to the 70% ethanol negative control (6.30±0.88 mm). However, the *V. rigidula* 70% ethanol extract was less effective than gentamicin positive control (16.45±4.53 mm). All other 70% ethanol extracts from *V. farnesiana*, *S. berlandieri*, and *S. greggii* were unable to inhibit microbial growth with a ZoI of 6 mm, which indicates no inhibition (Figure 3).

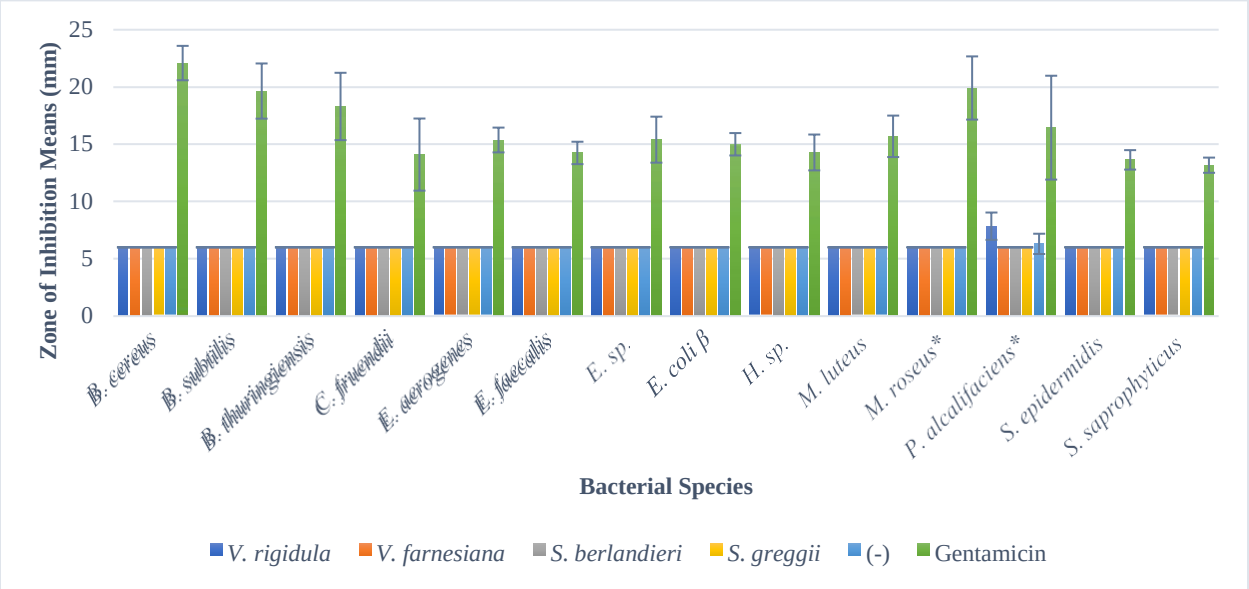

Figure 3. Clustered column graph illustrating the zone of inhibition (mm) of 70% ethanol extracts against all bacteria from the antimicrobial disc diffusion assay with standard deviation. Measurement of 6 mm = no inhibition. n = 2 for all bacterial species except *M. roseus* and *P. alcalifaciens*, where n = 3.

Table 1. Antibacterial activity of plant leaf extracts with respective solvents and antibiotic against bacterial species tested by disc diffusion assay

		Zone of Inhibition (mean ± SD, mm)															
		V. rigidula			*V. farnesiana*			*S. berlandieri*			*S. greggii*						
Bacterial sp.	*n*	70% EtOH	$CHCl_3$	EA	70% EtOH	$CHCl_3$	EA	70% EtOH	$CHCl_3$	EA	70% EtOH	$CHCl_3$	EA	Gentamicin (10 µg)	70% EtOH (-)	$CHCl_3$ (-)	EA (-)
B. cereus	2	6	6	6	6	6	6	6	6	6	6	6	6	22.08±1.50	6	6	6
B. subtilis	2	6	6	6	6	6	6	6	6	6	6	6	6	19.65±2.41	6	6	6
B. thuringiensis	2	6	6	6	6	6	6	6	6	6	6	6	6	18.30±2.94	6	6	6
C. fruendii	2	6	6	6	6	6	6	6	6	6	6	6	6	14.10±3.15	6	6	6
E. aerogenes	2	6	6	6	6	6	6	6	6	6	6	6	6	15.38±1.09	6	6	6
E. faecalis	2	6	6	6	6	6	6	6	6	6	6	6	6	14.24±0.98	6	6	6
E. sp.	2	6	6	6	6	6	6	6	6	6	6	6	6	15.41±2.01	6	6	6
E. coli β	2	6	6	6	6	6	6	6	6	6	6	6	6	15.00±0.98	6	6	6
H. sp.	2	6	6	6	6	6	6	6	6	6	6	6	6	14.28±1.56	6	6	6
M. luteus	2	6	6	6	6	6	6	6	6	6	6	6	6	15.69±1.81	6	6	6
*M. roseus**	3	6	6	6	6	6	13.54±5.44	6	6	6	6	6	6	19.92±2.76	6	6	6.71±1.79
*P. alcalifaciens**	3	7.85±1.19	6	6	6	6	6	6	6	6	6	6	6	16.45±4.53	6.30±0.88	6	6
S. epidermidis	2	6	6	6	6	6	6	6	6	6	6	6	6	13.64±0.85	6	6	6
S. saprophyticus	2	6	6	6	6	6	6	6	6	6	6	6	6	13.17±0.67	6	6	6

