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Evaluation of the Antimicrobial Activity of Vangueria Volkensii Bark, Fruit, Leaf, and Stem Extracts

Douglas Parnell Holland

EVALUATION OF THE ANTIMICROBIAL ACTIVITY OF *VANGUERIA VOLKENSII* BARK, FRUIT, LEAF, AND STEM EXTRACTS

A Thesis

by

DOUGLAS PARNELL HOLLAND

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

MASTER OF SCIENCE

August 2019

Major Subject: Biology

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Approved as to style and content by:

Chair of Committee,	Michael R. Kidd
Committee Members,	Alfred K. Addo-Mensah
	Monica O. Mendez
	Ruby A. Ynalvez
Head of Department,	Michael R. Kidd

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ABSTRACT

Evaluation of the Antimicrobial Activity of *Vangueria volkensii* Bark, Fruit, Leaf, and Stem Extracts (August 2019)

Douglas Parnell Holland, A.A., Western Texas College; B.S., University of Arkansas at Monticello;

Chair of Committee: Dr. Michael R. Kidd

Ethnopharmacological relevance: Medicinal plants have been used for thousands of years and continues to have a critical role in the healthcare system worldwide, and it is estimated that 80% of all pharmaceutical drugs are plant based in origin. Plants from the Rubiaceae family have gained the attention of scientist because they exhibit great medicinal potential and value. However, *Vangueria volkensii* has no published research on its pharmacological activity and properties.

Materials and Methods: In the present study, *Vangueria volkensii* bark, fruits, leaves, and stems were sequentially extracted *via* Soxhlet extraction using petroleum ether (PE), acetone (ACE), and ethanol (ETOH) solvents. Utilizing the diffusion method, the antimicrobial activity of each extract at concentrations 5, 15, 25, 50 mg/ml against six bacteria was determined via the size of the zones of inhibition. Gram-negative [*Escherichia coli* B strain, *Salmonella enterica* Subsp. *enteritidis*, *Shigella flexneri*] and gram-positive [*Enterococcus faecalis*, *Staphylococcus aureus*, methicillin-resistant *S. aureus*] bacteria were selected based on availability.

Results: *Salmonella enterica* Subsp. *enteritidis*, *S. flexneri*, *E. faecalis* and methicillin-resistant *S. aureus* were sensitive to all extracts prepared. E. *coli* B strain was resistant to bark ETOH and stem ACE extracts, while *S. aureus* was resistant to bark, leaf and stem ACE and PE extracts and the fruit ETOH extract. Comparing the extract zones of inhibition means for each microorganism revealed that the fruit extracts prepared from ACE had the most inhibitory activity for all microorganisms, except *E. faecalis*. For E. faecalis, the leaf extracts prepared from ETOH exhibited the most inhibitory activity.

Conclusions: This study shows that *V. volkensii* contains antimicrobial properties as indicated by inhibiting the growth of several microorganisms. The fruit and leaves of *V. volkensii* may be the most promising to explore for potential use as an antimicrobial drug. However, to determine the which active compound(s) are responsible the antimicrobial activity, further analysis and biomolecule isolation would prove beneficial in concluding the results of this study.

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INTRODUCTION

1.1 The Family Rubiaceae

The family Rubiaceae, commonly called the coffee family, is one of the most species-rich angiosperm families in terms of number of genera and species, with about 611 genera and more than 13,000 species (Aro et al. 2015; Karou et al. 2011; Verstraete et al. 2011). It is a predominantly tropical and subtropical family, but representatives occur on all continents, except Antarctica (Verstraete et al., 2011). Shrubs, trees, lianas or herbs are the growth forms of Rubiaceae, with shrubs being the most common. Species are mainly woody, and less than 20% of the genera are herbaceous (Karou et al., 2011). Members of the Rubiaceae family exhibit great medicinal potential and value. Numerous studies have documented the widespread use of Rubiaceae members throughout the world as treatment for a wide range of symptoms and diseases such as diarrhea, headaches, diabetes, malaria, and many more ailments. According to Karou et al. (2011), at least 34 different genera of Rubiaceae native to Sub-Sahara Africa have been documented for treating or managing over 70 different diseases, hence, Rubiaceae is considered a major component of sub-Saharan folk medicine. However, due to the vast amount of species, scientist have not been able to screen all of Rubiaceae for their ethnopharmacological uses and the identification of their medicinal properties is still ongoing.

