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EVALUATION OF ANTIMICROBIAL ACTIVITY & PHYTOCHEMICAL ANALYSIS OF LEAF EXTRACTS OF TWO SOUTH TEXAS SPECIES OF THE FABACEAE FAMILY

A Thesis

by

PETER CAVAZOS

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

MASTER OF SCIENCE

May 2020

Major Subject: Biology

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Approved as to style and content by:

Chair of Committee,	Ruby A. Ynalvez
Committee members,	Cord Eversole
	Monica O. Mendez
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Chair of Department,	Michael R. Kidd

May 2020

Major Subject: Biology

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ABSTRACT

Evaluation of Antimicrobial Activity & Phytochemical Analysis of Two South Texas Species of the Fabaceae Family (May 2020)

Peter Daniel Cavazos, A.A., Laredo College; B.S., Texas Tech University;

Chair of Committee: Dr. Ruby A. Ynalvez

Background: The emergence and spread of antibiotic resistance, as well as the evolution of new strains are of great concern to the global health community. The search for novel agents to combat the rapid evolution of bacterial pathogens becomes ever more important as current methods are proving ineffective. In the present study, the different solvent extracts (acetone, methanol, and acetic acid) of *Acacia berlandieri* and *Acacia rigidula* (leaves) were evaluated for phytochemical analysis, antimicrobial and antioxidant activities.

Methods: Plant leaves of *Acacia rigidula* and *Acacia berlandieri* were sequentially extracted using acetone, methanol and acetic acid that were then subjected to susceptibility testing against nine microorganisms. A qualitative phytochemical study was conducted to determine secondary metabolites of plant extracts. Extracts were analyzed using nuclear magnetic resonance spectroscopy (NMR), infrared spectroscopy (IR), and ultraviolet-visible spectroscopy (UV-Vis) to identify different substances within a test sample. Antioxidant activity was determined in *Acacia rigidula* using the FTC method.

Results: This study provided evidence of antimicrobial activity from the crude extracts of both species, with an activity higher from the leaves of species A. rigidula. Among the nine microorganisms, two gram-negative (*Providencia alcalifaciens* and *Yersinia enterocolitica*) and two gram-positive (*Staphylococcus aureus* and *Enterococcus faecalis*) bacteria were susceptible. Qualitative phytochemical

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testing revealed important secondary metabolites: phenols, flavonoids, saponins, terpenoids and tannins. Each of which have been shown to possess antimicrobial properties and exert different modes of action. NMR, IR, and UV-Vis spectroscopy revealed the basic structures within our unknown samples that are commonly found in flavonoids, saponins, and tannins, reinforcing the results of phytochemical testing. Results revealed antioxidant activity within selected *A. rigidula* extracts.

Conclusion: Analysis of the leaves extracts of *A. berlandieri* and *A. rigidula* have revealed the presence of medicinally valued bioactive components. This study confirmed the efficacy of selected plant extracts as natural antimicrobials and suggest the possibility of employing them in drugs for the treatment of infectious diseases caused by the test organisms.

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INTRODUCTION

Background

Throughout the world many people still rely on plants for medicinal purposes. This is especially true for countries with large indigenous populations where affordable healthcare and access to medicine can be nonexistent. Traditional health beliefs are most evident through the use of "bush medicine" by traditional healers. Traditional healers prepare "bush medicine" because of its easy access as compared to Western medicine (Maher, 1999). Thus, plants provide an essential support, mending the therapeutic gap in developing countries (Sen & Chakraborty, 2017). Plants possess valuable properties that prevent exploitation by other organisms, fight infections, and repair injuries. Secondary metabolites, which are not directly involved in the normal growth or reproduction, provide plant survival mechanisms which also are useful to humans. For example, secondary metabolites are a major source of antibiotics, food additives, and pesticides (Zhou, 2015).

Currently, there are ~20,000 species of known medicinally valuable plants; therefore, the development of novel drugs from natural sources will become more prevalent in the future because only an estimated 10% of the world's biodiversity has been evaluated for these purposes (Amor et al. 2008). Biosynthesized compounds derived from primary metabolites include: alkaloids, phenols, essential oils, steroids, lignins, tannins, etc. Each of these secondary metabolites have been the most successful source of potential drug leads (Dias et al. 2012). Therefore, the discovery of naturally synthesized medicinal compounds and the challenge of how to access them remains unanswered.

Previous studies have highlighted biological activities within the Fabaceae family. (González-Burgos et al. 2011; Hossain et al. 2012). It is the second largest family currently used for medicinal plant purposes with over 490 plant species (Dzoyem et al. 2014). A larger diversity of species

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suggests a greater supply of raw materials for drug discovery and biotechnology (Alvez & Rosa, 2007). These species possess important medicinal properties and have been widely used as components of pharmaceutical products. Species like *Glycyrrhiza uralensis*, *Glycyrrhiza inflata*, and *Glycyrrhiza glabra* have been identified for their inhibitory effects on HIV replication in vitro (Okamoto, 2000). Additionally, plant materials from nearly 290 species belonging to 100 different genera of Fabaceae have been reported to be toxic. For instance, many species within the genus *Crotalaria* contain pyrrolizidine alkaloids, and are toxic to mammals and birds (Williams & Molyneux, 1987). Plants from the Fabaceae family have high levels of biological activity, e.g. antimicrobial, antifungal, the medicinal value and a pattern of global ethnomedical knowledge will only broaden as more species within this family are further evaluated (Mattana et al. 2010; Jain et al. 2011; Cock, 2012; Jeyakumar, 2015; Zhou, 2015).

According to the World Health Organization (WHO), antibiotic resistance is rising to dangerously high levels across the globe (Avorn, 2001). This has been attributed to the overuse and misuse of medications within cultures. Because of these habits, many antibiotics have become ineffective due to the development of resistance in certain bacteria. The lack of new drug development by pharmaceutical industries due to a reduced economic incentives and challenges posed by regulatory requirements has also attributed to the antibiotic resistance crisis (Ventola, 2015). The search for new antimicrobial agents and their sources is an important line of research as the efficacy of antibiotics are being challenged by bacteria and their development of resistant genes (Ventola, 2015). The goal of this study is to contribute to the knowledge of potential novel plant sources with potential of antimicrobial activity towards medicinal drug development. My specific objectives are to (1) evaluate the antimicrobial potential of different crude plant extracts (leaves and bark) from the species *Acacia rigidula* and *Acacia berlandieri,* sequentially subjected to acetone, methanol and acetic acid via disc diffusion assays (2) qualitatively determine the phytoconstituent(s) responsible for the biological activities, and (3) to establish the profile of the bioactive components present in the extract that exhibited the most significant antimicrobial activity.

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For this experiment, BSL 1 organisms that were included in this study are: *Yersinia enterocolitica, Escherichia coli, Staphylococcus aureus, Providencia alcalifaciens, Klebsiella aerogenes, Serratia marcescens, Klebsiella pneumonia, Pseudomonas aeruginosa, Enterococcus faecalis*, in the disc diffusion assay to test for any antibacterial activity of our plant extracts. These microorganisms are both gram-negative and gram-positive bacterium (Table 1).

Table 1. List of gram-positive and gram-negative bacteria and respective mechanism of action.

Bacteria	Mechanism of Action
Gram-negative	
Yersinia enterocolitica ***Escherichia coli*** ***Providencia alcalifaciens*** ***Enterobacter aerogenes*** ***Serratia marcescens*** ***Klebsiella pneumonia*** ***Klebsiella aeruginosa***	• Enterobacteriaceae (*Y. enterocolitica, E. aerogenes, P. alcalifaciens)* and *E.coli*: release of endotoxins that cause enterocolitis, acute diarrhea, mesenteric lymphadenitis, and pseudoappendicitis and fatal sepsis. • *S. marcescens* secrete a cytotoxin that causes hemolysis of human and animal erythrocytes. • *K. pneumonia* causes community-acquired infections including pneumonia and sepsis • *K. aeruginosa* delivers the toxin ExoU to eukaryotic cells and causes lung injuries.
Gram-positive	
Staphylococcus aureus ***Enterococcus faecalis***	• *S. aureus* cause leading cause of bacteremia and infective endocarditis • *E. faecalis* causes bacteremia and abdominal and pelvic infections and other life-threatening diseases.

Hypotheses: Previous scientific studies reported antimicrobial properties of secondary metabolites from different plant species within the Fabaceae family. Therefore, the null hypothesis is: secondary metabolites in plant extracts from the leaves and bark of *Acacia rigidula* and *Acacia berlandieri* will not exhibit antimicrobial activity, while the alternative hypothesis is: secondary metabolites in the plant extracts from the two plant species and different plant parts will exhibit antimicrobial activity.

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REVIEW OF LITERATURE

Importance of microorganisms

Microorganisms are ubiquitous (Cushnie et al. 2014). Although some are beneficial to humans and can prevent diseases caused by other bacteria, viruses and fungi; others are pathogenic, that if left untreated may be detrimental to our health. Their acquisition of resistant genes makes them the ideal candidates for further research. Every bacterial organism is genetically and physiologically unique; however, they can easily share genetic material. Therefore, it is important that scientists develop innovative ways to remain ahead of any outbreaks that may result from mutations or the misuse of synthetic drugs.

Human populations are locked in evolutionary arms races with pathogens that invade our bodies. We must recognize that these are continuously evolving entities, developing better ways to fight and control defense mechanisms humans are designing (Avorn, 2001). The rise of harmful "superbug" bacteria challenges the effectiveness of antibiotics; thus, illnesses and deaths related to superbugs have surpassed those of more well-known diseases like Ebola or AIDS. The improper use and overuse of antibiotics by humans has given rise to the most well-known superbug, methicillin-resistant *Staphylococcus aureus* (MRSA) (Harmon, 2013). This evolutionary arms race between germs and medicines is a major threat to public health all over the world as the rate of bacterial resistance is much faster than the production of new treatments.

Due to the development of resistant bacterial strains, antibacterial activity of plant extracts is a heavily researched topic (Usman et al. 2009; Mattana et al. 2010; Cock, 2012). These studies present plant extracts that are tested against a variety of bacterial strains to be used in combating bacterial resistance. It is well documented that gram-positive organisms are more susceptible to drugs, which is why any results that would act as a bactericidal to gram-negative microbes in this experiment would be beneficial (Delcour 2008; Woodford & Livermore, 2009; Fair & Tor, 2014).

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The primary difference between gram-positive and gram-negative bacteria is the difference in the structure of their bacterial cell wall. Gram-positive bacteria lack an outer lipid membrane that is found in gram-negative bacteria. The cell wall of gram-positive bacteria is thick, with multiple layers of peptidoglycan that generally make them more susceptible to antibiotics. However, infections caused by multidrug-resistant gram-positive bacteria represent a major public health burden, not just in terms of death, but also an increase expenditure within the health sector as patient management and implementation of infection control measures are being taken (Woodford & Livermore, 2009). *Staphylococcus aureus* and *Enterococcus* spp. are gram-positive pathogens found in clinical settings, specifically in the hospital environment. The generation of superbugs, such as methicillin-resistant *S. aureus* (MRSA), regularly attracts the public's interest and, in many countries, there is ongoing research to prevent and reduce MRSA infection rates. Their ability to survive and persist in a broad range of environments makes these bacterial species dangerous to human health (Goh et al. 2017).

Gram-negative bacteria cause many types of infections which humans are often diagnosed with. Several species, including *Escherichia coli*, are common causes of foodborne diseases (Uzodi et al. 2017). Gram-negative bacteria can also cause respiratory infections, such as certain types of pneumonia, and sexually transmitted diseases, including gonorrhea (Sabina et al. 2011). Treating gram-negative bacterial infections can be difficult because of their extracellular membrane composition. It is this composition of the outer membrane that adds an extra layer of protection where efflux pumps are commonly found in gram-negative bacteria (Delcour 2008). By combining the hydrophobic lipid bilayer with channels that are highly selective, this barrier prevents the crossing of many antibiotic drug classes. Even so, they are still able to retain the ability to freely exchange genetic material with the external environment and possibly acquire antibiotic resistant genes from other microorganisms (Delcour 2008). Superbugs, such as vancomycin-resistant Enterococcus (VRE) and multidrug-resistant *Pseudomonas aeruginosa* have developed resistance to certain antibiotics. Examples of gram-negative bacteria that have demonstrated drug resistance to some

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antibiotics include *E. coli, P. aeruginosa and K. pneumoniae* (Mattana et al. 2010; Jeyakumar, 2015; Ventola, 2015; Uzodi et al. 2017). Therefore, new drugs to prevent gram-negative bacterial infections are in high demand.