Key: * = values measured in triplicate with three trials, duplicate with three trials otherwise, 6 mm = no inhibition

All extracts tested at 100 mg/mL concentration

The *V. farnesiana* ethyl acetate extract was shown to inhibit microbial growth of *M. roseus* with a ZoI of 13.54±5.44 mm. This was higher compared to the ethyl acetate negative control (6.71±1.79 mm). The *V. farnesiana* ethyl acetate extract was less effective than the gentamicin positive control (19.92±2.76 mm). All other ethyl acetate extracts from *V. rigidula*, *S. berlandieri*, and *S. greggii* did not show antimicrobial activity against other tested bacteria (Figure 4).

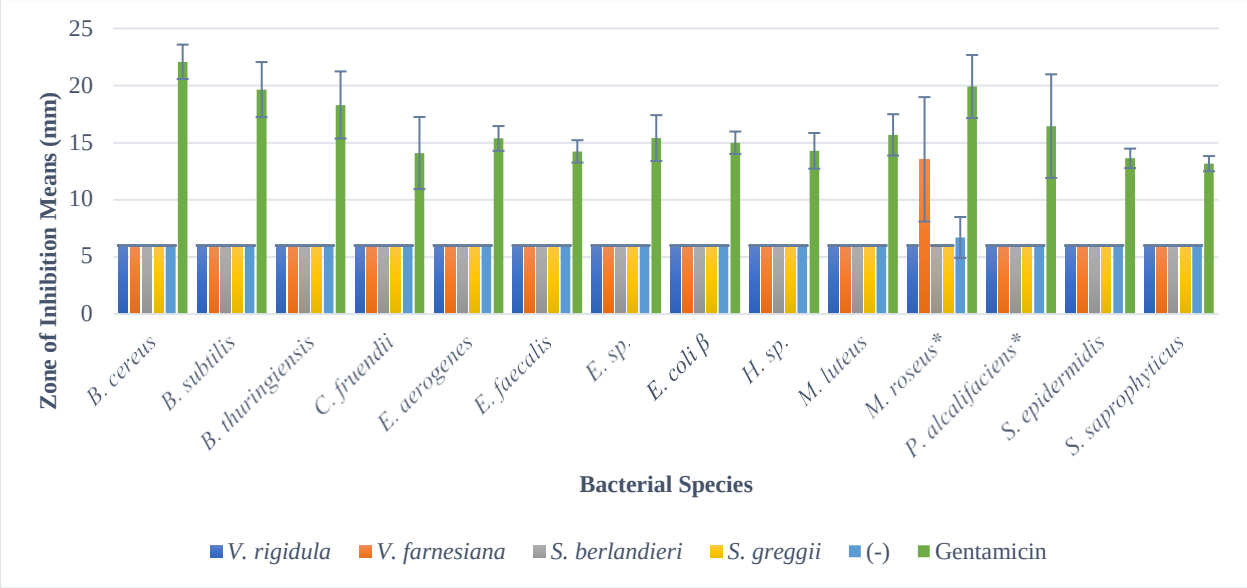

Figure 4. Clustered column graph illustrating the zone of inhibition (mm) of ethyl acetate extracts against all bacteria from the antimicrobial disc diffusion assay with standard deviation. Measurement of 6mm = no inhibition. n = 2 for all bacterial species except *M. roseus* and *P. alcalifaciens*, where n = 3.

All chloroform extracts from *V. rigidula*, *V. farnesiana*, *S. berlandieri*, and *S. greggii* did not exhibit antimicrobial activity against any of the tested bacteria (Figure 5). On the other hand, at least one 70% ethanol and ethyl acetate extract were able to inhibit at least one bacterium. Figure 6 illustrates and compares the different ZoI means between the *V. rigidula* 70% ethanol extract against *P. alcalifaciens* (7.85±1.19 mm), *V. farnesiana* ethyl acetate extract against *M. roseus* (13.54±5.44 mm), and *S. greggii* chloroform extract against *B. cereus* (6 mm). The *S. greggii* chloroform extract against *B. cereus* is a representative negative result. The *V. farnesiana*

ethyl acetate extract was more effective (13.54±5.44 mm) in inhibiting *M. roseus* than the *V. rigidula* 70% ethanol extract was in inhibiting *P. alcalifaciens* (7.85±1.19 mm).