1.2 Traditional Medicine

Medicinal plants have been used by humans for thousands of years and continues to play a critical role in the healthcare system in many areas in the world. The widespread use

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of herbal medicine in many African countries is not limited to just rural areas, but also in urban areas (Fennel et al. 2004; Kipkore et al. 2014; Muthee et al. 2011; Orwa et al. 2007). According to the World Health Organization (WHO), 80% of the global population, primarily those in developing countries, still rely on traditional medicine (Karou et al. 2011; Kipkore et al. 2014; Munodawafa et al. 2013; Muthee et al. 2011; Ndam et al. 2014; Pascaline et al. 2010; Pascaline et al. 2011). This is attributed to several reasons including: poverty, cultural acceptability, the lack of access to orthodox medicine and medical facilities, and the low ratio of trained medical doctors to patients. For instance, in Kenya there is one orthodox doctor to 7,142 people and one medical doctor to 50,000 people in Mozambique (Bekalo et al., 2009). Thus, traditional medicine is the primary healthcare treatment in such areas.

1.3 Traditional Healers

In developing countries, medicinal plants are the most important and are often the only source of therapeutics. Moreover, the importance of medicinal plants and role of those that administer traditional medicine, the traditional healer, cannot be stressed enough. In Uganda, it is estimated that there is one traditional healer for every 200-400 people compared to one orthodox practitioner to 20,000 people (Muthaura et al., 2011). Traditional healer practices vary by location but is generally linked to the different religious beliefs and culture of the people. Traditional healers tend to take a holistic approach to healing and complement medical doctors in the treatment of diseases, when available (Bekalo et al., 2009).

Some traditional healers will cultivate medicinal plants however, most will collect the medicinal plants in their natural habitat. The most widely used medicinal plant components for medical preparations is the leaves, followed by bark, roots, fruits, whole plant, and seeds (Bekalo et al. 2009; Kipkore et al. 2014). Oral absorption is the primary administration route of

medicinal remedies via decoctions or concoctions, followed by poultice/topical application and inhalation (Bekalo et al. 2009; Kipkore et al. 2014).

1.4 Traditional Knowledge

The reliance of traditional medicine is not confined to only the Third World, but also globally, primarily in terms of phytomedicine. As much as 33% of modern allopathic medicines are of plant origin (Amuka et al., 2014) and currently 74% of the plant-derived drugs on the market were derived from the traditional knowledge of indigenous people through ethnobotanical studies (Amusan et al., 2007). Therefore, ethnobotanical studies have been the driving force behind the discovery of crude drugs. According to Eloff (2000), when researchers select plants to be evaluated for medicinal purposes, those plants selected from ethnobotanical surveys yield up to five times more active leads than plants randomly selected. Despite the numerous roles played by traditional medicine, the existence of traditional medicine which is dependent on traditional knowledge of medicinal plants and practices, is at risk of being lost forever. Primarily because very little progress has been made in documenting the traditional knowledge of medicinal plants and practices (Giday et al. 2009; Giday et al. 2010; Kipkore et al. 2014).

The indigenous knowledge about the uses of plants has often evolved through trial and error (de Boer et al., 2005), and is transferred from generation to generation, largely orally (Giday et al. 2009; Giday et al. 2010; Muthee et al. 2011). In Ethiopia, the majority of traditional herbalist keep their medicinal knowledge a secret in the family and traditional knowledge is transferred to one of their children (either male or female) that they select via an apprenticeship starting at age 10 and last until the age of 18 (Giday et al., 2009). According to Kidane et al. (2014) and Giday et al. (2009), the traditional knowledge of males in Ethiopia is

more prevalent than females, because boys are usually more preferred than girls in the transfer of knowledge. In addition, the traditional knowledge of older individuals (40 years or older) is significantly greater than younger individuals because traditional knowledge is accumulated through time and the acculturation of the young generation has contributed to a loss of traditional knowledge (Giday et al. 2009; Giday et al. 2010; Kidane et al. 2014).