Antimicrobial activity of Fabaceae plant extracts

Several species of Fabaceae family have been effective for treating a cough (*Abrus precatorius*), diarrhea (*Acacia nilotica*), conjunctivitis (*Acacia karoo*), and toothaches (*Acacia modesta*) (Ashgar et al. 2003; Mazid et al. 2011; Mulaudzi et al. 2011; Deshpande 2013). In addition, these plant species possess antibacterial properties inhibiting the growth of microorganisms that include *E. faecalis*, *S. aureus*, and *B. subtilis* (Mulaudzi et al. 2011; Deshpande 2013). Cock (2012) examined the antimicrobial activity of methanolic extracts of *Acacia aulacocarpa* leaves and *Acacia complanta* leaves. Using the disc diffusion method, the antimicrobial activities of methanolic extracts were tested against a panel of bacteria where *A. aulacocarpa* leaf extract inhibited the growth of 6 of the 14 bacteria tested. In a similar study where acetone and methanol were used as the solvents in samples of *Acacia arabica* bark, extracts were found to be the most potent against all the selected bacterial pathogens. In this study, the researchers sequentially extracted secondary metabolites with acetone being the first solvent followed by methanol. The sequential extraction was based on the increasing polarity of the solvents (Jeyakumar, 2015). The results of this study show that extracts prepared from *A. arabica* bark show varying degrees of antimicrobial activity against both gram-positive and gram-negative multi-drug resistant organisms selected for the study.

Contrary to the synthetic drugs, antibacterial activities of plant origin are associated with lesser side effects and have an enormous therapeutic potential to heal many infectious diseases (Demain, 2009). Plants are continuously being challenged by herbivores and microbes, but they also continuously survive. Plants, like humans, are at an evolutionary arms race with pathogens, however, the inducible defenses they possess deems them better fit to adapt. Plants belonging to the Fabaceae are commonly located in

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harsh environments and the production of certain chemicals, such as anthraquinones, has allowed them to compete for the survival of their species. The production of anthraquinones and many other structurally different secondary metabolites allow plants to evolve during plant development as a mechanism of herbivory, bacterial, and fungal defense (Zhou, 2015). For example, in *A. arabica* bark extracts, phytochemical screening revealed the presence of terpenoids, phenols, tannins, flavonoids, and cardiac glycosides in both the acetone and methanol plant extracts (Jeyakumar 2015). Studies suggest that *Acacia* species possess phytochemical constituents with antibacterial properties that can be used to inhibit the growth of bacteria (Mattana et al. 2010; Jain et al. 2011). Although much has been reported on other species belonging to the Fabaceae family, to our knowledge, there is no report on the antimicrobial activity of the species in this study. Identifying potential antibacterial agents through phytochemical analysis is necessary as results of such studies will lead to the development of drugs against microbes. Thus, biomolecules of plant origin appear to be a possible alternative for the control of these antibiotic resistant human pathogens (Kumaraswamy et. al. 2008; Singh & Sharma, 2015).

Sample preservation and choice of solvents for extraction

There are two typical protocols of plant sample preparation prior to solvent extraction: sun drying and freeze drying. Freeze drying plant samples has been shown to be more advantageous that sun drying for conserving secondary metabolites. An important aspect of freeze drying is that it limits oxidative changes of secondary metabolites taking place because the oxygen concentration is very low under vacuum (Sarker, 2006). The decision of sample preparation in this study was chosen based on the amount of preservation of phytochemical constituents, which was deemed a preferred method (Saifullah et al. 2019) when determining antimicrobial and antioxidant activity in leaves. Through the process of freeze drying, enzymes become inactive, microbial growth is inhibited, there is no presence of heat, and hydrolytically-unstable compounds remain stable under such conditions (Asami et al. 2003; Sarker, 2006;

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Papoutsis et al. 2017). In addition, the dried material can be stored in a freezer for long periods without significant degradation of phytochemicals.

The extraction procedure, which is an important step in deriving bioactive constituents from plant material, has been continuously studied (Azwanida 2015). Traditional methods such as maceration and Soxhlet extraction are commonly used. However, extraction yield and phytochemical constituents actively recovered not only depend on the extraction method but are also largely dependent on the type of solvent used during extraction procedures. The literature suggests that the most suitable solvents used for extraction are aqueous mixtures containing ethanol, methanol, acetone, and some form of acid (Do et al. 2014). Therefore, the choice of solvents that will extract the desired secondary metabolites is critical.

Previous studies have reported that acetone, methanol and acetic acid extractions recovered soluble components that inhibited growth of several bacteria. For example, methanol has been generally found to be more efficient in extracting lower molecular weight phenols, whereas acetone is good for extraction of flavonoids with higher molecular weights (Do et al. 2014). These results are similar to those of Mattana (2010) where their extracts, using alcoholic solvents, exhibited a higher degree of antibacterial activity. They further reported that antimicrobial activity of flavonoids was displayed against methicillin-resistant *Staphylococcus aureus*. Other studies have noted that acids are a great solvent for separating alkaloids on the basis of their solubility in immiscible solvents (Sarker, 2006).

Biological functions of selected secondary metabolites

Phenols

Phenols are compounds with an aromatic benzene ring produced by plants mainly for protection against stress (Bhattacharya et al. 2010). Phenolics play other important roles in plants, from development to structural integrity and scaffolding support for plants. There are approximately 8,000 known different structures of plant phenolics, each with different activities. Modes of action include denaturation of bacterial

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proteins and lysis of the cell membrane. Different groups of phenolic compounds have different biological characteristics, and very little is known about the mechanisms by which they contribute to the prevention of diseases. For example, phenolic phytoalexins, secreted by wounded plants, repel or kill many microorganisms (Bhattacharya et al. 2010). In addition to the many roles that phenols play in plants, phenolic metabolites exhibit a series of biological properties that promote human health (Dzialo et al. 2016).

Other studies have demonstrated a wide range of possible biologic mechanisms of action from the phenols produced in the fruits of plants. Such mechanisms include the protection against cancer, oxidative stress, anti-inflammatory activity, and anti-estrogenic effects. In addition, polyphenols also act on certain skin disorders to help prevent or attenuate the progression of such skin conditions (Dzialo et al. 2016).

Tannins

Tannin, also called tannic acid, is a secondary metabolite that is ubiquitous and widely distributed in plants that serves a purpose in various medical applications. Tannins are synthesized in the rough endoplasmic reticulum of plants and can easily interact with most proteins. These compounds have been reviewed to possess antibacterial, antiviral, antitumor and inhibitory effects on enzymes (Nakashima et al. 1992). A study performed on six tannins against *Staphylococcus aureus* determined that tannins interfered with fibrin formation in *S. aureus* (Akiyama et al. 2001). It is also suggested that tannins affect the stability of enzymes by forming complexes and altering their action and disrupt their overall integrity. This has been observed in substrates that are required by many microorganisms, in addition to an altering of cell membranes by bacterium. (Scalbert, 1991). Thus, tannins serve an important role in the defense of plants against many organisms including bacterium and viruses.

Terpenoids

The fragrance of plants is carried in the essential oil fraction where they are highly enriched in compounds based on an isoprene structure, otherwise known as terpenes. Along with the wide variety of

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plant flavors and aromas which are produced by these compounds, terpenes also serve other functions for plants. Terpenoids are another beneficial phytochemical with more than 40,000 individual compounds known to exist. Plants have used these secondary metabolites for basic functions like growth, repair and development. Interestingly, the medicinal use of these compounds has resulted in the production of the cancer drug Taxol and the antimalarial and a potential anti-cancer drug, artemisinin, both of which are terpenoid-based drugs. These drugs and their synthetic derivatives are still currently being used as well as continuously being studied (Luo et al. 2014; Weaver, 2014; Konstat-Korzenny et al. 2018; Li et al. 2018). Of all the secondary metabolites, terpenoids have been identified for their potential to prevent and treat liver cancer (Singh & Sharma, 2015). In addition to their anti-cancer properties, terpenoids also have been used for treatment of lung infections caused by *P. aeruginosa* (Cowan, 1999). Terpenoids' chemical composition and their many biological activities in nature has made them a resource for traditional and modern human exploitation.

Alkaloids

Application of alkaloid-containing plants dates back to 2000 BCE with the primary use of medicines for humans and animals, as well as a source of poison (Aniszewski, 2007). The first medically useful example of an alkaloid was morphine. Alkaloids are a heterocyclic nitrogen compound that serves as a protective agent for the plant against attack by predators, along with other functions such as end products of metabolism or waste products, storage reservoirs of nitrogen, and growth regulators (Amirkia & Heinrich, 2014). The applications of alkaloids are not limited to the biological control to repel predators from plants but also have pharmacological, veterinary and medical importance.

These compounds belong to the beta-carboline group which possess anti-HIV and antiparasitic activities (Patel et al. 2012). Because alkaloids possess a nitrogen atom with an unshared pair of electrons, they are able to bind with a wide variety of proteins, enzymes and receptors and influence the integrity of

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both bacterial and viral enzymes (Cushnie et al. 2014). Finally, several phytochemicals possess neuroprotective potential and manage neurodegenerative diseases (NDDs). Plant derived components have been used from the ancient times against related diseases and alkaloids are one of the most reliable agents against NDDs (Amirkia & Heinrich, 2014).

Flavonoids

Flavonoids are ubiquitous and found in many plant parts. Flavonoids and their metabolites are also thought to exert modulatory actions in cells through actions on protein kinase and lipid kinase signaling pathways (Mansuri et al. 2014). More than 6,500 different compounds that fall into flavonoids have been identified. From the protection against stress and other biotic factors, flavonoids play an important role between the plant and their environment. In plants, flavonoids have been known to protect them from harmful UV radiation and subsequent cellular damage (Samanta et al. 2011). Along with the protective properties for plants, these compounds have been exploited for decades to treat human diseases. For example, they have favorable biochemical effects associated with diseases that include cancer, Alzheimer's disease, and atherosclerosis (Panche, 2016). Ongoing research has resulted in the isolation and identification of flavonoid structures possessing antifungal, antiviral and antibacterial activity. Resistance to antimicrobial agents continues to become a global pressing issue of concern. Improving our efforts of finding antimicrobial agents in established classes promotes future discovery of additional compounds that also possess such capabilities.

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METHODS

Plant collection

Plant collection for the preliminary study

Fresh leaves and stem bark of *Acacia berlandieri* and *Acacia rigidula* were collected at Texas A&M International University campus with the respective coordinates of (27° 34' 27" N 99° 25' 56" W) and (27°34'26''N 99°25'55''W). For this study, two trees (one from each species) were sampled whereby each tree was sampled for both its leaves and stem barks at three distinct frontages (i.e., northern, western, and eastern frontage of the tree). For every sample, 30 grams of plant leaves and 30 grams of stem bark were collected and stored in labeled bags. In total, six samples were collected from each tree for sequential extraction. After plant samples were collected, they were stored at -20°C, freeze dried and grounded into a powder consistency using a coffee blender.

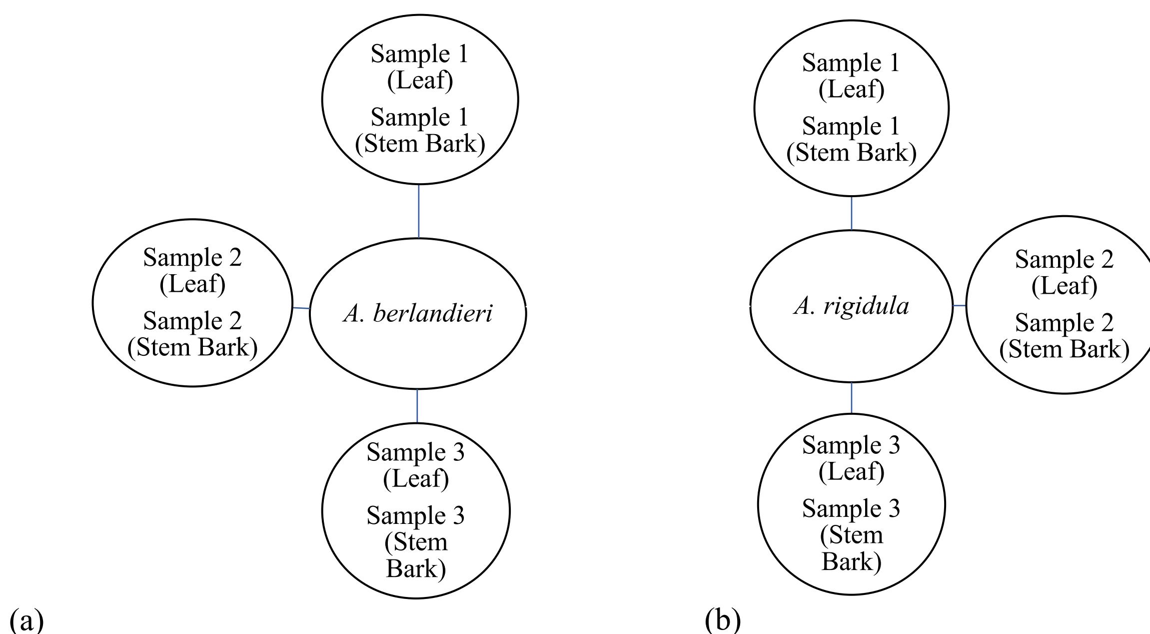

Figure 1 Schematic diagram to demonstrate sampling of leaves and bark from (a) *Acacia berlandieri* and (b) *Acacia rigidula*.