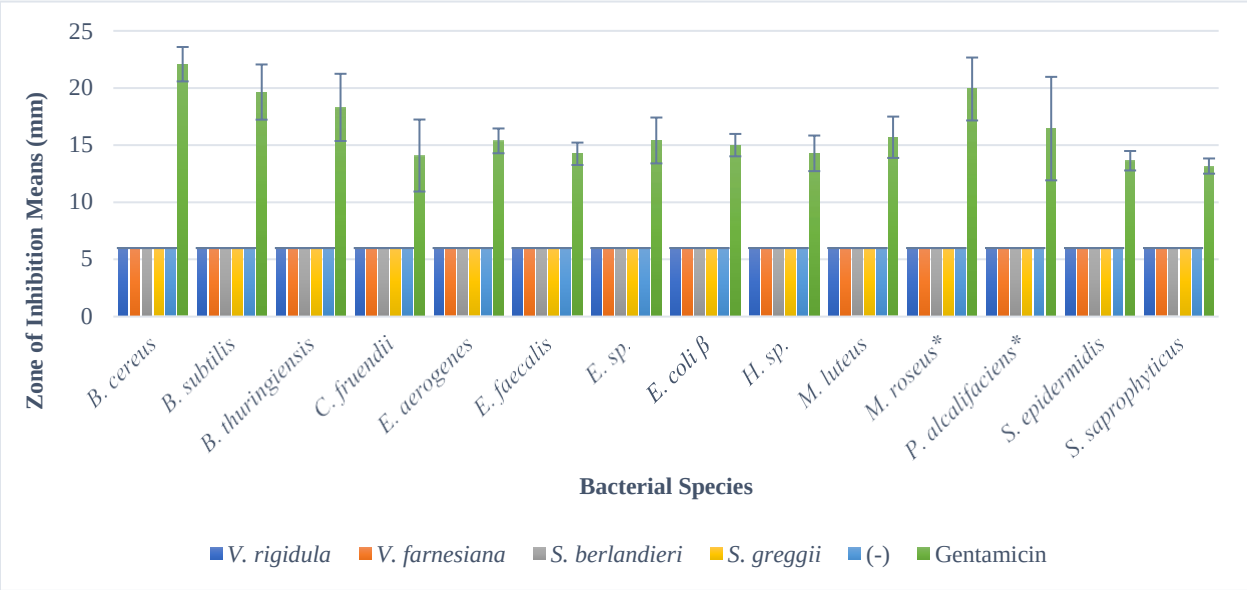

Figure 5. Clustered column graph illustrating the zone of inhibition (mm) of chloroform extracts against all bacteria from the antimicrobial disc diffusion assay with standard deviation. Measurement of 6mm = no inhibition. n = 2 for all bacterial species except *M. roseus* and *P. alcalifaciens*, where n = 3.

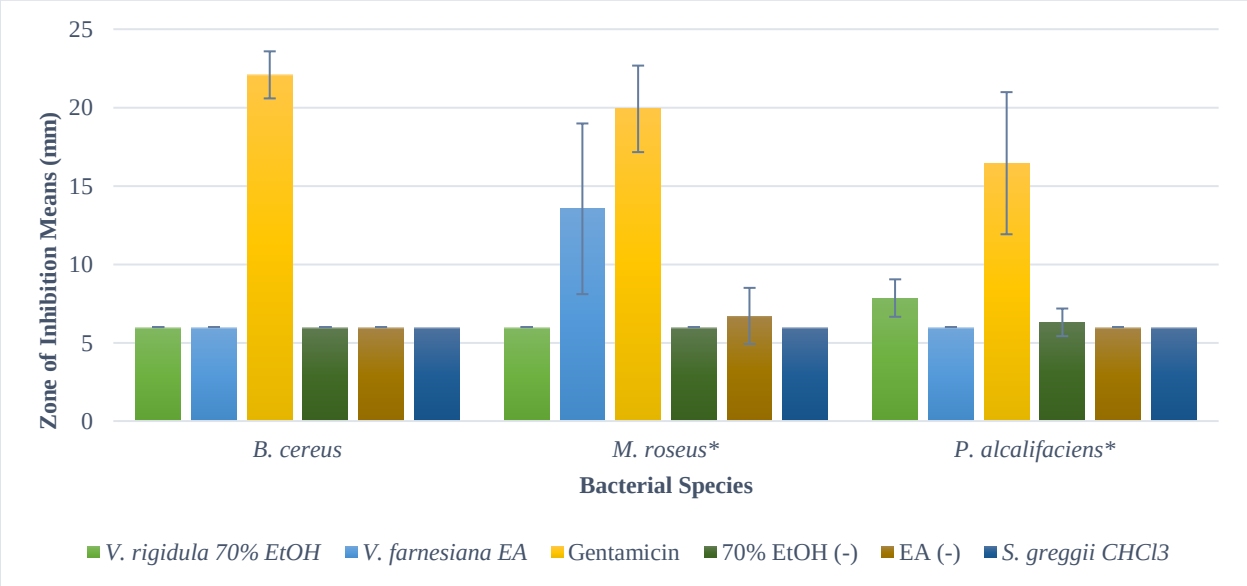

Figure 6. Clustered column graph comparing the zone of inhibition (mm) means of *V. rigidula* 70% EtOH, *V. farnesiana* EA, and *S. greggii* $CHCl_3$ extracts against respective bacteria from the antimicrobial disc diffusion assay with standard deviation. Measurement of 6mm = no inhibition. n = 2 for *B. cereus*. n = 3 for *M. roseus* and *P. alcalifaciens*.