Traditional knowledge is vanishing at an alarming rate due to changes towards a more western lifestyle, migration from rural to urban areas, and rapid loss of natural habitats due to anthropogenic and natural factors (Nanyingi et al. 2008; Njamen et al. 2008). For instance, overgrazing and overexploitation of plant resources, industrialization, droughts, and bush fires (Nanyingi et al. 2008; Njamen et al. 2008). As part of an ethnopharmacological survey of the Samburu district of Kenya, Nanyingi et al. (2008) interviewed 100 individuals for the local uses of medicinal plants, and the management and extinction threats. Forty-seven percent of those interviewed were keenly aware and actively conserving medicinal plants via building fences to protect natural habitats, refraining from overexploiting, taking measures to protect plants from fires, and some individuals were cultivating medicinal plants in nurseries.

1.5 Plant Poisoning and Gousiekte

Ethnopharmacological studies are conducted to determine new bioactive compounds from plants, however, the safety and efficiency of these compounds should not be assumed. Although medicinal plants are often presumed to be safe and efficient, medicinal plants are continuously screened via in vitro bacterial and mammalian cell assays (i.e., Ames test, micronucleus test, comet assay, etc.) for their safety to ensure rational use in traditional medicine (Fennel et al., 2004; Karou et al., 2011). Some medicinal plants are used as antidotes for snake bites and/or arrow poison, and there are some reports on poisoning from medicinal plants,

depending on routes of administration or the different plant parts used. For instance, traditional healers in Mali reported that diarrhea and/or vomiting was observed in individuals that were given a decoction of either the roots of *Gardenia ternifolia* (Rubiaceae) and *Nauclea latifolia* (Rubiaceae) or the leaves of *Mitragyna inermis* (Rubiaceae) for the treatment of malaria, thus indicating that these medicinal plants and parts are toxic (Maiga et al., 2005). Interestingly, while the roots of *Gardenia ternifolia* were reported to be toxic causing diarrhea and/or vomiting, the maceration of the fruit and/or leaves, which are abundant in tannins, can be given to treat for persistent vomiting. In addition, it is known that tannins in food can act as digestion inhibitors, resulting in the suppression of food intake, thereby supporting the claim of treating persistent vomiting. Additionally, the use of toxic plants for tribal defense purposes, such as poison arrows and criminal poisoning, is still present in many indigenous communities. For instance, the root and/or stem bark of *Nauclea latifolia* are used as ingredients of the Senufo arrow poison in areas of Burkina Faso and Ivory Coast. Whereas the leaves of *Gardenia ternifolia* are put in baths and lotions against arrow-poisoning in Ivory Coast and the fruits are used as fishing poison (Maiga et al., 2005).

Gousiekte, a cardiac disease of domestic ruminants caused by ingestion of certain poisonous plants, is one of the six most common plant poisonings in Africa (Van Elst et al. 2013; Verstraete et al. 2011). It is characterized by acute heart failure within two months of initial ingestion and six species of the Rubiaceae family identified as the causative agents. These species are: *Vangueria pygmaeum*, *Vangueria thamnus*, *Vangueria latifolium, Pavetta harborri*, *Pavetta schumanniana*, and *Fadogia homblei*. The active compound responsible for the cardiotoxicosis is a polyamine known as pavettamine, which is the only naturally occurring polyamine that is responsible for the poisoning of livestock. Interestingly, the leaves of all

gousiekte-causing plants contain bacterial endosymbionts and they all belonging to the genus *Burkholderia*. The physiological aspects of pavettamine is lacking, thus, at the present, it is unknown whether the plants or the endophytic *Burkholderia*, or perhaps both are responsible for the origin of this putative toxin, but promising research is ongoing (Van Elst et al. 2013; Verstraete et al. 2011).