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Plant collection for the main study

From the results of the preliminary study described above, results were averaged across samples. Based on these results, the species with the higher antimicrobial activity, and the plant part with the higher antimicrobial activity was selected to become the focus of the main study. The overall protocol for plant part collection described in the preliminary study was used, with the exception that this time there were six (6) trees from which samples were collected. Using the one species, the one plant part, and the one frontage that yielded the highest antimicrobial activity was used as the treatment combination in which to compare the zone of inhibition (ZOI) of the four bacteria that showed the largest (ZOI) in the preliminary study.

Crude extraction

Each powdered plant sample of *A. rigidula* and *A. berlandieri* was extracted separately with different organic solvents based on the increasing polarity order of 30% acetone < 20% methanol < 7% acetic acid; and a 1:10 mass: volume ratio. Each sample was homogenized separately in acetone with shaking (VWR S-500 Orbital Shaker) for 48 hours at 350 rpm. Following the 48 hours shaking, the homogenate was centrifuged at 3000 rpm for 20 minutes (Beckman GS-15 Series Centrifuge) and the supernatant was collected and evaporated for the respective solvent using a Hei-VAP Precision rotary evaporator and finally lyophilized (Labconco 2.5 L). The product after lyophilization was designated as the extract. The extract was stored in a -20 °C freezer for future use. After centrifugation, the remaining pellet was added to the next solvent for extraction. The extracts were then tested for their antimicrobial activity.

Microbial cell culturing

The microorganisms tested for antibacterial activity included: *Y. enterocolitica, E. coli, S. aureus, P. alcalifaciens, E. aerogenes, S. marcescens, K. pneumoniae, P. aeruginosa* and *E. faecalis.* Bacteria were obtained from the microbiology laboratory of the Department of Biology and Chemistry, Texas A&M

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International University. Following the instructions of the manufacturer, Mueller Hinton (MH) agar (HiMedia No. 2) was prepared after being autoclaved. Streak plating was performed to prepare master plates and a single colony of each microorganism was used to prepare overnight cultures for the disc diffusion assay.

Bacterial inoculum preparation

Miller's LB Broth (Amresco) was prepared following the instruction of the manufacturer and 2 mL were added to each test tube that were sterilized using the autoclave. A single colony of each bacterium, grown in our MH culture plates, was transferred into each LB broth tube. Cultures were incubated in a water bath shaker at 37 °C for 16-18 hours. Following incubation, the turbidity of each LB Broth was diluted with sterile water to an absorbance level of 0.132 ± 0.005 at 625 nm. This level is optically comparable to the 0.5 McFarland standards. A spectrophotometer (Bausch and Lomb, Model Spectronic 20) was used to adjust the absorbance of the suspension. This yielded a bacterial suspension of approximately 0.5-1.0 x 10^8 CFU/mL (Ynalvez et al. 2018).

Testing for antibacterial activity

Antibacterial activity of bark and leaf extracts were tested by using the disc diffusion method as described by Tendencai (2004) with some modifications. Using Mueller Hinton agar, the presence of a zone of inhibition (mm), indicated antibacterial activity by the plant extracts. From our diluted bacteria in the LB broth tubes, 100 uL were inoculated to Mueller Hinton agar plates. Bacteria were spread onto the plate using a sterilized L-shaped rod, while being rotated 15 times clockwise on a platform, to ensure equal distribution of the inoculum. Sterile Whatman 6 mm antibiotic assay discs were impregnated with 20 μL of the lyophilized plant extracts dissolved in dimethyl sulfoxide (DMSO) at a concentration of 0.5 mg/ μL. The solvent to prepare extract solutions was DMSO. DMSO served as the negative control due to DMSOs inability to inhibit bacterial growth.

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The following antibiotic discs were used as the antibiotic positive controls for the following bacteria: *P. aeruginosa, K. pneumoniae, S. aureus,* and *E. faecalis* 10 µg of vancomycin (Carolina Biological); *E. coli*, *Y. enterocolitica*, *E. aerogenes, P. alcalifaciens and S. marcescens*- 10 µg of ciprofloxacin (Carolina Biological). After completing the disc diffusion assay, plates were incubated at 37°C for 18-20 hours. After incubation, presence of a zone of inhibition (ZOI) and the diameter of the ZOI (largest and smallest) were measured in millimeters using a Vernier caliper. The test was replicated three times and each replicate with three trials for the determination of antibacterial activity.

Statistical Analysis

Statistical analysis for the preliminary study

For each of the nine bacteria, an analysis of variance (ANOVA) associated with a 2 x 3 factorial experiment was performed. In this experiment, species (factor 1) had 2 levels, namely: *A. berlandieri and A. rigidula*; and solvents (factor 2) had 3 levels namely: acetone, methanol and acetic acid. To compare the 2 x 3 treatment combinations to both positive (+) and negative (-) controls, a mean comparison was performed among the 2 x 3 = 6 treatment combinations and the two controls. The plant species x solvent extracts were randomly allocated within each of the three frontages. Each treatment was given a number and using a random number generator, this designated the placement of each extract. In all analyses, a type-I error rate of $\alpha = 0.01$ (or 1%) was used.

Statistical analysis for the main study

For this experiment, three bacteria that showed the largest ZOI during the preliminary experiment were compared along with the three extraction solvents. An ANOVA associated with a 3x3 factorial experiment was performed whereby the six trees served as blocks. By blocking, we reduce the variability within blocks from external factors and to produce a better estimate of treatment effects. To compare the 3 x 3 = 9 treatment combinations to both the positive (+) and the negative (-) controls, a mean comparison

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was performed among 9 treatment combinations and the two controls. The bacteria x extracts combinations were randomly allocated within each of the six trees. In all analyses, a type-I error rate of α= 0.01 (or 1%) was used.

Qualitative phytochemicals analysis

Each extract was subjected to a standard protocol for phytochemical screening to identify the active chemical constituents. The results were graded on a scale that ranges from the absence (symbolized by a "----") of the respective metabolite, to the presence + (which defines minimal), ++ (intermediate) and +++ (definite).

Testing for Alkaloids

Dragendorf & Wagner's test: to prepare Dragendorf reagent, two solutions were prepared. To the first solution, 0.17 g of bismuth nitrate, 2 mL of acetic acid, and 8 mL of water were added. To the second solution, 4.0 g of potassium iodide, 10 mL of acetic acid, and 20 mL of water were added. The solutions were mixed and diluted with distilled water to prepare 100 mL of the Dragendorf's reagent. Additionally, to prepare Wagner's reagent, first 1.27 g of iodine was sublimed. Then, 2.0 g potassium iodide dissolved in 20 mL water, was added to the mixture. Subsequently, the mixture was diluted with distilled water to prepare 100 mL of Wagner's reagent (Lu et al. 2014). Two hundred mg of extract was dissolved in 10 mL of ethanol. To the plant extract, 5 mL of 2 M HCl was added. Then, the mixture was heated in a boiling water bath for 5 minutes. After cooling, the mixture was filtered by centrifugation, and the filtrate was divided into two equal portions. One portion was treated with 4 drops of Dragendorf reagent and the other with equal amounts of Wagner's reagent. The samples were then observed for the presence of turbidity or precipitation, which indicated the presence of alkaloids. A (+) score was recorded if the reagent produces only a slight opaqueness; a (++) score was recorded if a definite turbidity, but no flocculation was observed; and a (+++) score was recorded if a definite heavy precipitate or flocculation was produced (Lu et al. 2014).

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Testing for Phenols

Ferric chloride test: two mL of the 20mg/mL extract was treated with 3 drops of 10% ferric chloride solution. Formation of a bluish-black colored solution indicated the presence of phenols (Panti et al. 2014).

Testing for Tannins

Gelatin test: 200 mg of freeze-dried extract was dissolved in 10mL of hot distilled water and later filtered by centrifugation. The solution was divided into three test tubes. The following was added to the respective test tube: (1) 0.9% solution of sodium chloride; (2) 0.9% sodium chloride and 1% gelatine solution; and (3) 4 drops of 1% ferric chloride. Formation of a precipitate in the second treatment and a characteristic blue, blue-black, green, or blue-green color for the third treatment, indicated the presence of tannins (Lu et al. 2014; Evans, 2009).

Testing for cardiac glycosides

Legal's test: 40 mg of freeze-dried extract was dissolved in 2 mL of ethanol and then filtered using centrifugation. The filtrate was then treated with 1 mL of glacial acetic acid, 4 drops of 10% ferric chloride, and concentrated 18 M sulfuric acid. The presence of cardiac glycosides was indicated by the green-blue color indicates (Lu et al. 2014).

Testing for anthranol glycosides

Modified Borntrager's test: two mL of the 20mg/mL extract was treated with 4 drops of 10% ferric chloride solution and then immersed in boiling water for 5 minutes. The resulting solution was extracted with an equal volume of concentrated benzene. Next, the benzene layer was separated by the addition of 10% ammonium hydroxide. The subsequent solution was mixed, and the immediate formation of rose-pink color solutions indicated the presence of anthranol glycosides (Panti et al. 2014).

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Testing for Diterpenes

Copper acetate test: two mL of the 20mg/mL extract was treated with 3 drops of copper acetate solution. Formation of an emerald green solution indicated the presence of diterpenes (Panti et al. 2014).

Testing for Sterols/ Triterpenes

Forty mg of freeze-dried extract was dissolved in 2 mL of chloroform and filtered through centrifugation. The filtrate was then added to 1 mL of concentrated 18M sulfuric acid. The presence of sterols was indicated by a two-phase formation, with a red color in the chloroform phase (Lu et al. 2014).

Testing for Saponins

Froth test: Two mL of the 20mg/mL extract was diluted with 18mL of distilled water to make up a 20mL solution. The solution was later mixed, where a formation of about 1 cm layer of foam indicated the presence of saponins (Panti et al. 2014).

Testing for Flavonoids

Four mL of the 20mg/mL extract, with the addition of 2mL of distilled water, was boiled for 10 minutes. The solution was later filtered by centrifugation. Four drops of 10% ferric chloride solution was added to 2 mL of the filtrate. A green-blue or violet coloration indicated the presence of a phenolic hydroxyl group (Usman et al. 2009).

Testing for resins

Precipitate test: 200 mg of the freeze-dried extract was treated with 15 mL of 96% ethanol. The alcoholic extract was then poured into a beaker that contained 20 mL of distilled water. The presence of a precipitate indicated a positive result for resins. (Usman et al. 2009).

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Determination of minimum inhibitory concentration (MIC)

Extracts with the greatest potential of inhibition were further analyzed to determine the minimum inhibitory concentration of the extracts as described by Chorianopoulos et al. (2006) with modifications. The extracts were tested in the following concentrations: 0.5 mg/uL, 0.25 mg/uL, 0.1 mg/uL and 0.0625 mg/uL along with the addition of growth medium inoculated with the selected bacteria that were susceptible to the extracts. The resulting turbidity was observed after 24 hours, MIC were determined where growth was no longer visible by assessment of turbidity by optical density readings at 600nm. This was done in triplicates for each assay.

Chemical Analysis

Standard protocols for chemical analysis were performed by Dr. Jocelyn Lanorio, Department of Chemistry, Illinois College. Reagents and deuterated NMR solvents were obtained from commercial sources and used as received. ^{1}H NMR spectra were recorded on a Bruker 300 MHz AVANCE I spectrometer with a quattro nucleus probe (QNP). Proton chemical shifts were given in δ values (ppm) using D_2O as solvent at room temperature.

UV-Vis single beam spectrophotometer Agilent Cary 60 UV-Vis Spectrophotometer was used for the absorbance measurements in the range 200-800 nm using water as solvent at room temperature. All measurements were repeated at least thrice to ensure reproducibility.

Solid state IR spectra were recorded on a Varian 640-IR with single bounce ZnSe ATR cell spectrometer and υ_{max} are partially reported (cm^{-1}). The solid samples were pressed against the diamond crystal using the attached pressure clamp.

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Antioxidant Assay

The antioxidant activity of leave extracts was also determined in terms of measurement of % inhibition of peroxidation in linoleic acid system following a reported method of Sultana et al (2007). Extracts (5 mg) of each treatment were added to a solution mixture of linoleic acid (0.13 ml), 99.8% ethanol (10 ml) and 10 ml of 0.2 M sodium phosphate buffer (pH 7). Total mixture was diluted to 25 ml with distilled water. The solution was incubated at 40 C for 15 days and the degree of oxidation was measured following thiocyanate method with 10 ml of ethanol (75%), 0.2 ml of an aqueous solution of ammonium thiocyanate (30%), 0.2 ml of sample solution and 0.2 ml of ferrous chloride ($FeCl_2$) solution (20 mM in 3.5% HCl) being added sequentially. After 3 min of stirring, the absorption values of mixtures measured at 500 nm were taken as peroxide contents. A control was performed with linoleic acid but without extracts. Percent inhibition of linoleic acid peroxidation, 100 – [(Abs. increase of sample at 360 h/Abs. increase of control at 360 h) 100], was calculated to express antioxidant activity.