Qualitative screening of secondary metabolites

The plant extracts that consistently exhibited antimicrobial activity across three replications were chosen for subsequent phytochemical analysis. These extracts were the *V. rigidula* 70% ethanol and *V. farnesiana* ethyl acetate extracts. The secondary metabolite screening results of the *V. rigidula* 70% ethanol extracts and *V. farnesiana* ethyl acetate extracts are shown in Table 2. The *V. rigidula* 70% ethanol extract tested positive for the following secondary metabolites: phenols, tannins, diterpenes, sterols, triterpenes, and saponins. Of these secondary metabolites found present in the extract, high qualitative scores were given to phenols, tannins, and saponins. The *V. rigidula* 70% ethanol extract tested negative for alkaloids, cardiac glycosides, and resins. On the other hand, the *V. farnesiana* ethyl acetate extract tested positive for phenols, tannins, diterpenes, sterols, and triterpenes. High qualitative scores were given only to phenols for the *V. farnesiana* ethyl acetate extract. The *V. farnesiana* ethyl acetate extract tested negative for alkaloids, cardiac glycosides, saponins, and resins.

Table 2. Results from the qualitative phytochemical screening tests of *V. rigidula* 70% EtOH and *V. farnesiana* EA extracts

Phytochemical Screening					
Secondary Metabolite Test	Secondary Metabolite	*V. rigidula* 70% EtOH Extract	Observations	*V. farnesiana* EA Extract	Observations
Dragendorf Test	Alkaloids	Negative (-)	NR	Negative (-)	NR
Ferric Chloride Test	Phenols	Positive (+++)	Bluish-black	Positive (+++)	Bluish-black
Gelatin Test	Tannins	Positive (-++)	Precipitate & dark green/black soln.	Positive (--+)	No precipitate, green black soln.
Legal's Test	Cardiac glycosides	Negative (-)	NR	Negative (-)	NR
Copper Acetate Test	Diterpenes	Positive (+)	Green-brown precipitate	Positive (+)	Emerald green soln.
Phase Test	Sterols/Triterpenes	Positive (+)	Two phases, dark green/black and clear	Positive (+)	Two phases, dark green/black and clear
Froth Test	Saponins	Positive (+++)	>1cm layer	Negative (-)	<1cm foam layer
Precipitate Test	Resins	Negative	NR	Negative	NR

Key: NR = No reaction
(+) = Minimal reaction
(++) = Moderate reaction
(+++) = Strong reaction
Representative of three replications

DISCUSSION

This was an observational study, and so results have been reported using descriptive statistics. Results from the antimicrobial disc diffusion assay showed antimicrobial activity in the *V. rigidula* 70% ethanol and *V. farnesiana* ethyl acetate leaf extracts. The *V. rigidula* 70% ethanol extract had a mean zone of inhibition (ZoI) of 7.85±1.19 mm against *P. alcalifaciens*, while the *V. farnesiana* ethyl acetate extract had a mean ZoI of 13.54±5.44 mm against *M. roseus*. These ZoI values are clearly different from the negative controls and other non-inhibitory plant extracts. Furthermore, the *V. farnesiana* ethyl acetate extract seems to be more promising due to the higher ZoI mean measurement. The presence of antimicrobial activity in these specific plant extracts is a novel discovery and contribution to the ethnopharmacological field since they have not previously been investigated. Therefore, the hypothesis in this study is supported, as at least one plant extract from *V. rigidula*, *V. farnesiana*, *S. berlanieri*, and *S. greggii* exhibited antimicrobial activities.

The effect of plant extracts on antimicrobial activity

The antimicrobial activity exhibited by *V. rigidula* and *V. farnesiana* is expected. Other species of the *Acacia*, *Vachellia*, and *Senegalia* genera were reported to possess antimicrobial activity (Priyanka *et al.* 2014; Mattana *et al.*, 2010; Negi and Dave, 2010). Different plant material is used in antimicrobial investigations of ethnopharmacological plant species, such as leaves, bark, fruits, and roots. Different methods of plant extractions were also employed by other researchers. Previous studies on antimicrobial activities of leaf extracts of the previously mentioned plant genera served as the rationale for investigation of leaves in this study. For instance, Solomon-Wisdom and Shittu (2010) extracted *A. nilotica* leaves with ethanol and observed antimicrobial activity against *Campylobacter coli*. The 70 mg/mL concentration of the

ethanolic *A. nilotica* extract was shown to inhibit *C. coli* with a ZoI of 15 mm. Coincidentally, the MIC was also found to be 70 mg/mL. This finding is interesting, because it not only validated the ethnopharmacological use of *A nilotica*, but also revealed the potential of the leaf extract to address bacterial infection in mammals. The *Campylobacter* genus is moderately responsible for foodborne illnesses, specifically, diarrheal illnesses (Solomon-Wisdom and Shittu 2010).