1.6 Plant Extractions

Due to feasibility, traditional healers tend to use aqueous extractions, for medicinal purposes without understanding or knowing the mode of action, while the objective of scientists is to identify the medicinal compounds of plants and deduce their mechanism of action. Unlike traditional healers, scientists commonly employ the Soxhlet extraction method using solvents with increasing polarity (Eloff, 1998). Scientists select solvents based on the desired compounds to be extracted. For instance, acetone can extract alkaloids, but not tannins and methanol can extract tannins but not alkaloids, but both flavonoids can be extracted from both solvents. In addition, other factors should be considered when selecting the solvent to be used, these include; the rate of extraction, amount of extract produced, volatility, and the toxicity of the solvent during the bioassay process. Acetone is one of the preferred solvents used by scientists, due to its low toxicity in antimicrobial assays and its ability to extract a wide range of compounds (Eloff, 1998).

1.7 Plant Chemistry

Since the beginning of humankind, plants have been used to manage various illnesses and have been an important source of drugs and chemotherapeutic agents (Ademola et al. 2007; Amuka et al. 2014; Muthaura et al. 2011). The primary focus of ethnopharmacology is to discover novel compounds derived from plants based on traditional knowledge, with the

intention to develop new pharmaceuticals. Plants produce primary metabolites (lipids, carbohydrates, proteins), which are essential for growth and metabolism, and secondary metabolites, which are products of primary metabolism and act as plant defense mechanisms (Ndam et al., 2014). Some of the most common secondary metabolites of Rubiaceae species include: alkaloids, glycosides, terpenes, steroids, saponins, flavonoids, tannins, coumarins, fatty acids, and carbohydrates. The alkaloids and flavonoids exhibit a broad spectrum of biological activities, including antimicrobial, antiviral, anti-cancer, and anti-inflammatory properties.

Alkaloids, the most potent therapeutic compounds of natural origin (Okwu and Uchenna, 2009), are organic heterocyclic nitrogen compounds. Alkaloids' chemical structures are extremely variable, but in general they contain nitrogen derived from an amino acid. The primary classes of alkaloids, based on their chemical structures, include the pyrrolidines, pyrrolizidines, tropanes, pyridines, indoles, isoquinolines, quinolines, and the terpenoids and steroids (Savoia, 2012). The occurrence of indolique alkaloids in the Rubiaceae family seems to be the rule in this family and they can occur in tetracyclic or pentacyclic rings (Karou et al., 2011).

Flavonoids are phenolic structures that widely distributed in plants including the fruits, stems, and flowers, and they have several functions. They are found abundantly in photosynthesizing cells and they are the most important plant pigment for flower coloration which to attract pollinator animals. Currently, there are 14 different classes of flavonoids, all of which have the general structural of a 2-phenyl-benzopyrane or flavine nucleus, consisting of two benzene rings linked through a heterocyclic pyrane ring. In addition, each class is differentiated based on the chemical nature and position of substituents on the different rings (Savoia, 2012).

The distribution and function of metabolites varies between species and plant parts. In addition, the chemical composition of constituents can be affected by climatic conditions (droughts, floods, etc.), growing location, and the season of harvest (Fiot et al. 2005; Muthaura et al. 2011). For instance, the leaves of *Mitragyna inermis* from Ghana and Mali have different proportions of alkaloids. In Ghana, isorhynchophylline and rotundifoline were the dominant alkaloids present in the leaves, while in Mali these two alkaloids were present, but in low proportions and uncarine D was the dominate alkaloid (Fiot et al., 2005).