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RESULTS AND DISCUSSION

Preliminary Study

Determination and comparison of means of zone of inhibitions between leaf and stem extracts *of the two Acacia species*

Plant extracts in the Fabaceae family such as *Acacia aroma*, *Acacia arabica*, and *Mentha spicata* have been reported to possess antimicrobial properties (Mattana et al. 2010; Jain et al. 2011; Jeyakumar, 2015). In this regard, the potential of the presence of antimicrobial activity of six crude extracts (*A. berlandieri*'s leaf extracts: acetone, methanol, acetic acid and *A. rigidula*'s leaf extracts: acetone, methanol, acetic acid) was investigated. The extracts were subjected to antibacterial assays against nine different bacteria to determine and to compare the antimicrobial activities between species and plant parts - - stems and leaves (Tables 2 and Table 3).

Table 2. Antimicrobial activity of acetone, methanol, and acetic acid stem extracts of *Acacia berlandieri* and *Acacia rigidula* against human pathogenic bacterial strains.[1]

	A. berlandieri			***A. rigidula***			**Antibiotic**
Human Pathogens	**Acetone**	**Methanol**	**A.A.**	**Acetone**	**Methanol**	**A.A.**	
P. alcalifaciens	-	+	-	-	-	-	+++
E. aerogenes	-	-	-	-	-	-	+++
S. marcescens	-	-	-	-	-	-	+++
K. pneumoniae	-	-	-	-	-	-	+++
P. aeruginosa	-	-	-	+	-	-	+++
Y. enterocolitica	-	+	-	-	+	-	+++
E. coli	-	-	-	-	-	-	+++
S. aureus	+	+	-	-	+	-	+++
E. faecalis	-	-	-	-	-	-	+++

[1] Three positives (+++) were recorded for extracts showing an average zone of inhibition (ZOI) of greater than 10mm. Two positives (++) for extracts showing an inhibition of between 8-10mm and one positive (+) for extracts with a ZOI between 6-8 mm. A negative (-) was given if nothing was observed around the disc. Antibiotics: Vancomycin (*P. aeruginosa,* and *K. pneumoniae S. aureus* and *E. faecalis)* Ciprofloxacin: (*E. coli*, *Y. enterocolitica*, *E. aerogenes, P. alcalifaciens and S. marcescens*). A.A. is acetic acid. DMSO was used as a negative control and did not inhibit any bacteria, the data was not included.

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Table 3. Antimicrobial activity of acetone, methanol, and acetic acid leaf extracts of *Acacia berlandieri* and *Acacia rigidula* against human pathogenic bacterial strains.[1]

	A. berlandieri			*A. rigidula*			Antibiotic
Human Pathogens	**Acetone**	**Methanol**	**A.A.**	**Acetone**	**Methanol**	**A.A.**	
P. alcalifaciens	+	+	-	+++	++	+	+++
E. aerogenes	-	-	-	-	-	-	+++
S. marcescens	-	-	-	-	-	-	+++
K. pneumoniae	-	-	-	-	-	-	+++
P. aeruginosa	+	-	-	-	-	-	+++
Y. enterocolitica	+	-	++	+++	++	++	+++
E. coli	++	+	+	+	+	+	+++
S. aureus	+	-	-	+++	++	++	+++
E. faecalis	+	-	+	++	++	++	+++

[1] Three positives (+++) were recorded for extracts showing an average zone of inhibition (ZOI) of greater than 10mm. Two positives (++) for extracts showing an inhibition of between 8-10mm and one positive (+) for extracts with a ZOI between 6-8 mm. A negative (-) was given if nothing was observed around the disc. Antibiotics: Vancomycin (*P. aeruginosa,* and *K. pneumoniae S. aureus* and *E. faecalis)* Ciprofloxacin: (*E. coli*, *Y. enterocolitica*, *E. aerogenes, P. alcalifaciens and S. marcescens*). A.A. = Acetic acid extract. DMSO was used as a negative control and did not inhibit any bacteria, therefore is not included.

Of the nine bacterial pathogens exposed to the plant extracts, six of them (*P. alcalifaciens*, *P. aeruginosa*, *Y. enterocolitica*, *E. coli*, *S. aureus* and *E. faecalis*) showed inhibition, while the other three bacteria were not inhibited. The results demonstrated differences in antimicrobial activities as shown by the differences in mean zone of inhibitions among plant parts and solvents used for extraction (Tables 2 and 3). The results from this experiment were used to assess the antimicrobial potential of each crude extract and to determine which plant would be used for further studies. During the qualitative analysis data were not statistically analyzed. The results demonstrated that stems from both species, *A. rigidula* and *A. berlandieri* did not exhibit promising antimicrobial activity and were not used in future experiments. Therefore, leaves were again sampled and tested in order to provide further evidence of the antimicrobial activities present in leaf extracts of *A. berlandieri* and *A. rigidula*.

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Comparison of means of zone of inhibitions between leaf extracts of two Acacia species

The three leaf extracts (acetone, methanol, acetic acid) per species (*A. rigidula* and *A. berlandieri*) were subjected to antibacterial assays against nine different bacteria where the mean zones of inhibition were measured (Table 4). Both *Acacia* species exhibited differences in antimicrobial activities based from mZOIs. *A. berlandieri* displayed minimal inhibitory effects (mZOI, 6.00 -8.99 mm) against three of the nine bacterial species (*P. alcalifaciens*, *P. aeruginosa*, and *Y. enterocolitica*). Six of the nine bacterial species were susceptible to the *A. rigidula* extracts (acetone, methanol, and acetic acid): four gram-negative (*P. alcalifaciens*, *P. aeruginosa*, *Y. enterocolitica* and *E. coli*) and two gram-positive (*S. aureus* and *E. faecalis*). The ZOIs ranged from 8.70 to 17.56 mm for acetone extract, 7.83 to 14.43 mm for methanol extract, and 6.00 to 12.33 mm for acetic acid extract. Surprisingly, the reports from this study provided different results against *Pseudomonas aeruginosa* (Cock, 2012).The remaining gram-negative pathogens (*S. marcescens* *E. aerogenes*, and *K.* pneumonia) were not inhibited by any of the extracts, consistent with results of the preliminary experiment.

Table 4. Average zone of inhibitions in millimeters (mm) of acetone, methanol, and acetic acid leaf extracts of *Acacia berlandieri* and *Acacia rigidula*.[1]

	A. berlandieri			***A. rigidula***			**Antibiotic**
Human Pathogens	**Acetone**	**Methanol**	**A. A.**	**Acetone**	**Methanol**	**A. A.**	
P. alcalifaciens	**8.05**	**8.99**	**7.61**	**17.56**	**13.57**	**12.33**	34.40
E. aerogenes	6.00	6.00	6.00	6.00	6.00	6.00	36.00
S. marcescens	6.00	6.00	6.00	6.00	6.00	6.00	35.29
K. pneumoniae	6.00	6.00	6.00	6.00	6.00	6.00	33.56
P. aeruginosa	**8.50**	**8.66**	6.00	**12.21**	**11.91**	**9.55**	31.31
Y. enterocolitica	**8.62**	6.00	6.00	**16.50**	**14.43**	**12.19**	34.79
E. coli	6.00	6.00	6.00	**8.70**	**8.40**	6.44	34.69
S. aureus	6.00	6.00	6.00	**11.37**	**9.48**	**9.55**	21.01
E. faecalis	6.00	6.00	6.00	**12.07**	**7.83**	6.00	16.13

[1]Measurements of 6.00 mm were recorded as not having activity (size of disc) while anything above 6.00 mm was recorded as having antimicrobial activity and given the respective value. A.A. is acetic acid. DMSO was used as a negative control and did not inhibit any bacteria (zone of inhibitions were 6.00 mm), therefore is not included.

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Similar results were reported from two studies of acetone and methanol extracts of two species belonging to the Fabaceae family, *Acacia arabica* and *Acacia aulacocarpa* (Cock, 2012; Jeyakumar, 2015). Jeyakumar (2015) extracts of *A. arabica*, using different solvents, showed varying degrees of antimicrobial activities against gram-positive and gram-negative organisms selected for the study. Three microorganisms (*Escherichia coli*, *Staphylococcus aureus* and *Pseudomonas aeruginosa*) used in Jeyakumar's (2015) study were among three of the six susceptible bacteria in this study. Cock's study of *A. aulacocarpa* revealed that both gram-positive and gram-negative bacteria were inhibited by *A. aulacocarpa* methanol extracts. Of the six susceptible bacteria in this study, *Yersinia enterocolitica* and *Staphylococcus aureus*, were also susceptible to *A. rigidula's* methanol extract. Furthermore, the three gram-negative bacteria (*K. pneumonia*, *E. aerogenes* and *S. marcescens*) were not inhibited by neither *A. rigidula* nor *A. berlandieri*, nor were they affected in Cock's study (2012). Finally, the two gram-positive bacteria included in this present study (*S. aureus* and *E. faecalis*) were both susceptible to our extracts and in agreement with Cock (2012).

Of the two categories, gram-positive are known to be in general more inhibited than gram-negative bacteria. Gram-positive bacteria lack the outer membrane, exposing them to antibiotics or in this case, secondary metabolites. Without the outer membrane, secondary metabolites are able to penetrate the peptidoglycan envelope and reach the cell membrane of gram-positive bacteria easier (Azzam et al. 2014; Dzoyem et al. 2014; Ani et al. 2015). In this study, both *S. aureus* and *E. faecalis* were inhibited by *A. rigidula* leaf extracts. *S. aureus* and *E. faecalis* are commonly used gram-positive bacteria which are susceptible to extracts in other studies as well (Cock, 2012; Azzam et al. 2014; Dzoyem et al. 2014; Jeyakumar, 2015). The susceptibility of *E. faecalis* has been reported by the Jaidka et al. (2017) as phenol derivatives have the ability to partition the lipids of the bacterial cell membrane and mitochondria causing extensive leakage from bacterial cells leading to death. Conversely, gram-negative bacterial cell wall outer membrane (a lipopolysaccharide) is thought to act as a barrier to many substances including antibiotics.

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This was evident by the fact that 5 of the 7 gram-negative bacteria conferred resistance to extracts in this study and Cock's study (2012).

Comparison of the antibacterial activity between both species and solvents

The mZOI observed in the plants extracts against *P. alcalifaciens*, *Y. enterocolitica*, *E. faecalis*, and *S. aureus* were significantly different to those of DMSO (negative control) at an α = .01 (Table 5). The analysis of variance determined significance, between plant species (Figure 2) and among solvents to establish the most effective treatment (Figure 3). The negative control (DMSO) in all assays did not exhibit any inhibition, thus indicating that the inhibition observed was attributed to the positive control (antibiotics) or the antibacterial properties exhibited by active components of the extract. The four most susceptible microorganisms to *Acacia* extracts are shown by results from the disc diffusion assay (Figure 2). For all four bacteria, *A. rigidula* extracts showed significant differences in mean ZOI (α = .01) when compared to *A. berlandieri* extracts. Although the extracts of *A. berlandieri* were not significantly different from DMSO (negative control), *A. rigidula* did show a significant difference (Table A1-A6). Lines extending from the colored bars (blue and green) represent 95% confidence intervals (CIs). Non-overlapping CIs for side-by-side colored bars indicate significant difference in mean ZOI.

Of the six susceptible bacteria, *P. alcalifaciens*, *Y. enterocolitica*, *E. faecalis*, and *S. aureus* were the most inhibited by *A. rigidula*. Specifically, acetone extracts showed a higher ZOI than methanol and acetic acid extracts; however, there was no significant difference among the three solvents (Figure 3). The results from this study are comparable to that of two other studies (Dzoyem et al. 2014; Jeyakumar, 2015). Both studies used acetone for extraction and reported varying degrees of antimicrobial activity. Dzoyem et al. (2014) tested nine different plant species within the Fabaceae family, six of them reporting significant activity against at least one bacterial species. Jeyakumar's (2015) acetone extracts of *A. arabica* displayed promising results, specifically against *S. aureus* with a mZOI ranging between 17.67 to 22.67 mm. These

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results set the baseline to use *A. rigidula's* acetone extract for further analysis or in the main study. In this study more replications were used and aimed to determine any significant difference among the ZOI of the three bacteria that have shown the largest ZOI.