The use of leaves as plant material for extraction is further supported by Mattana *et al.* (2010) by testing *A. aroma* ethanolic and ethyl acetate leaf extracts against methicillin-resistant *Staphylococcus aureus* (MRSA), methicillin-sensitive *S. aureus* (MSSA), and methicillin-resistant *S. epidermidis*. The *A. aroma* leaf extracts were found to exhibit antimicrobial activity against all the *Staphylococcus* species tested. The 20 mg/mL ethanolic and ethyl acetate extracts were shown to possess the most antimicrobial activity, with ZoI measurements ranging from 14-16 mm (Mattana *et al.*, 2010). This is a notable finding, as MRSA is an incredibly pathogenic bacteria and has developed significant antibiotic resistance. Gumgumjee and Hajar (2015) have also reported antimicrobial activity of a leaf extract, specifically from *A. saligna*. The *A. saligna* leaves were extracted with ethanol and tested against three gram-negative (*E. coli*, *Klebsiella pneumoniae*, and *Pseudomonas aeruginosa*) and four gram-positive (*B. subtilis*, MRSA, *S. aureus*, and *Micrococcus luteus*) bacteria. The 200 mg/mL *A. saligna* ethanolic leaf extract was shown to exhibit antimicrobial activity against all the tested bacteria. However, the *Micrococcus* strains were most susceptible to the *A. saligna* ethanolic extract with a ZoI of 27.66±0.33 mm and 25.66±0.00 mm against *E. coli*. Furthermore, the *A. saligna* ethanolic extract was able to inhibit *B. subtilis* growth with a ZoI of 23.33±0.33 mm (Gumgumjee and Hajar 2015). In this

study, *M. roseus* was the most susceptible bacteria with a ZoI of 13.54±5.44 mm with the *V. farnesana* ethyl acetate extract.

Although the use of plant leaves for extraction in this study has been supported, an acknowledgement must be made regarding antimicrobial activity investigations of other plant material. Antimicrobial activity has been reported on different plant material from the genera under investigation in this study. For instance, Smitha *et al.* (2012) utilized the bark of *A. caesia* for extraction with methanol and tested for antimicrobial activity against *E. coli*, *B. subtilis*, *B. cereus*, *S. aureus*, *Pseudomonas aeruginosa*, *Proteus vulgaris*, and *Salmonella sp.* The methanol *A. caesia* bark extract exhibited antimicrobial activity against all but *E. coli* and *Salmonella sp* bacteria. Of these bacteria tested, the *A. caesia* bark extract was found to be most effective against *B. cereus* and *P. aeruginosa*. The 100 mg/mL *A.* caesia methanolic extract inhibited *B. subtilis* with a ZoI of 7±0.7 mm, while *B.* cereus saw a ZoI of 10±0.6 mm (Smitha *et al.* 2012). In this study, no plant extract was able to inhibit growth of *B. subtilis* or *B. cereus*. Khalid *et al.* (2011) has reported antimicrobial activity of methanolic and aqueous extracts from *A. modesta* stems. However, *A. modesta* leaves were also extracted using the same solvents. Interestingly, the *A. modesta* stem extracts exhibited higher antimicrobial activity against tested bacteria compared to the leaf extracts. The methanolic *A. modesta* stem extract had a ZoI of 10 mm against *E. faecalis*. On the other hand, the leaf extract had a ZoI 8 mm against *E. faecalis* (Khalid *et al.* 2011). This finding demonstrates differences in antimicrobial activity of different plant material originating from the same plant species, suggesting that such investigations and comparisons are a worthwhile endeavor. Designation of preferred plant material of a respective plant species may streamline and simplify future antimicrobial investigations.

There are several implications from the results of the present study. *Providencia alcalifaciens* was inhibited by the *V. rigidula* 70% ethanol extract with a 7.85±1.19 mm ZoI. *Providencia alcalifaciens* has previously been identified as being a causative agent of gastroenteritis (Murata *et al.* 2001) and has more recently been the cause of a foodborne illness outbreak in Kenya (Shah *et al.* 2015). Therefore, this study contributes a novel source for potential treatment of diarrhea-inducing *P. alcalifaciens* bacterial infections with the *V. rigidula* 70% ethanol extract. Additionally, *M. roseus* was shown to be inhibited by the *V. farnesiana* ethyl acetate extract. The *Micrococcus* species is not usually pathogenic but has been linked to rare cases of septic arthritis, meningitis, and prosthetic valve endocarditis infections (Miltiadous and Elisaf, 2011). *Micrococcus kristinae* may be an opportunistic pathogen in immunocompromised patients and may cause catheter-related bacteremias (Basaglia *et al.*, 2002). Although the *V. farnesiana* ethyl acetate extract was not tested against these specific *Micrococcus* species, it showed antimicrobial activity against *M. roseus* with a 13.54±5.44 mm ZoI. *Micrococcus roseus* may also be an opportunistic pathogen and cause infection in immunocompromised patients. Therefore, the *V. farnesiana* ethyl acetate extract may be another novel source for treatments of potential infections by *M. roseus*.

Secondary metabolites: presence and mode of action

The qualitative phytochemical screening tests in this study detected the presence of secondary metabolites in the *V. rigidula* 70% ethanol and *V. farnesiana* ethyl acetate extracts (Table 2). The *V. rigidula* 70% ethanol extract tested positive for phenols, tannins, diterpenes, sterols, triterpenes, and saponins. The *V. farnesiana* ethyl acetate extract tested positive for phenols, tannins, diterpenes, sterols, and triterpenes. This study is the first to report the presence and classes of secondary metabolites in these specific plant extracts.

Although numerous studies successfully identify secondary metabolite classes and specific compounds, rarely is the mechanism of their antimicrobial action discussed. Identification of secondary metabolites is immensely important, since they can later be targeted for isolation and purification, potentially leading to pharmaceutically relevant drugs. However, it is also important to understand the mechanisms of inhibitory action secondary metabolites use against microorganisms. Plants used as a phytomedicine in phytotherapy are often used as an extract and usually have a high concentration of phenolic compounds and terpenoids (Wink 2015). Phenolic compounds include flavonoids and tannins. Terpenoids include diterpenes, triterpenes, steroids (sterols), saponins, and cardiac glycosides. These secondary metabolites possess general mechanisms to mediate biological activities, including the biological activities of bacteria. Therefore, the antimicrobial activity of a plant extract depends on the ability of the active secondary metabolites to disrupt bacterial biological activities.