Secondary metabolites (alkaloids, flavonoids, tannins, etc.) and their derivatives are usually the bioactive compounds in plant-derived drugs. According to Ngari et al. (2010), medicinal plants tend to contain above average concentrations of secondary metabolites compare to plants that are considered non-medicinal. History has shown that plant secondary metabolites have a wide range of medicinal activities and they are major sources of drugs. For instance, it has been known for more than three centuries that the bark of *Cinchona*, a genus in the Rubiaceae family, contains antimalarial properties, which led to the isolation of the alkaloid quinine, which was the first effective and still most widely used agent in treating malaria (Ademola et al. 2007; Muthaura et al. 2011).

1.8 Antimicrobial Resistance

The "golden age" of antibiotic research began in 1928 with the discovery of penicillin, the first true antibiotic, and continued into the second half of the 20th century. The discovery of most of the antibiotic drug classes still used today can be attributed to this period (Butler and Cooper, 2011). Currently, the development of new antimicrobials is lagging, while the emergence of multi-drug-resistant microorganisms are occurring at an alarming rate. Thus,

antimicrobial resistance is one of the world's most pressing public health issues (Butler and Cooper, 2011; Mthethwa et al., 2014).

The uncensored use of antimicrobial agents has led to microorganisms becoming resistant to almost all known antimicrobials, due to the development of new resistance mechanisms. Resistance can be transferred from microorganisms in the same species, different species, and sometimes related general (Okwori et al., 2008). The biochemical composition of bacterial cell walls can provide insight as to whether the bacteria will or will not be susceptible to the antimicrobial agent. Generally, gram positive bacteria tend to be more susceptible to antimicrobial agents compared to gram negative bacteria because their cell wall is composed of a single peptidoglycan layer, which is a less effective barrier (Karou et al., 2011). *Staphylococcus* species have been reported as the most commonly emerging resistant bacterial species and infections are very difficult to treat due to their great pathogenic potential (Mthethwa et al., 2014). On the other hand, gram negative bacteria are significantly harder to kill because their cell wall is composed of an additional phospholipidic outer membrane, which often contain multiple efflux pumps, as well as antibiotic and target-modifying enzymes (Butler and Cooper, 2011; Savoia, 2012).

Plant-derived antimicrobial compounds can have a variety of action mechanisms depending on the plant, the class compounds and the cellular properties of the target microorganism. Rubiaceae family contains diverse phytochemicals however, those of particular interest possess known antimicrobial properties such as; alkaloids, flavonoids, and tannins. Quinolone alkaloids have been known to act as an DNA intercalator targeting DNA gyrase and DNA topoisomerase IV, RNA polymerase, and nucleic acids (Butler and Cooper, 2011; Savoia, 2012). Flavonoids are known to form irreversible complexes with bacterial membranes and

extracellular and soluble proteins, resulting in the inhibition of cytoplasmic membrane function, nucleic acid synthesis and energy metabolism (Savoia, 2012). Tannins are polyphenolic compounds that causes the inactivation of bacterial adhesions, membrane-bound proteins, and enzymes via hydrogen bonding or act as an iron-chelating agent, thereby causing their inactivation (Savoia, 2012).

1.9 Studies on Antibacterial Properties

The disc (or agar) diffusion assay is an efficient method for quantifying the antimicrobial activity of compounds (Karou et al., 2011) by applying antimicrobial solutions to paper discs, placing them on bacterial inoculated agar plates and measuring the diameter of the zones of inhibition (ZOI), or the area around the discs that inhibit bacterial growth (Bonev et al., 2008). The susceptibility of bacteria to an extract is indicated by an inhibition zone diameter of 9 mm or more around the disc (Akomo et al. 2009; Sarker et al. 2014; Zongo et al. 2009). According to Sarker et al. (2014), the ZOI diameter measured to the nearest whole millimeter is commonly used to define the sensitivity status of bacteria to antibiotic, which categorizes the bacteria as susceptible (inhibition of growth and the antibiotic dose is therapeutic), moderately susceptible (lower inhibition and dose may not achieve therapeutic response), or resistant (little to no inhibition and no therapeutic response to the antibiotic).