Table 5. Analysis of variance for *P. alcalifaciens*, *Y. enterocolitica*, *S. aureus*, and *E. faecalis*.[1]

Human Pathogens	Source	SS	DF	MS	F Value	P>F	
P. alcalifaciens	Block	0.111348	2	0.055674	0.02	0.9836	
	Species	142.361345	1	142.361345	42.38	<.0001	***
	Extract	3809.853758	4	952.463442	283.51	<.0001	***
	Species*Extract	78.523976	4	19.630994	5.84	0.0034	**
Y. enterocolitica	Block	10.304587	2	5.152294	2.84	0.0845	
	Species	16.815053	1	16.815053	9.28	0.007	**
	Extract	1250.401728	4	312.600432	172.49	<.0001	***
	Species*Extract	47.544595	4	11.886149	6.56	0.0019	***
S. aureus	Block	8.4296867	2	4.2148433	3.47	0.0533	
	Species	38.5333333	1	38.5333333	31.69	<.0001	***
	Extract	697.7986348	4	174.4496587	143.49	<.0001	***
	Species*Extract	30.4724481	4	7.618112	6.27	0.0024	**
E. faecalis	Block	45.928165	2	22.964083	6.47	0.0076	**
	Species	137.723471	1	137.723471	38.79	<.0001	***
	Extract	3229.575826	4	807.393956	227.4	<.0001	***
	Species*Extract	113.759614	4	28.439904	8.01	0.0007	***

[1]*,**, and ***Denote significance at the 0.05, 0.01 and 0.001 level, respectively

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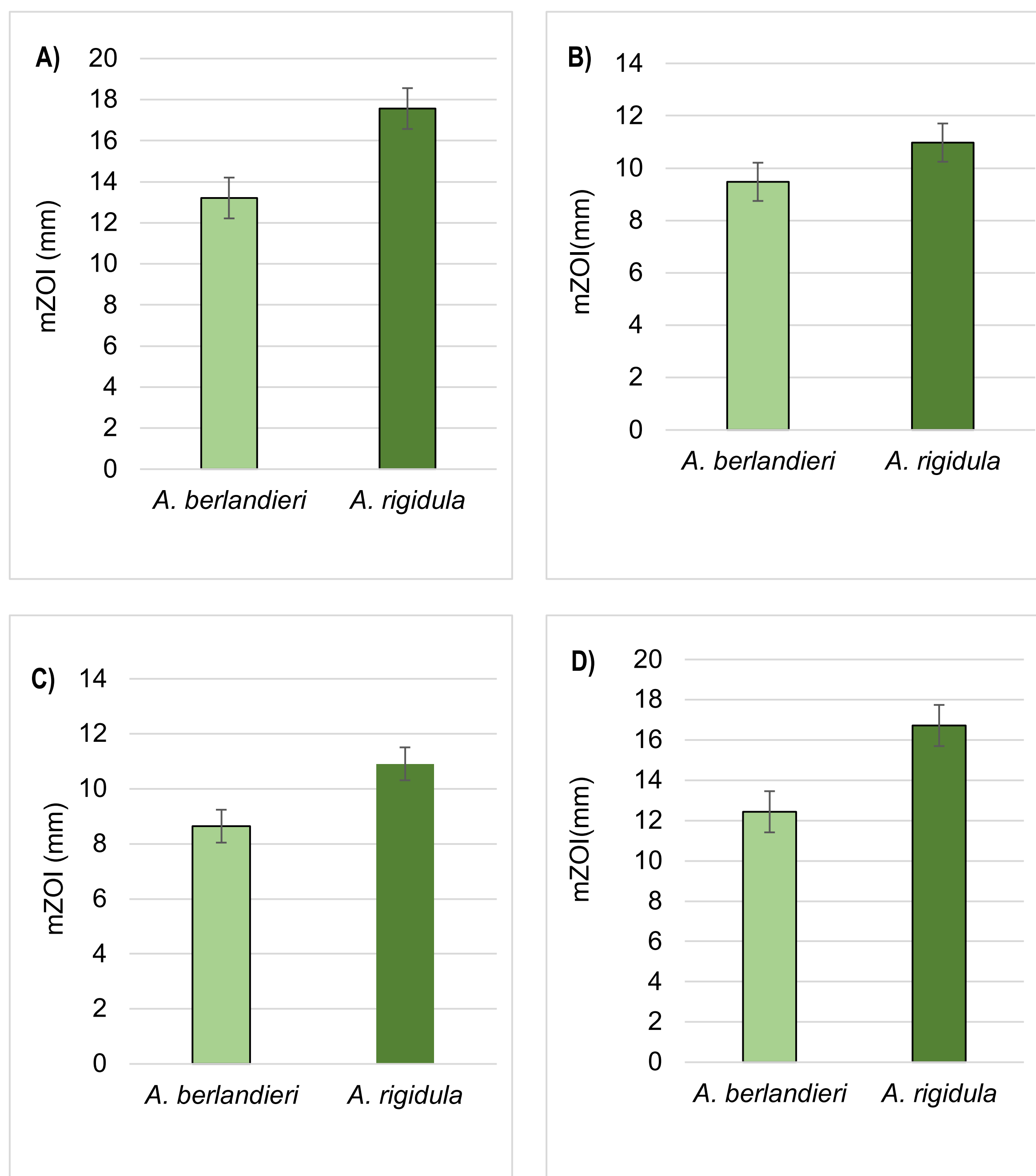

Figure 2. Comparison of the mean zone of inhibitions of *Acacia berlandieri* and *Acacia rigidula* leaf extracts against four human pathogens. A) *P. alcalifaciens* ($p<0.001$), B) *E. faecalis* ($p=0.007$), C) *S. aureus* ($p<0.001$), and D) *Y. enterocolitica* ($p<0.001$).

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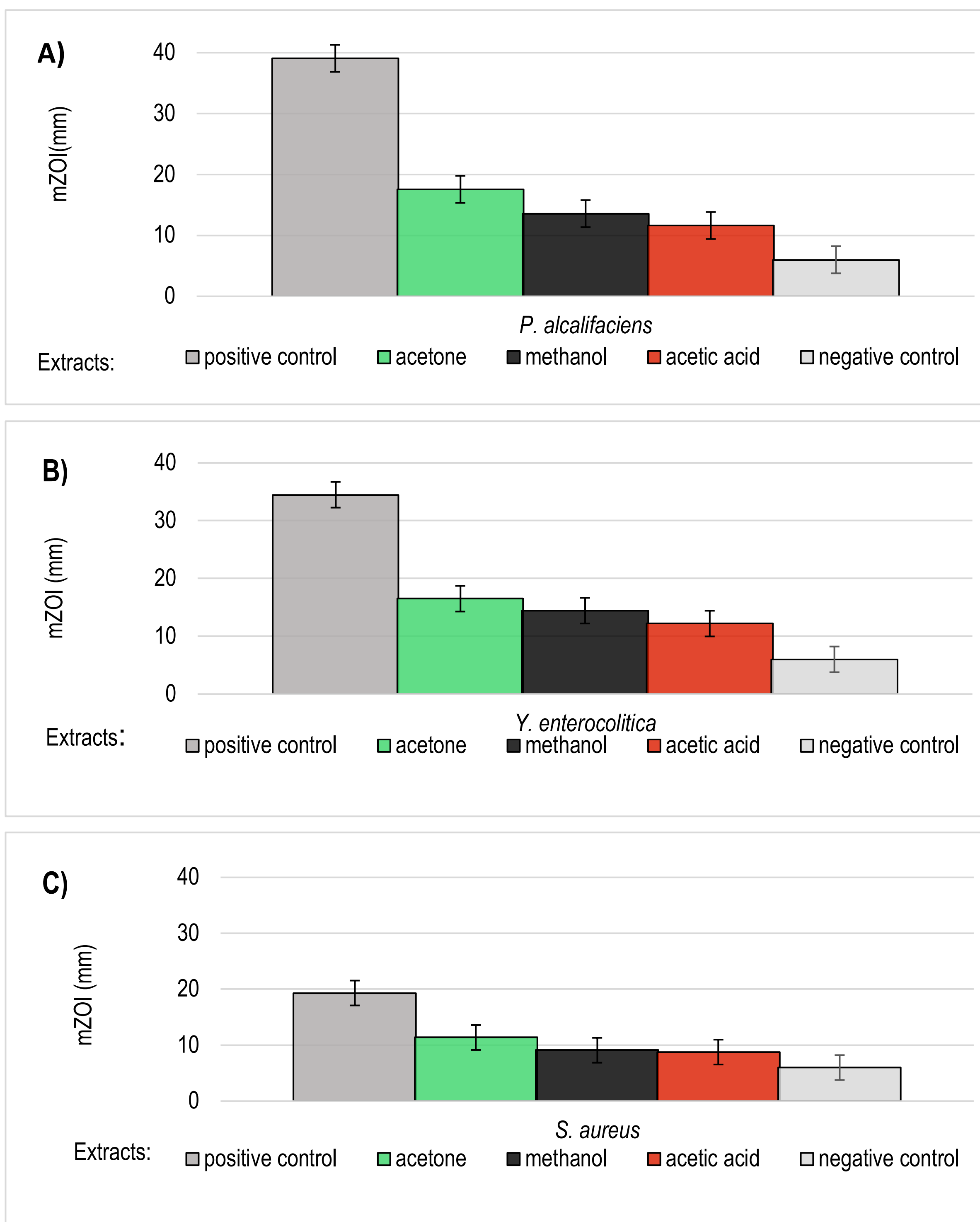

Figure 3. Comparison of mean zone of inhibitions of acetone, methanol, and acetic acid *Acacia rigidula* leaf extracts. Notes: A) *P. alcalifaciens*, B) *Y. enterocolitica*, and C) *S. aureus*. Bars within a bacterial treatment, non-overlapping confidence intervals indicate significant difference at α= .01. See appendices (pages 54-55) for least mean squares and respective p values.

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Main Study

Comparison of the effect of *A. rigidula* acetone leaf extracts on mean zone of inhibitions among four bacterial pathogens

Four bacterial pathogens were subjected to acetone extracts of *Acacia rigidula* from six different samples (n=72). Mean ZOIs of acetone extracts against *P. alcalifaciens, E. faecalis, S. aureus,* and *Y. enterocolitica* ranged from 10.41 to 14.65mm, 9.51 to 13.17mm, 12.44 to 14.97mm, 11.72 to 16.44mm, respectively. Each assay was replicated in quadruplets and showed consistent antimicrobial activity (Figure 4). Neither bacterial species was statistically significant from the others but *Y. enterocolitica* did display the highest mZOI compared to the other microorganisms (Table A7 and A8). None of the six extracts showed a significant difference amongst one another but were significantly different when compared to DMSO (negative control) (Table 6). The antibiotics used as positive controls (Ciprofloxacin and Vancomycin) were significantly more active against the four bacterial species when compared to all the plant extracts and DMSO.

Table 6. Analysis of variance for all six samples using acetone as the extraction solvent.

Source	SS	DF	MS	F Value	P>F	
Block	17.124672	5	3.424934	0.97	0.4436	
Bacteria	54.356074	3	18.118691	5.14	0.0033	**
Extract	3313.970685	2	1656.985342	469.86	<.0001	***
Bacteria*Extract	116.705236	6	19.450873	5.52	0.0002	***

***Denote significance at the 0.001 level, **Denote significance at the 0.01 level.

After comparing the six different samples to one another, pooling the data provided the analysis to compare the efficacy per treatment against the four microorganisms (Figure 4). As expected, the antibiotics displayed a significantly higher ZOI compared to the acetone extract and the negative control. In addition, when comparing each bacterium, neither of them were statistically significant from one another. However, there is a significant difference in the ZOI of the acetone extract against all four microorganisms when

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comparing it to the negative control. A review of literature revealed a few studies whereby acetone extracts exhibited antimicrobial activity (Patel et al. 2009; Sharma et al. 2014; Jeyakumar 2015). Each study included different solvents and microorganisms subjected to disc diffusion assays, but all reported a potent acetone extract displaying maximum activity against *S. aureus*, one of the species in this study as well. This study is one of few when identifying the efficacy of *A. rigidula* extracts and inhibiting specifically *P. alcalifaciens.* As of now, no other study have provided data inhibiting the growth of *P. alcalifaciens.* This provides evidence into the potential pharmacological applications of this species.

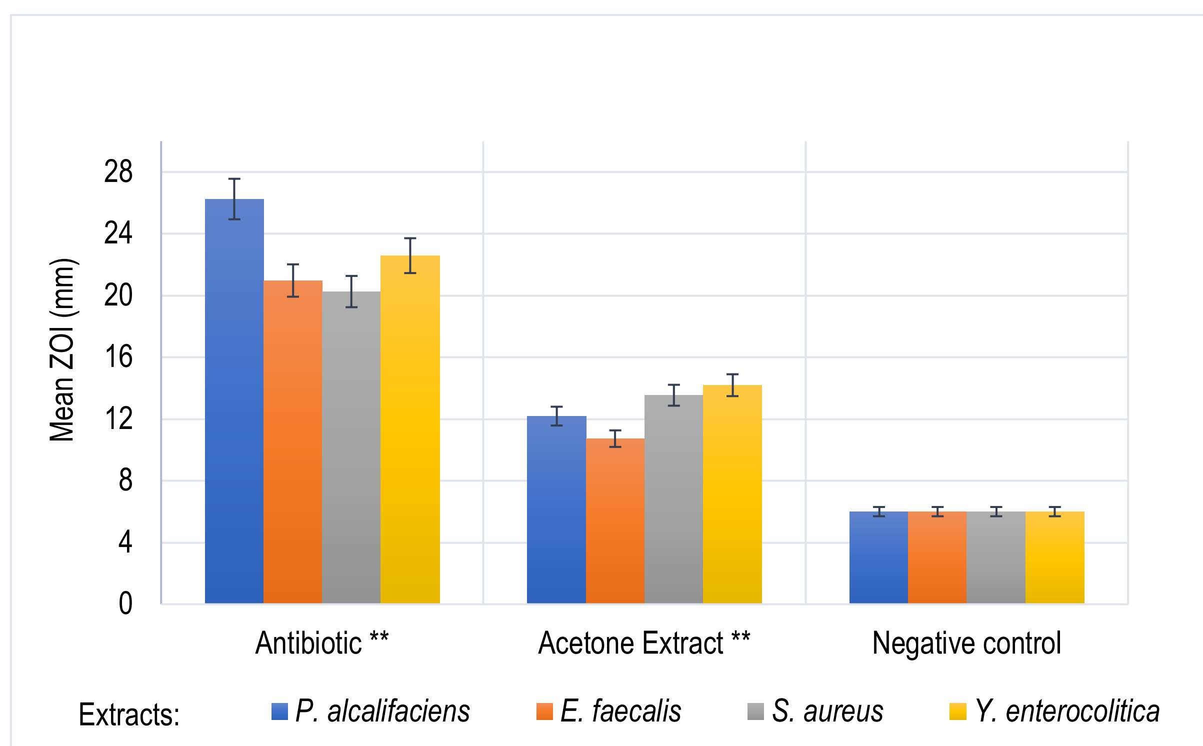

Figure 4. Efficacy of *Acacia rigidula's* acetone extract against *P. alcalifaciens, E. faecalis, S. aureus,* and *Y. enterocolitica*. ** Denote significance at 0.001 level.