One manner secondary metabolites may disrupt bacterial cellular activity is through covalent modification of proteins and DNA bases (Wink 2015). Secondary metabolites usually have reactive functional groups such as aldehydes and SH-groups, which form covalent bonds with proteins and DNA. These covalent bonds ultimately induce conformational changes to a protein, compromising function. For instance, if secondary metabolites bond covalently to bacterial enzymes, the enzyme's active site may be altered, preventing substrate binding and reducing catalytic activity. Additionally, if Na^+ ion channels on the cellular membrane of a bacteria are altered, the cell's ability to maintain ion flux is impaired. Transcriptional factors may also be covalently bonded to secondary metabolites, resulting in modification of gene regulation. Secondary metabolites may also bond with proteins via non-covalent bonds, yet again resulting in conformational changes. Phenolic compounds, such as flavonoids and tannins, contain one or

multiple hydroxyl groups, which form non-covalent hydrogen bonds with proteins and amino acids (Wink 2015; Tiwari and Rana 2015). As the case with covalent protein modification, secondary metabolites binding non-covalently to proteins may alter their structure and compromise function.

All living cells are surrounded by a cellular membrane that primarily serves as protection but is also semi-permeable to facilitate diffusion. Furthermore, cell surface receptors are proteins studded on the cellular membrane that is essential for cell to cell communication. Secondary metabolites may interact and disrupt bacterial cellular membranes and their activities. Lipophilic secondary metabolites have an affinity for cellular membranes and bind to the lipophilic inner layer of the cellular membrane (Wink 2015). Such lipophilic secondary metabolites include terpenoid, including diterpenes, triterpenes, sterols, and saponins. Lipophilic secondary metabolites attachment to the inner layer of cellular membrane bilayer results in alterations to membrane fluidity, thereby increasing permeability. Saponins for instance can create cholesterol complexes in animal membranes and bind to glycoproteins with their sugar side chains, resulting in cellular membrane lysing (Wink 2015).

Phenolic compounds were identified in the phytochemical analysis for both *V. rigidula* 70% ethanol and *V. farnesiana* ethyl acetate extracts (Table 2). The phenols and tannins present in both extracts may be non-covalently binding to proteins and amino acids necessary for bacterial function, changing their confirmation and compromising function. Terpenoids were also identified in both extracts. Both extracts tested positive for diterpenes, triterpenes, and sterols. However, only saponins were identified in the *V. rigidula* 70% ethanol extract. Since these terpenoids are lipophilic, they may be attaching to the inner phospholipid bilayer of bacterial cellular membranes or to glycoproteins, resulting in lysis of the cellular membrane. It is

possible that the antimicrobial activity observed for these extracts is due to synergistic interactions among the secondary metabolites present in the extracts. This is strongly suggested in the case of the *V. rigidula* 70% ethanol extract due to identification of saponins. The presence of saponins in an extract may facilitate extraction of polar secondary metabolites, thereby increasing the likelihood of antimicrobial activity through synergistic actions (Wink 2015). Both extracts tested negatively for alkaloids, cardiac glycosides, and resins. However, alkaloids are often specific in mechanism of inhibitory action and are therefore not usually found in phytotherapy extracts, especially toxic alkaloids. Likewise, cardiac glycosides are not typically present in phytotherapy extracts (Wink 2015). Therefore, it is highly likely that phenols, terpenoids, or synergistic interactions of these secondary metabolites are responsible for the observed antimicrobial activity in the *V. rigidula* 70% ethanol and *V. farnesiana* ethyl acetate extracts.

The mean ZoI differences observed between the *V. rigidula* 70% ethanol extract against *P. alcalifaciens* (7.85±1.19 mm) and the *V. farnesiana* (13.54±5.44 mm) ethyl acetate extract against *M. roseus* may be due to the classification of bacteria. *Providencia alcalifaciens* is a gram-negative bacterium, while *M. roseus* is gram-positive. Gram-negative bacteria are more resistant to lipophilic inhibitors compared to gram-negative bacteria. The outer membrane barrier of gram-negative bacteria often accounts for the greater intrinsic resistance to antibiotic compounds when compared to gram-positive bacteria (Nikaido, 1996). Terpenoids are lipophilic secondary metabolites that attach to the inner membrane layer and alter membrane fluidity (Wink, 2015). Terpenoids were identified in both the *V. rigidula* 70% ethanol and *V. farnesiana* ethyl acetate extracts and may be responsible for the observed antimicrobial activity. However, *P. alcalifaciens* may be more resistant to the terpenoid lipophilic secondary metabolites due to its

outer membrane barrier. This may explain why the *V. rigidula* 70% ethanol extract had a lower mean ZoI when compared to the *V. farnesiana* ethyl acetate extract against *M. roseus*. It is also possible that the terpenoid concentration present in the extract is not high enough to further inhibit *P. alcalifaciens.*

The effect of solvent extraction method on secondary metabolite extraction

The method of extraction is important when discussing the presence of secondary metabolites. Sequential solvent extraction of plant material may yield different secondary metabolites when compared to individual solvent extractions. Furthermore, different solvents used to extract the plant material will result in different secondary metabolites being extracted. Ultimately, the presence of secondary metabolites in a plant extract is dependent upon the polarity of solvents used for extractions. Polar secondary metabolites will more readily be extracted with polar solvents. Likewise, non-polar secondary metabolites will be extracted with non-polar solvents (Sultana *et al.* 2009). However, there are varying degrees of solvent and secondary metabolite polarity.