Using the disc (or agar) diffusion assay, Akomo et al. (2009) screened *Canthium multiflorum* (Rubiaceae) aqueous and methanol extracts for antimicrobial properties against thirteen bacterial strains. Results showed that all bacteria, except *P. aeruginosa* and *S. pyogenes* (clinical isolates), were susceptible to both extracts. The zones of inhibition (mm) ranged from 11 to 18 mm for methanol extract and 9 to 16 mm for aqueous extract. The methanol extract

exhibited the highest zones of inhibition (18 mm) when tested against *Bacillus cereus* and *Salmonella enterica* (Akomo et al. 2009).

Chatterjee et al. (2007) found that the antibacterial activity of aqueous and methanol leaf extracts of *Vangueria spinosa* (Rubiaceae) was determined by the disc diffusion method against four bacterial strains (*Staphylococcus aureus*, *Bacillus subtilis*, *Escherichia coli*, and *Pseudomonas aeruginosa*). Results showed that both aqueous and methanol extracts were active against all bacterial strains, but methanol extract was comparatively more active. The zones of inhibition (ZOI in mm) against *S. aureus*, *B. subtilis*, *E. coli*, and *P. aeruginosa* for the aqueous extract were 25.2, 20.2, 20.0, and 20.3 respectively, and for the methanol extract they were 20.5, 23.0, 20.6, and 23.4, respectively (Chatterjee et al., 2007).

The disc diffusion technique has been used for determining the minimum inhibitory concentration (MIC), the lowest concentration of extract that is bacteriostatic, by using linear fitting of the square radius (diameter) of the inhibition zones to the natural logarithm of extract concentration (Bonev, et al., 2008). MIC values lower than 0.1 mg/ml are significantly active, values from 0.1 to 0.625 mg/ml are said to be moderately active, and values > 0.625 mg/ml have weak activity (Eloff, 2000). In Shai et al. (2013), the MICs of *Vangueria infausta* (Rubiaceae) acetone leaf extracts were determined against eight Gram positive bacterium and eleven Gram negative bacterium. Results showed that *Vangueria infausta* was more active against Gram positive bacteria than Gram negative bacteria, with average MIC values around 0.2 mg/ml and over 0.6 mg/ml, respectively. *Vangueria infausta* had the most activity against *Steptococcus pyogenes*, *Bacillus stearothermophilus* and *Staphlylococcus aureus* with an MIC values of 0.04 mg/ml, 0.08 mg/ml, and 0.08 mg/ml, respectively (Shai et al., 2013).

The use of many Rubiaceae species in the treatment of TB-related symptoms (i.e. coughing, fever, chest and respiratory pain) has been well documented. In Aro et al. (2015) acetone leaf extracts from fifteen species of Rubiaceae were screened for antimycobacterial activity against pathogenic *M. tuberculosis* and three non-tuberculous mycobacteria (*M. smegmatis*, *M. aurum*, *M. bovis* BCG). Antimycobacterial assay results showed that seven of the extracts had good antimycobacterial activity against the four mycobacterial test species and all fifteen species had MIC values that were moderately active for at least two species each. The best MIC values of 0.04 mg/ml were from extracts of *Cephalanthus natalensis* and *Keetia* sp. against *M. tuberculosis* and *Psychotria zombamontana* against *M. smegmatis*. In addition, MIC values for *Keetia* sp. and *Psychotria capensis* extracts against *M. smegmatis* were 0.06 mg/ml, while *Oxyanthus speciousus* and *Psychotria zombamontana* extracts against *M. aurum* was 0.06 mg/ml and 0.08 mg/ml, respectively (Aro et al., 2015).

1.10 Other Studies

The discovery of novel antifungal compounds has gained worthy attention due to the growing resistance to azole compounds, the active compounds in amphotericin B, and the difficulty of eradicating fungal diseases in immunocompromised individuals. Flavonoids, saponins, and tannins as antifungal compounds, is well documented. Munodawafa et al. (2013) screened methanol leaf and root extracts of *Vangueria infausta* for its phytochemical properties. Phytochemical screening was carried out using thin layer chromatography (TLC) with detection by UV and confirmed with standard tests. Results showed that the leaf extract contained high amounts of flavonoids and saponins, and moderate amounts of tannins. Whereas the root extract contained high amounts of saponins and trace amounts of tannins (Munodawafa et al., 2013).