Determination of minimum inhibitory concentration (MIC)

Acetone extracts of *Acacia rigidula* leaves were tested using four different concentrations ranging from 0.0625 to 0.5mg/uL, along with three controls: (1) negative, (2) double negative and (3) positive, to assess the minimum inhibitory concentration (MIC). The negative (-) control was a sterilized blank to

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ensure no contamination, the double negative (--) included the respective antibiotic's MIC along with the bacterium and the positive (+) was the bacteria alone in broth medium. The three microorganisms (*P. alcalifaciens, Y. enterocolitica and S. aureus*) were replicated in triplicates by assessing the turbidity by optical density readings at 600nm at 0 hours and 16 hours. The mean difference was then taken between 0 hours and 16 hours (Table 7). Aside from the MIC of the microorganism's respective antibiotic, all three bacteria were affected at the highest concentration, 0.5 mg/uL. When comparing all three bacteria, extracts at different concentrations displayed higher activity against *S. aureus*.

Table 7. Determination of minimum inhibitory concentrations using the acetone extract of *Acacia rigidula* against the three *P. alcalifaciens*, *Y. enterocolitica*, and *S. aureus* at OD_{600} nm.

Treatments	**Test organisms**		
	P. alcalifaciens		
	0 hours	**16 hours**	**Mean difference**
Negative (-)	0.014	0.013	-0.001
Double negative (--)	0.022	0.022	0.00
Positive (+)	0.017	0.581	0.564
0.5 mg/uL	0.971	1.017	0.046
0.25 mg/uL	0.073	0.247	0.174
0.125 mg/uL	0.050	0.288	0.230
0.0625mg/uL	0.034	0.311	0.277
	Y. enterocolitica		
	0 hours	**16 hours**	**Mean difference**
Negative (-)	0.014	0.013	-0.001
Double negative (--)	0.016	0.015	-0.001
Positive (+)	0.018	0.875	0.857
0.5 mg/uL	1.211	1.264	0.053
0.25 mg/uL	0.071	0.188	0.117
0.125 mg/uL	0.043	0.310	0.267
0.0625mg/uL	0.025	0.523	0.498

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Table 7 continued

	S. aureus		
	0 hours	**16 hours**	**Mean difference**
Negative (-)	0.017	0.015	-0.002
Double negative (--)	0.025	0.023	-0.002
Positive (+)	0.026	0.928	0.902
0.5 mg/uL	1.248	1.263	0.015
0.25 mg/uL	0.074	0.157	0.083
0.125 mg/uL	0.049	0.255	0.206
0.0625mg/uL	0.032	0.481	0.449

For extracts to be designated as the MIC, a difference of 0.005 or below between 16 hours and 0 hours must be recorded. From the results, no extract showed a difference of 0.005, but 0.05 mg/µL concentration against *S. aureus* produced the nearest results. This does not mean that the MIC could not be determined. Our extracts have not been purified and characterized to isolate the fraction with the greatest activity; therefore, it is not as potent as the respective antibiotics. A study performed by Chorianopoulos et al. (2006) tested to determine the MIC of *Satureja spinosa* oils against *E. coli*. Reports from that study displayed an effective MIC at low concentrations against *E. coli*. Using the spectrophotometer provides an alternative rapid method for screening the biocide activity of novel antimicrobial agents (Chorianopoulos et al. 2006).

Qualitative phytochemical analysis

All extracts were analyzed for the presence of secondary metabolites using standard procedures for phytochemical analysis. The intensity of color developed and/or the appearance of precipitation in the reactions were observed. Medicinal plants are still the most preferable source of new bioactive chemical compounds. The plants used in the present study proved as a good source of bioactive compounds in inhibiting a broad array of microorganisms. Of the six extracts, most contained flavonoids, saponins, phenols, tannins and terpenoids (Table 8).

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Table 8. Determination of the presence of secondary metabolites for the *Acacia* extracts through qualitative phytochemical tests.[1]

Tests	*Acacia berlandieri*			*Acacia rigidula*		
	Acetone	Methanol	Acetic Acid	Acetone	Methanol	Acetic Acid
Cardiac Glycosides	-	-	-	+ light green	-	+ light green
Diterpenes	++ green color solution	++	+++ emerald green	+++	++	+
Sterols/ Triterpenes	+ two phase formation, no red color	+++ two-phase formation, with red precipitate	+ two-phase formation, no red color	+ two-phase formation, slight red	+++ two-phase formation, with red precipitate	+ two-phase formation, no red color
Phenols	+++ dark blue green color	+++ dark blue green color	+++ dark blue green color	+++ dark blue green color	+++ dark blue green color	+++ dark blue green color
Tannins	+++ blue-black color	– no color change	+ slightly dark color	+++ blue black color	+++ blue black color	+++ blue black color
Flavonoids	+++ blue green color	+++ blue green color	+++ blue green color	++ blue green color	+++ blue green color	+++ blue green color
Saponins	+++ 1 cm foam layer	+++ 1 cm foam layer	++ 1 cm foam layer	+++ 1 cm foam layer	++ 1 cm foam layer	+++ 1 cm foam layer

[1]The compounds, which were clearly present was symbolized as (+++), fairly present as (++), slightly present as (+), whereas negative reactions (−) represent the absence of those particular compounds in respective extracts. All extracts were negative for resins and alkaloids and therefore are not included in the table.

Saponins have several biological effects, some of which are antibacterial, antifungal, and antiviral (Sparg et al. 2004; Mandal et al. 2005; Freiesleben and Jager, 2014). The presence of saponins in plants have been related to a defense role against human pathogenic microbes and this was also reported by Mandal et al. (2005). Of the saponins isolated from *Acacia auriculiformis*, one of the microorganisms

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inhibited from the study was *Pseudomonas aeruginosa*. Similarly, *P. aeruginosa* was inhibited by extracts from both species from the data report as all six extracts displayed signs of saponins. Surprisingly, *A. berlandieri* exhibited most of its inhibitory effects against gram-negative bacteria. Saponins have been studied to have antimicrobial mechanisms, which include disruption of the cell membrane integrity. Saponins have a steroidal or triterpenoid aglycone which is able to form complexes with cholesterol. When doing so, these surface-active properties increase membrane permeabilization allowing for cells to lyse (Freiesleben and Jager, 2014).

Tannin compounds have been noted to possess antibacterial, antiviral and inhibitory effects on enzymes (Nakashima et al. 1992). Studies have suggested that tannins possess antibacterial capabilities, more importantly on *S. aureus* (Scalbert, 1991; Akiyama et al. 2001). When exposed to several tannins, *S. aureus* is unable to coagulate plasma. The qualitative phytochemical analysis revealed that *A. rigidula* extracts exhibited higher amounts of tannins when compared to *A. berlandieri. A. berlandieri* provided little to no evidence when testing for tannins in the methanol and acetic acid extract. This can attribute to the inhibitory effects of all three extracts from *A. rigidula*, which showed anti-*S. aureus* activity. Specifically, the acetone extract of *A. rigidula* showed the highest activity against *S. aureus* and displayed greater positive results of tannins when comparing the three solvents used for extraction.

Terpenoids have been shown to be active against bacteria, fungi, viruses and protozoa and their mechanism of action is speculated to involve membrane disruption by the lipophilic compounds (Cowan, 1999; Singh and Thakur 2016; Zhou, 2015). All extracts showed different degrees of positive results containing diterpenes and triterpenes, both of which are a class of terpenoids. Methanol extracts of *A. berlandieri* and *A. rigidula* displayed greater positive results when comparing them to the other solvents. According to Jeyakumar (2015) and the phytochemical screening of *A. arabica*, the presence of terpenoids was confirmed. As mentioned previously, three inhibited test subjects in their study were also affected in this study. The membrane interactions of terpenoids to modify membrane property and organization have

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been implicated and could correlate to such antibacterial properties (Cowan 1999). The possibility of terpenoid compounds having a range of polarity and the differences in concentration in each species could suggest the degree of positivity among all three solvents.

Flavonoids constitute one of the most ubiquitous groups of phytochemicals contained in medicinal plants. They cover a very broad pharmacological spectrum and the mode of action is only partially understood. Research indicates that flavonoids interact with functional proteins as their primary target, but bioactive flavonoids have been presumed to act on lipid bilayers modifying the membrane's physicochemical properties (Mansuri et al. 2014). In addition, flavonoids from some medicinal plants have been found to cause permeability of the inner bacterial membrane in gram-negative and gram-positive bacteria (Cushnie and Lamb, 2005). The phytochemical analysis displayed higher positive results for flavonoids but revealed no difference among species nor solvents. According to a study by Singh and Thakur (2016), *A. nilotica* leaves found high amounts of flavonoids within their extracts, of which their methanol extract exhibited antimicrobial activity against *E. faecalis*. Along with flavonoids, their methanol extract showed the presence of saponins and tannins, both of which were present in our *A. rigidula* extract (Singh and Thakur 2016). This study provides further confirmation that medicinal properties are found within the Fabaceae family. However, with no conspicuous differences among extracts, no assertions can be made when comparing species or solvents and the same follows for phenols.

Plant phenolics are chemically heterogeneous with different biological activities. Phenolics have been known to play a variety of roles in the plants, one of which is serving as defense against pathogens. The mode of action is said to include denaturation of the bacterial proteins and lysis of the cell membrane (Bhattacharya et al. 2010). The presence of phenols in all extracts can attribute to the antimicrobial effects among the six susceptible bacteria. These reports are in congruence with two other studies that exhibited high phenols (Hossain et al. 2012; Jeyakumar, 2015). Both studies have exhibited high amounts of phenols in plants species belonging to the Fabaceae family where they showed to have antimicrobial activity

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against both gram-positive and gram-negative microorganisms. As with the flavonoids, no difference was exhibited between species or solvents.

Alkaloids have been found to have antimicrobial properties with microbicide effects (Cowan 1999). However, in this study, no alkaloids were extracted from either species. It is suggested that alkaloids were not soluble in the following immiscible solvents when extracting alkaloids due to the nature of their polarity (Sarker, 2006). Singh and Thakur (2016) provided similar findings where *A. nilotica* leaves extracted with butanol, ethanol and methanol did not reveal any alkaloids. Singh and Thakur used both polar and nonpolar solvents for extraction while this study focused primarily on polar solvents (2016). A previous study performed by Clement et al. (1998) identified several alkaloids in the stems of *A. rigidula* using chloroform, a polar solvent but having a lesser dipole moment as the solvents used in this study. They identified several alkaloid constituents but concluded that early and late seasons of the plant detects varying concentrations of these derivatives. Therefore, absence of alkaloids could be due to the choice of polar solvents, the age of the species, and the plant part selected.

Chemical analysis

Nuclear Magnetic Resonance (NMR)

Nuclear Magnetic Resonance provides a sensitive and powerful method of detecting presence of different protons depending on their chemical environment. It can be used to monitor reaction progress and detects the presence of different compounds. Due to the high polarity of the extracts, deuterated water (D_2O) was used as the NMR solvent. The extracts were found to be insoluble in common NMR solvents such as chloroform-d ($CDCl_3$) and benzene-d_6 (C_6D_6). Since, phytochemical analysis provided clues for the structure of the compounds; the chemical shifts observed were used in order to access different types of protons that are indicative of the presence of the class of metabolites present in the extract. Representative ^1H NMR spectra of extraction solvents of *A. rigidula* are reported in Figure 5.