In this study, 70% ethanol, chloroform, and ethyl acetate were the solvents selected for leaf extraction from *V. rigidula, V. farnesiana*, *S. berlandieri*, and *S. greggii*. Ethanol and ethyl acetate are polar solvents and should extract polar secondary metabolites. Chloroform, however, is moderately polar, and should extract moderately polar secondary metabolites (Ramos Mendonca-Filco, 2006). Antimicrobial activity of ethanolic leaf extracts from these plant genera have already been discussed. Notably, the *V. rigidula* 70% ethanol extract was found to exhibit antimicrobial activity against *P. alcalifaciens* in the current study. Regarding secondary metabolites, however, use of ethanol for plant extractions has been shown to be effective for extraction of secondary metabolites with antimicrobial activity (Ramos Mendonca-Filco, 2006).

Ethanolic *V. farnesiana* leaf extracts have previously been reported to exhibit antimicrobial activity against *B. cereus* (Ramli *et al.* 2011). Broth microdilution was performed to assess antimicrobial activity, rather than the disc diffusion test. Ramli *et al.* reports the ethanolic *V. farnesiana* leaf extract as having MIC = 0.8 mg/mL against *B. cereus*. Contrary to this study, however, the *V. farnesiana* 70% ethanol extract did not inhibit *B. cereus*. This is likely due to differences in extraction methodology. Sequential extraction of *V. farnesiana* leaves using petroleum ether, dichloromethane, and pure ethanol solvents was performed. This is in comparison to the single extraction using 70% ethanol in this study. The use of petroleum ether in the sequential Soxhlet extraction may have yielded more secondary metabolites of lesser polarity that may be responsible for antimicrobial activity. Interestingly, however, putative flavonoids were reported as being the active secondary metabolite found in the ethanolic, sequential *V. farnesiana* plant extract. Although secondary metabolite analysis was not performed on the *V. farnesiana* 70% ethanol extract, analysis was done on the *V. farnesiana* ethyl acetate extract. Both ethanol and ethyl acetate solvents are highly polar, therefore flavonoids should theoretically have been extracted using 70% ethanol and ethyl acetate. This may be the case in the current study, as the *V. farnesiana* ethyl acetate extract tested positive for phenols, which may include flavonoids (Table 2). It is possible that the phenol or flavonoid concentration present in the sequential ethanol *V. farnesiana* extract was higher and thus exhibited antimicrobial activity in *B. subtilis* compared to the theoretical concentration of the single 70% ethanol *V. farnesiana* extract tested in this study. The presence of alkaloids has been reported in *V. rigidula* by Clement *et al.* (1997). Compared to the present study, the *V. rigidula* 70% ethanol extract and *V. farnesiana* ethyl acetate extracts tested negative for alkaloids. This contradictory finding is interesting considering alkaloids in this study were not identified from

the same species. However, Clement *et al.* (1997) utilized methanol to extract a combination of leaves and stems. The difference in detection of alkaloids may be attributed to the use of stems in the extraction. It is possible that leaves of *V. rigidula* do not contain alkaloids, while the stems do. Furthermore, it is possible that, compared to 70% ethanol and ethyl acetate, methanol can extract alkaloids from the leaves due to variation of solvent polarity (Clement *et al.* 1997).

Ethyl acetate has also been reported as being one of the most suitable polar solvents for which to extract active secondary metabolites. Specifically, ethyl acetate has been demonstrated to effectively extract phenolic and flavonoid compounds from plant material (Sultana *et al.* 2009; Shon *et al.* 2004). Amoussa *et al.* (2014) investigated successive extraction involving ethyl acetate of *A. ataxacantha* and performed secondary metabolite screening tests like the ones in the current study. The *A. ataxacantha* ethyl acetate bark extract was able to inhibit microbial growth of *S. epidermidis* and *E. faecalis*, two bacterial strains tested in this study. However, contrary to this study, the *V. farnesiana* ethyl acetate leaf extract was not able to inhibit growth of *S. epidermidis* and *E. faecalis*. Furthermore, the secondary metabolites identified included flavonoids, alkaloids, saponins, triterpenes and tannins (Amoussa *et al.* 2014). Like the *A. ataxacantha* ethyl acetate bark extract, the *V. farnesiana* ethyl acetate leaf extract tested positive for tannins and triterpenes. Saponins and alkaloids were not detected in the *V. farnesiana* ethyl acetate leaf extract. The differences are likely attributed to the use of leaves versus bark plant material in extraction. However, it is also highly likely that the differences in present secondary metabolites are simply due to being different species, despite being closely related taxonomically.