In Mahlo et al. (2010) leaf extracts (acetone, hexane, dichloromethane, and methanol) from six South African tree species, including two Rubiaceae species (*Breonadia salicina*, *Vangueria infausta*), were examined for antifungal activity against seven plant pathogens (*Aspergillus niger*, *Aspergillus parasiticus*, *Collectotricum gloeosporioides*, *Fusarium oxysporum*, *Penicillium expansum*, *Penicillium janthinelleum*). Using the microdilution assay, all extracts of *B. salicina* and *V. infausta* were active against all seven plant pathogen fungi and compared to the other solvents, acetone extracts produced on average the highest total antifungal activity concentrations for all plant pathogen fungi. *B. salicina* showed high antifungal activity against *P. janthinellum* and *F. oxysporum*, with minimum inhibitory concentration (MIC) values as low as 0.08 mg/ml and 0.16 mg/ml. *B. salicina* showed moderate to low antifungal activity against the other five plant pathogen fungi, with MIC values ranging from 0.32 mg/ml to 2.50 mg/ml. *Vangueria infausta* showed moderate antifungal activity against *A. parasiticus*, *P. janthinellum*, *T. harzianum*, and *F. oxysporum*, with MIC values as low as 0.32 mg/ml and 0.63 mg/ml. *Vangueria infausta* had low antifungal activity against *A. niger*, *C. gloeosporiodies*, and *P. expansum*, with MIC values ranging from 1.25 mg/ml to 2.50 mg/ml (Mahlo et al., 2010).

Boer et al. (2005) used crude ethyl acetate and methanol extracts of *Vangueria infausta* to study the inhibition of the microbial growth of *Aspergillus fumigatus*, *Fusarium culmorum*, and C*andida albicans* via microtiter plate assay. The extraction of 5.0 g of plant material for each extract, ethyl acetate and methanol, yielded 1.25 mg/ml and 1.80 mg/ml, respectively. The ethyl acetate extracts showed the most promising results with varying inhibition of growth of all three microorganisms. At the test concentration of 10 times the original concentration, the extract completely inhibited the growth of *Aspergillus fumigatus* and showed intermediate inhibition of growth (50-80%) of *Fusarium culmorum* and *Candida albicans*. The methanol extract was less

effective at the same concentration, showing intermediate inhibition of growth of *Aspergillus fumigatus* and *Fusarium culmorum*, and no inhibition of growth of *Candida albicans* (de Boer et al., 2005).

In Asase et al. (2008) the antifungal activity of n-hexane, acetone and 50% aqueous methanol extracts of leaves, stem bark and roots of *Mitragyna inermis* from Ghana was tested against *Cladosporium herbarum*. The antifungal activity was determined using TLC direct-autobiographic methods with 10 µg of the crude extracts and the antifungal positive control Nystatin (1 µg). The 50% aqueous methanol solvents used for *Mitragyna inermis* leaf and stem bark extracts showed the inhibition of *Cladosporium herbarum*, while the acetone solvent for *Mitragyna inermis* root extract showed strong inhibition of *Cladosporium herbarum* (Asase et al., 2008).