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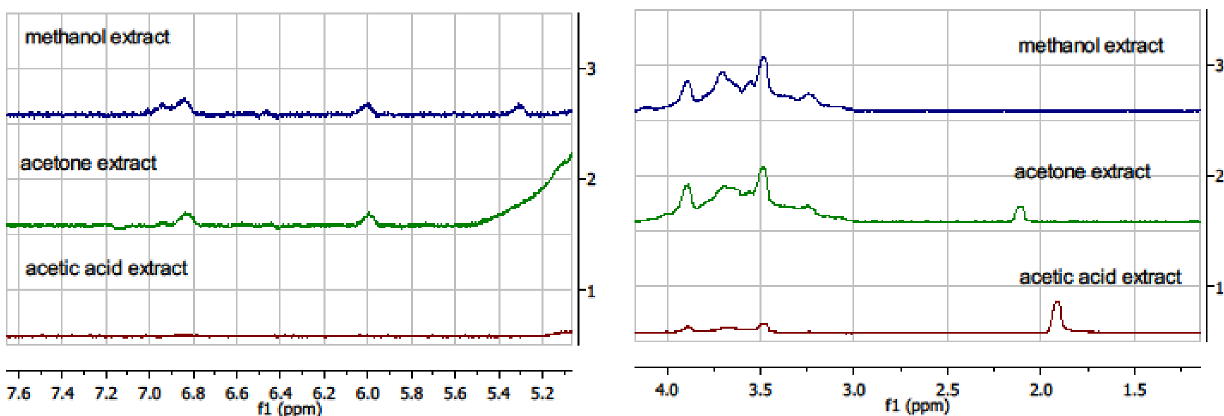

Figure 5. ^{1}H NMR spectra of *Acacia rigidula's* acetone, methanol, and acetic acid extracts in deuterated water (D_2O).

The broad ranges are typical of hydrogen bonded protons e.g. OH and NH. The broad peaks associated with OH groups seen in the ^{1}H NMR of the acetone and methanol extract may be due to the exchangeable nature of such protons. Because a protic deuterated solvent was used (D_2O), then the NH and OH protons exchanged with the deuterium and the peaks will either appear too broad or disappeared entirely, as a result D(^{2}H) did not show up in the ^{1}H NMR spectrum (Brown et al. 2018). In this regard, the acetone and methanol spectra that displayed signals in the 4-7 ppm region are likely to be contributed by hydrogens in alcohol (O$\underline{H}$) groups. Although peaks were not displayed for the acetic acid extracts, this could be the results of how polar the constituents in the extract are (OH being an exchangeable proton in deuterated solvents). In the region between 3.0-5.0 ppm, peaks likely originate from the methylene protons ($C\underline{H}_2$) of hydrocarbon chains belonging to high molecular-weight n-alkanes. The peaks are found in all three extracts but more so in the acetone and methanol extract. Signals between the regions of 6.5-7.5 ppm are associated with the aromatic protons and are present in both the acetone and methanol extracts. The results from this study were able to identify potential chemical structures belonging to all five classes of secondary metabolites qualitatively determined in the phytochemical analysis. Although, the results of this experiment were comparable to those of Buchanan et al. (2007), where the presence of aromatic rings in

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Acacia confusa were detected using ^{1}H NMR spectroscopy; the isolation and purification of the extracts is highly recommended in order to confirm the results of this experiment. The next two analytical techniques further support the presence of the chemical constituents reported in all three extracts, as qualitatively determined by the phytochemical analysis.

Ultraviolet-Visible spectroscopy

Ultraviolet-Visible spectroscopy allows for the determination of conjugated organic compounds in unknown extracts. The qualitative UV-Vis spectroscopy profile of all three extracts of *A. rigidula* was selected at a wavelength between 200 to 450 nm (Figure 6). The UV-Vis profile of the acetone extracts showed peaks at 215, 235, 260, 270, 290, 310, and 325 nm with the absorption 4.523, 10.000, 10.000, 10.000, 10.000, 3.676, and 3.232, respectively (Figure 6). The absorbance values of each peak is greater than 1 and this can be attributed to how concentrated the extracts were. As a result, in the UV-VIS spectra there is an appearance of one or more peaks in the region from 200 to 400 nm with high absorbance values that is indicative of the presence of unsaturated groups and heteroatoms such as S, N, O (Jain et al. 2016). The results of this profile provide characteristics for phenols and its derivatives such as flavonoids and saponins.

The spectrum for *A. rigidula* acetone extract shows multiple peaks within these exact parameters. More so, flavonoids typically consist of two absorption maxima in the ranges 240-285 nm (band I) and 300-350 nm (band II) which are represented in the acetone spectra. UV-Vis analysis reported on two species belonging to the Fabaceae family, *Acacia raddiana* and *Meizotropis pellita,* support the findings from this study (Rani et al. 2016; Elgubbi et al. 2019). Results on these two species presented similar chemical characteristics that relate to those found in flavonoid derivatives (Rani et al. 2016; Elgubbi et al. 2019). UV-Vis spectra of *M. pellita* displayed peaks at 235 and 270 nm, while the spectra for *A. raddiana* showed a peak at 272 nm, both of which are associated with the bands that correspond with flavonoids and are

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shown in the results of this study. The findings from UV-Vis analysis were further supplemented with the analytical technique known as infrared (IR) spectroscopy.

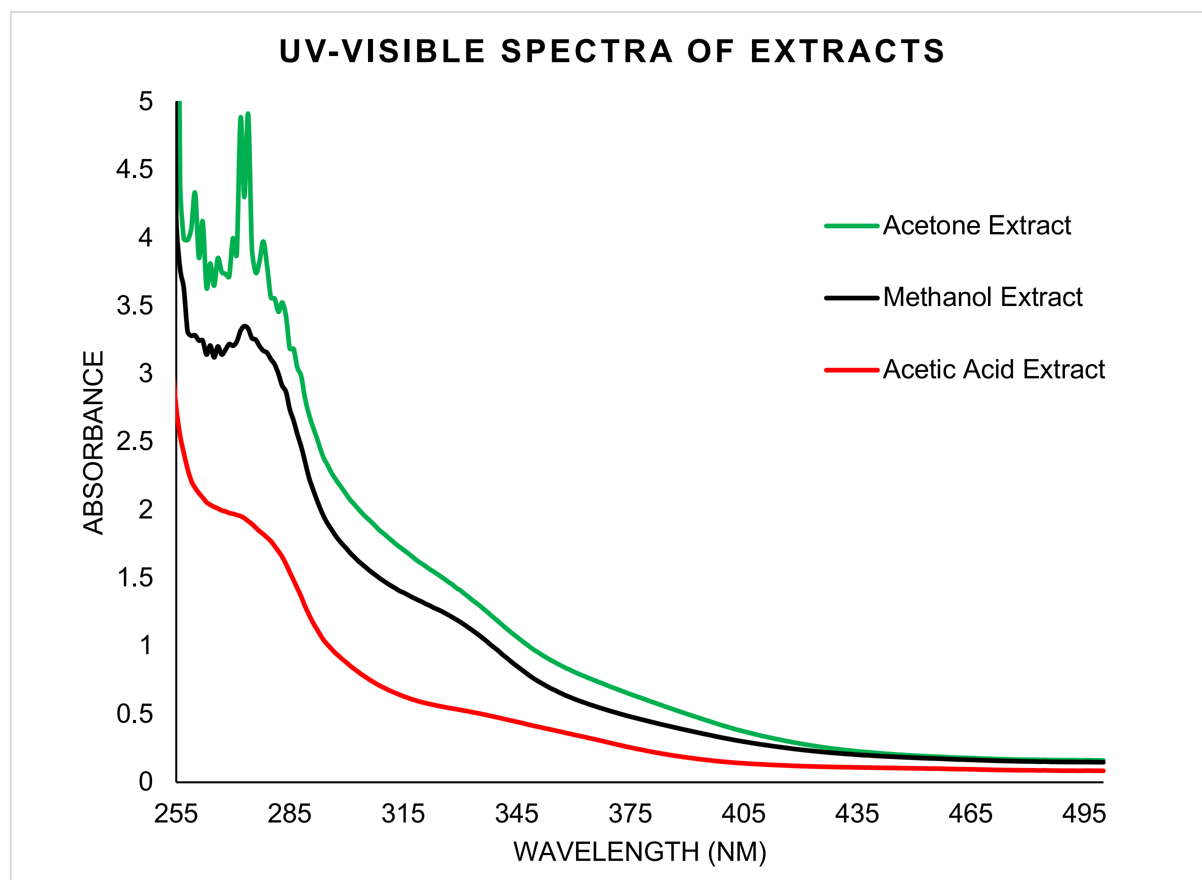

Figure 6. Ultraviolet-visible spectra of Acacia rigidula's acetone, methanol, and acetic acid extracts.

Infrared Spectroscopy

Infrared spectroscopy was performed to determine the functional groups present in all three extracts of leaves of *A. rigidula*. The results obtained in the infrared region enable the identification of the chemical constituents and elucidation of the structures of compounds. The results of IR peak values and functional groups are represented in Table 9. The IR spectrum profile is illustrated in Figure 7. The acetone extract displayed an absorption at 3263.03 cm^{-1} that is due to the stretching of hydroxyl groups present in the extract. It gave a strong peak at 2927.47 cm^{-1} which indicated the presence of C-H group that is due to

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the stretching of saturated (sp^3) carbons. The peaks obtained 1702.87 cm^{-1} indicates the presence of C=O stretching in carboxylic acids, while the peak at 1031.75 cm^{-1} is associated with C-O of esters. The peaks at 1606.44 cm^{-1} and 1442.52 cm^{-1} is due to C=C stretching associated with the aromatic skeletal mode of the extracts.

Table 9. Infrared absorption values of common functional groups found in *A. rigidula* extracts.

Wavelength (cm^{-1})	Interpretation
3263.03 cm^{-1}	-OH stretch
2927.47 cm^{-1}	C-H stretch
1702.87 cm^{-1}	C=O (carbonyl)
1606.44 cm^{-1}	Alkene C=C
1442.52 cm^{-1}	Aromatic C=C
1031.75 cm^{-1}	C-O (ester)

The results from other studies of *M. pellita* and *A. raddiana* both confirmed the presence of alcohols, carboxylic acids, aromatic, and halogen compound through the use of infrared spectroscopy to elucidate structural compounds in their extracts (Rani et al. 2016; Elgubbi et al. 2019). IR spectra of both *A. raddiana* and *M. pellita* displayed stretches produced by hydroxyl groups between 3269.21 cm^{-1} and 2249.81 cm^{-1}, respectively. Methylene stretches in both species were seen between the wavenumbers of 2927-2031 cm^{-1}, and C=O stretches between 1649.80-1691.20 cm^{-1}.

Characterization of secondary metabolites using fingerprinting analytical techniques provides valuable information by spectroscopy and other methods. Chemometry allows for the recognition of plant's chemical composition through the use of qualitative and quantitative formulations that were previously unknown. Employing NMR, UV-Vis, and IR spectroscopy, the results of each separate method reinforce

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one another and allow for the confirmation of characteristic functional groups such as carboxylic acids, alcohols, phenols, esters, and unsaturated hydrocarbons such as alkenes and arenes (C=C). All of which are necessary for bioactive compounds that could be responsible for the various medicinal properties of *Acacia rigidula*.

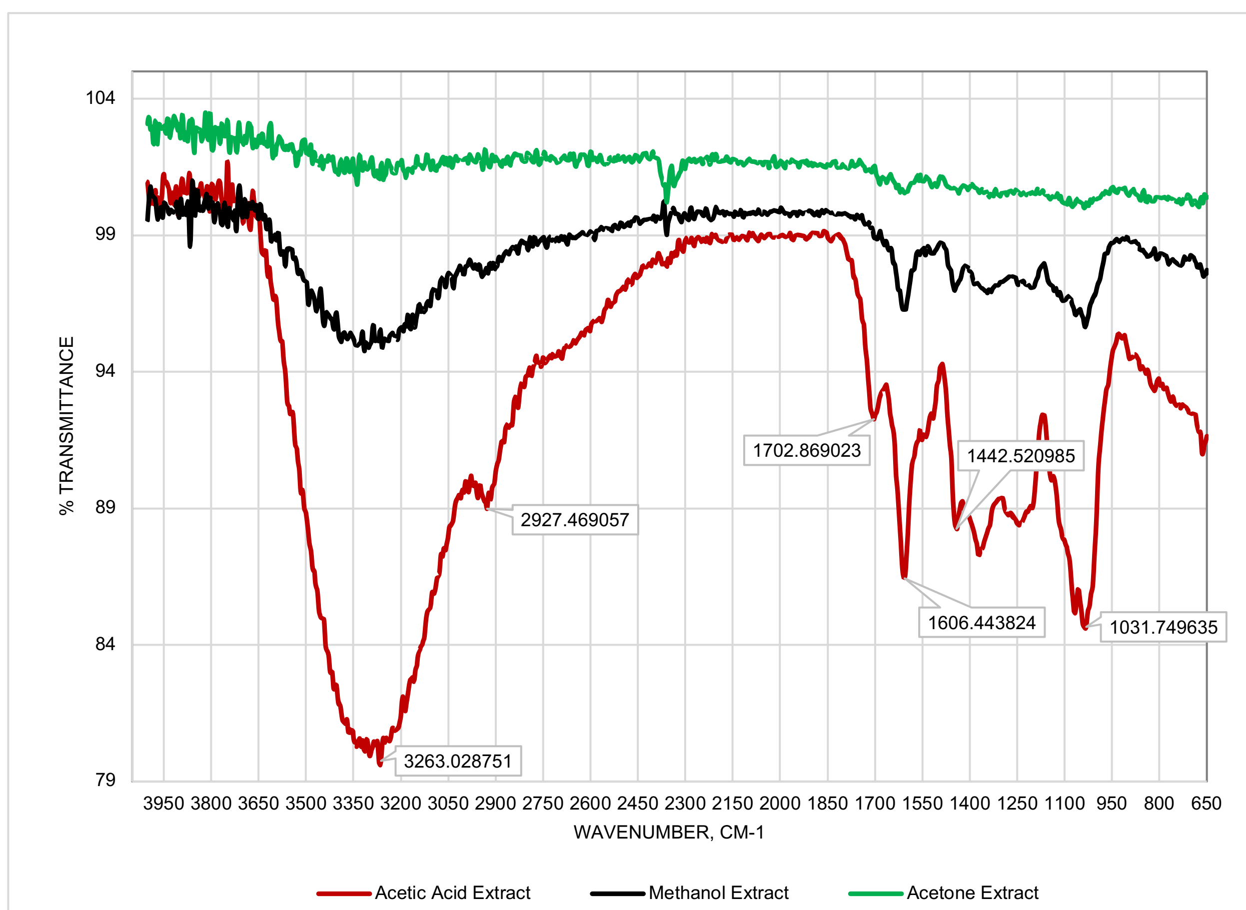

Figure 7. Infrared spectra of *Acacia rigidula* extracts: Acetone, Methanol, and Acetic Acid. Peak values are given for distinct functional groups absorbed at their respective value.