Chloroform extracts from the fruit of *A. nilotica* have previously been evaluated. Mustafa *et al.* (1999) extracted *A nilotica* fruit with aqueous, ethanol, chloroform, and *n*-hexane solvents

and found varying antimicrobial activity between extracts. The aqueous and ethanol extracts were found to be more effective against tested pathogens compared to chloroform and *n*-hexane extracts. This finding is in line with the current study, as none of the chloroform extracts were found to exhibit antimicrobial activity. In another study evaluating chloroform extracts, Patel *et al.* (2009) reports similar findings. The *S. catechu* resin part was extracted with aqueous, chloroform, methanol, and petroleum ether solvents. Petroleum ether and chloroform extracts of *S. catechu* were found to have minimal antimicrobial activity against all tested pathogens (Patel *et al.* 2009) Again, this is relatively consistent with the results found in this study. Mutai *et al.* (2009) tested the antimicrobial activity of several *S. mellifera* chloroform extracts of increasing polarity (pure chloroform, chloroform in 50% methanol, and chloroform in 75% methanol). The pure chloroform extract of *S. mellifera* was unable to inhibit microbial growth of any tested pathogens, compared to both chloroform in 50% and 75% methanol extracts (Mutai *et al.* 2009). These results are equivocal to the present study. This suggests that chloroform is likely unable to extract the more polar secondary metabolites found in the *Vachellia*, *Acacia*, and *Senegalia* genera that are responsible for antimicrobial activity. Alternatively, chloroform should be used in combination with a more polar solvent, such as methanol, to extract plant material.

The use of 70% ethanol and ethyl acetate as solvents for extracting plant material in this study has been supported. Performing sequential Soxhlet extractions rather than single extractions of the same solvents used in this study may yield different classes of secondary metabolites in individual plant extracts. Additionally, investigation of plant material extracted with other solvents of varying polarity may prove useful for identifying other secondary metabolite classes that may exhibit antimicrobial activity. Although findings suggest that using increasingly polar solvents may yield active secondary metabolites for antimicrobial activity,

investigation of plant material extracted with non-polar compounds may yield interesting results. These potential results may shift the current focus of utilizing more polar solvents for extraction of plant material in the ethnopharmacological field.

One must question the potential effect of tree age on secondary metabolite production and by proxy, antimicrobial ability. Most antimicrobial investigations of the *Vachellia, Acacia,* and *Senegalia* species assume there is no effect of tree age on secondary metabolite production and antimicrobial activity. This is also the case with the present study. However, there have been scarce reports on this variable. In a study by Brooks and Owen-Smith (1994), *A. nilotica* thorns were generally longer and closer in proximity in juveniles compared to adults. Similarly, *A. tortili* had longer curved thorns in juveniles compared to adults. There may be a correlation between secondary metabolite production and physiological plant defense mechanisms that has yet to be reported. Younger trees may produce more classes of secondary metabolites as defense mechanisms against predators compared to older trees or may produce higher concentrations of a secondary metabolite. Researchers should procure plants of interest, harvest plant material at different growth intervals, and perform antimicrobial and secondary metabolite tests. Admittedly, these experiments would require a significant time investment, however, results may suggest preferred plant material harvesting times for increased extract efficacy. Moreover, results may refute considerations on the effect of tree age on the antimicrobial activity of plant extracts.

CONCLUSION AND RECOMMENDATIONS

This study has demonstrated the ability of the *V. rigidula* 70% ethanol and *V. farnesiana* ethyl acetate extracts to inhibit microbial growth against *P. alcalifaciens* and *M. roseus*, respectively. Furthermore, the presence and classes of secondary metabolites in these specific plant extracts have been identified. These findings are vital towards progressing the knowledge of ethnopharmacologically relevant medicinal plants and contributes to the dearth of information on the *Vachellia*, *Acacia*, and *Senegalia* genera endemic to Texas. However, further investigation is needed to fully elucidate potential antimicrobial activity and in order to use these plant species as novel sources for antimicrobial treatment.

Firstly, other plant material such as the bark, stem, flowers, and roots of *V. rigidula*, and *V. farnesiana* should be investigated with antimicrobial assays. Studies have shown differences in secondary metabolite classes identified when performing phytochemical analysis on different plant material, even when originating from the same species. Because of such potential variation in active secondary metabolites, degree of antimicrobial activity may vary as well (Priyanka *et al.* 2014; Saini *et al.* 2008; Arias *et al.* 2004). Use of different solvents aside from 70% ethanol, chloroform, and ethyl acetate is recommended to extract secondary metabolites of different polarities. Although it seems using higher polarity solvents are more effective in extracting active secondary metabolites, investigation of non-polar solvents may also yield interesting results (Mutai *et al.* 2009).

High performance liquid chromatography (HPLC), liquid chromatography-mass spectrometry (LC-MS), and gas chromatography-mass spectrometry (GC-MS) should be performed on *V. rigidula* 70% ethanol and *V. farnesiana* ethyl acetate extracts. These analytical methods can isolate and characterize the secondary metabolites found to be present in the current

study. The GC-MS method is becoming incredibly important for its ability to rapidly characterize biological samples and their metabolites (Schauer *et al.* 2005). Lastly, cytotoxicity assays of these isolated secondary metabolites should be performed to validate the use of these plant species and their secondary metabolites as novel treatments for bacterial infections.

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