Antioxidant assay

Oxidative damage by free radicals plays a critical role in the pathogenesis of human diseases, including cancer, emphysema, and neurodegenerative diseases such as Alzheimer's disease. Therefore, there is an increasing demand for identifying exogenous antioxidants to overcome the toxicity generated by

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free radicals (Subhaswaraj et al. 2017). This assay is of interest to determine the antioxidant activity in *A. rigidula* extracts. The ferric thiocyanate method was used to assess the ability of all extracts to prevent oxidation. Therefore, the antioxidant activity of all three extracts of *Acacia rigidula* was determined using ferric thiocyanate by measuring the percent inhibition of linoleic acid oxidation (Figure 8). Linoleic acid is a polyunsaturated fatty acid and under peroxidation, super radicals are formed that oxidizes Fe^{2+} to Fe^{3+} which forms a complex with SCN^- and its concentration can be determined by measuring its absorbance at 500nm. The higher the absorbance of each treatment is the result of the concentration of peroxides formed during the reaction and results in a lower antioxidant activity (Sultana et al. 2007). Significance was found between the six treatments (Table 10).

Table 10. Analysis of variance determining antioxidant potential of *A. rigidula* extracts.[1]

Source	SS	DF	MS	F Value	P>F	
Block	5.86701	2	2.93350	2.30	0.1504	
Treatment	13857.24693	5	2771.444939	2175.92	<.0001	***

[1] ***Denote significance at the 0.001 level.

Of the three extracts, the acetone extract was the only one to exhibit an observable inhibition of peroxidation having an average of 42%, followed by the methanol extract at 25% and the acetic acid extract at 6%. All three extracts are being compared to the positive controls, BHT and ascorbic acid, where both showed inhibition of peroxidation of 70% and 69%, respectively. All three extracts exhibited a significant difference (p <0.001) of percent inhibition of oxidation compared to one another and the negative control, linoleic acid. Interestingly, the acetone extract was also the most effective extract at preventing oxidation, as well as being the best extract for displaying the highest zone of inhibition.

A study performed by Sultana et al. (2007) found antioxidant activity in *Acacia nilotica's* acetone and methanol extract displaying inhibition of peroxidation between the ranges of 75%– 86%, however they were using bark instead of leaves. The decrease in antioxidant activity among extracts reinforces the

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results of both the phytochemical analysis and fingerprinting analysis where more secondary metabolites and structural compounds where identified in the acetone extract. The results of this experiment set the baseline whereby the secondary metabolites that were extracted confer antioxidant properties, specifically phenols and flavonoids.

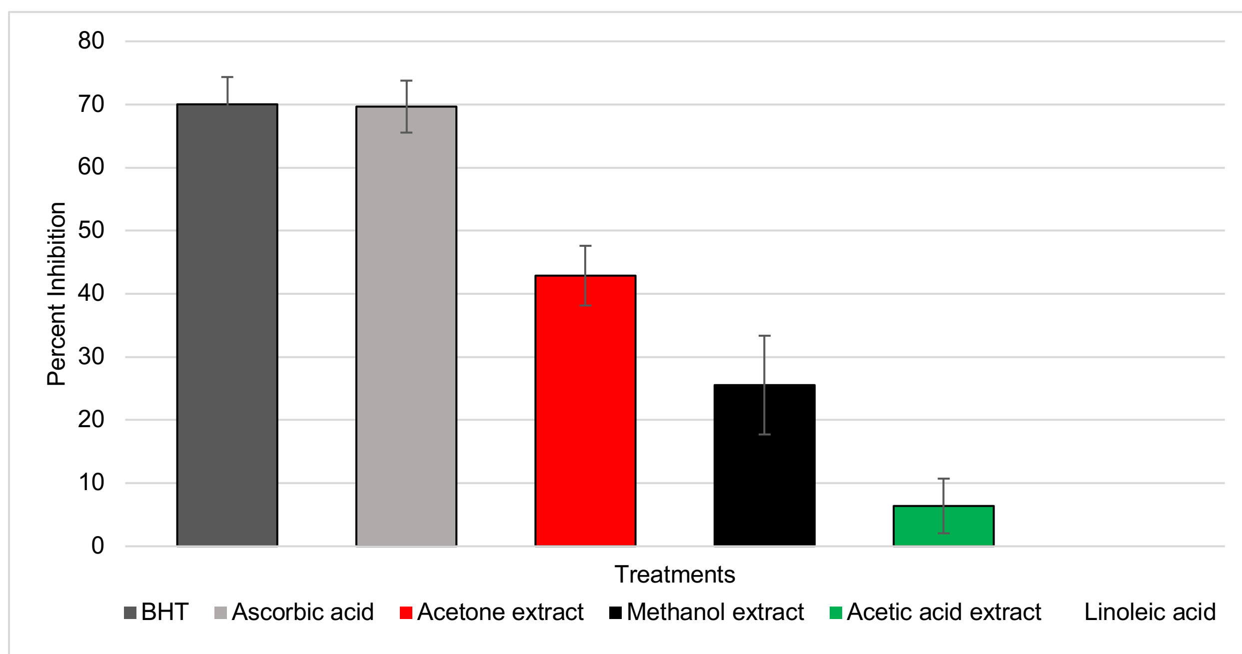

Figure 8. Antioxidant properties of *Acacia rigidula* extracts (5mg/25mL) determined by ferric thiocyanate method with their 95% confidence intervals. Positive controls (BHT and Ascorbic acid) and negative control (Linoleic acid) were used to compare results of each extract. Duncan's Multiple Range Test indicated a significant difference as shown by the non-overlapping confidence intervals at α= .05. Mean values are from three replicates run in quadruplets.

Phenols and flavonoids are important components in the mechanism of preventing oxidation by deactivating free radicals by having the ability to donate protons to super radicals. In unfavorable conditions, plants generate phenolic compounds and flavonoids for the process of growth in stressful environments or to defend itself (Tungmunnithum et al. 2018). Their ideal structural composition and configuration of multiple hydroxyl groups allows for free radicals to scavenge protons to form more stable

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compounds and preventing the denaturing of fatty lipids or other necessary proteins that undergo oxidative damage. By specifically extracting phenols and flavonoids of *A. rigidula* and removing any impurities, we can then determine a more accurate representation of the antioxidant potential in *A. rigidula*.

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CONCLUSION

The increase in resistance to many commercially produced synthetic antimicrobial agents by microorganisms has increased, hence the need of searching for new antimicrobial agents. Previous studies of the Fabaceae family have reported properties of antimicrobial and secondary metabolites (Mattana et al. 2010; Jain et al. 2011; Jeyakumar, 2015). This study, with the use of antimicrobial susceptibility testing and phytochemical analysis, has provided a novel discovery that *Acacia berlandieri* and *Acacia rigidula* exhibit antimicrobial activity. Although stem samples did not provide promising results, leaf samples of both species did. When comparing both species, extracts of *Acacia rigidula* ($p<0.001$) were significantly different from DMSO (negative control) at inhibiting the growth of several microorganisms. Furthermore, key secondary metabolites (phenols, flavonoids, saponins, terpenoids and tannins) are responsible for the antimicrobial activity by extracts from plants were discovered via qualitative analysis (Sparg et al. 2004; Mandal et al. 2005; Mujeeb, 2014). Each of these secondary metabolites has been shown to have antimicrobial properties along with other applications (Nakashima et al. 1992; Cowan, 1999; Bhattacharya et al. 2010; Mansuri et al, 2014). Extracts of both species did exhibit bactericidal activity; therefore, partially supporting the alternative hypothesis. These extracts have not been purified but set the foundation for further analysis. NMR, UV-Vis and IR spectroscopy did elucidate functional groups found in the classes of secondary metabolites confirmed through qualitative phytochemical analysis. By continuing to use more fingerprinting techniques, such as GC-MC and HPLC, this will lead to the isolation of those bioactive compounds that allow for the inhibition of microorganisms and others that have antioxidant activity. Further studies are needed before these extracts can be applied as antibiotics and antioxidants but there is potential in these extracts.

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RECOMMENDATIONS AND FUTURE STUDIES

The results of the antibacterial evaluation of the two medicinal plants suggests that these plants, specifically *A. rigidula,* have the potential to be a good natural source as an antibacterial agent. The future work should focus on investigating bioactive compounds and fractionating them to isolate those that exhibit antimicrobial activity. Another potential idea for future work could be using other extraction techniques with more extracting solvents or different concentrations of acetone to retrieve as many active compounds as possible. An in-depth analysis on the effectiveness of other plant parts such as roots is recommended. This study focused primarily on inhibiting the growth of bacteria alone; however, toxicity studies of the effective extracts should be done to determine how safe these plant extracts are when treating infectious diseases. Finally, further experimentation is needed to evaluate the antioxidant activity within these plant species.

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APPENDICES

Table A1. Least square mean of *A. rigidula* extracts and controls when subjected to *P. alcalifaciens*.

Extract	Treatment	mZOI LSMEAN	LSMEAN Number
1	Acetone Extract	12.6233333	1
2	Methanol Extract	10.9483333	2
3	Acetic Acid extract	9.8644444	3
4	Antibiotic	37.5008333	4

Table A2. Least square means for the effect of *A. rigidula* extracts and controls when subjected to *P. alcalifaciens*.

i/j	1	2	3	4	5
1	----------	0.5261	0.1109	<.0001	<.0001
2	0.5261	----------	0.8409	<.0001	0.0016
3	0.1109	0.8409	----------	<.0001	0.0138
4	<.0001	<.0001	<.0001	----------	<.0001
5	<.0001	0.0016	0.0138	<.0001	----------

Table A3. Least square mean of *A. rigidula* extracts and controls when subjected to *Y. enterocolitica*.

Extract	Treatment	mZOI LSMEAN	LSMEAN Number
1	Acetone Extract	12.5594444	1
2	Methanol Extract	10.35	2
3	Acetic Acid extract	9.0961111	3
4	Antibiotic	34.8925	4

Table A4. Least square means for the effect of *A. rigidula* extracts and controls when subjected to *Y. enterocolitica.*

i/j	1	2	3	4	5
1	----------	0.2915	0.0363	<.0001	<.0001
2	0.2915	----------	0.777	<.0001	0.0066
3	0.0363	0.777	----------	<.0001	0.0708
4	<.0001	<.0001	<.0001	----------	<.0001
5	<.0001	0.0066	0.0708	<.0001	----------

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Table A5. Least square mean of *A. rigidula* extracts and controls when subjected to *S. aureus*.

Extract	Treatment	mZOI LSMEAN	LSMEAN Number
1	Acetone Extract	8.6838889	1
2	Methanol Extract	7.545	2
3	Acetic Acid extract	7.3811111	3
4	Antibiotic	19.27	4

Table A6. Least square means for the effect of *A. rigidula* extracts and controls when subjected to *S. aureus.*

i/j	1	2	3	4	5
1	----------	0.4094	0.2848	<.0001	0.0041
2	0.4094	----------	0.9989	<.0001	0.1531
3	0.2848	0.9989	----------	<.0001	0.2353
4	<.0001	<.0001	<.0001	----------	<.0001
5	0.0041	0.1531	0.2353	<.0001	----------

Table A7. Least square mean of six samples of *A. rigidula* acetone extracts against the four most susceptible bacteria.

Bacteria	Name	mZOI LSMEAN	LSMEAN Number
1	*P. alcalifaciens*	14.8129167	1
2	*E. faecalis*	12.5669444	2
3	*S. aureus*	13.2658333	3
4	*Y. enterocolitica*	14.2581944	4

Table A8. Least square means for the effect of four microorganism when subjects to six different replicates of *A. rigidula* acetone extracts.

i/j	1	2	3	4
1	----------	0.0038	0.0758	0.8120
2	0.0038	----------	0.6810	0.0440
3	0.0758	0.6810	----------	0.3953
4	0.8120	0.0440	0.3953	----------

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