Viral diseases pose some of the greatest threats to human health, contributing to a significant portion of morbidity and mortality world-wide. Medicinal plants, including members of the Rubiaceae family, have a long-standing history of treating and managing viral diseases (Lawal et al. 2010; Vlietinck et al. 1995), including HIV/AIDS and the related opportunistic infections (Chinsembu and Hedimbi 2010; Munodawafa et al. 2013; Okwori et al. 2009). HIV/AIDS continues to be a major problematic heath concern world-wide with 80% of cases occurring in Asia and Africa (Aro et al., 2015). Two thirds of all individuals infected world-wide reside in sub-Saharan Africa (Kisangau et al., 2007) including South Africa which is estimated that 5.5 million individuals were infected at the end of 2006 (Shai et al. 2013; Stafford et al. 2008). Managing the HIV/AIDS pandemic is a global challenge because infected individuals are susceptible to secondary fungal and bacterial opportunistic infections. In addition, the HIV/AIDS pandemic is further complicated due to the uncensored and repeated use

of anti-infective agents to treat secondary infections resulting in the rapid emergence of resistant pathogens (Mthethwa et al. 2014; Munodawafa et al. 2013; Shai et al. 2013).

In Mthethwa et al. (2014) methanol root extract of *Vangueria infausta*, a Rubiaceae species from South Africa, was evaluated for cytotoxic and anti-HIV-1 activity by MTT assay. The MTT assay resulted in a CC_{50} of 100 µg/ml for the root extract, which was exceptionally higher than the positive control, berberine, which yielded a CC_{50} of 27 µg/ml. The observed anti-HIV and cytotoxic activity of *V. infausta* offer promise for its phytotherapeutic application in managing HIV/AIDS (Mthethwa et al., 2014).

Vlietinck et al. (1995) screened 100 Rwandese medicinal plants for antiviral properties, 4 of which were Rubiaceae plants (*Mitragyna rubrostipulata*, *Pavetta ternifolia*, *Rubia cordifolia* subsp. *conotricha*, *Virectaria major*). Leaf, stem, root and whole plant extracts were obtained by 80% ethanol maceration and screened against one DNA virus (herpes simplex type 1) and four RNA viruses (coxsackie B2, measles Edmonston A, poliomyelitis type 1, Semliki forest L10), all of which had a viral titer of 10^7 TCD_{50}/ml, except for measles (10^5 TCD_{50}/ml). All extracts showed either low or moderate antiviral activity against all viruses, with reduction factors of 1 to 10 and 10^2 to 10^3, respectfully. Of the 4-plant species, only *M. rubrostipulata* and *P. ternifolia* showed moderate antiviral activity to one or more viruses. *M. rubrostipulata* stem extract had a 10^2 reducing factor for the coxsackie virus, *P. ternifolia* root extract had a 10^2 reducing factor for the Semliki forest virus, and *P. ternifolia* leaf extract for poliomyelitis and Semliki forest viruses had reducing factors of 10^2 and 10^3, respectively (Vlietinck et al., 1995).

1.11 Species for Investigation

In recent years, many Rubiaceae species have been extensively examined for their phytochemical composition and biological activities. *Vangueria volkensii*, locally referred to as

"Kimuluet," is a species of Rubiaceae native to Kenya. *V. volkensii* (bark, fruit, leaves, roots, and stems) is known to treat and manage venereal diseases (Jeruto et al. 2015; Pascaline et al. 2010) and malaria (Orwa et al. 2007; Pascaline et al. 2011); however, there is no published scientific literature on its pharmacological activity and properties. Therefore, the aim of this study is to investigate the antibacterial activity of *V. volkensii* with the intent of establishing the antimicrobial potentials of *V. volkensii* extracts on bacterial pathogens. Species in the genus *Vangueria* have been shown to have high antibacterial activity against several microorganisms (Chatterjee et al. 2007; Shai et al. 2013); thus, *V. volkensii* is predicted to have moderate to high antibacterial activity against several different microorganisms. The antibacterial activity of *V. volkensii* against both gram-negative and gram-positive bacteria will be conducted via the disc diffusion method by measuring the zone of inhibitions (ZOI in mm).

METHODS

2.1 Plant Material

Collection of *V. volkensii* bark, fruit, leaves, and stems was contracted out to Caleb Rugut and Haron Koech, local natives, who excavated the plant material from naturally growing scrubs located near Kapkoros Village, Nandi North District, Rift Valley Province, Kenya. The plant parts were shipped to Texas A&M International University (TAMIU) and authenticated by Amede Rubio, an ecologist associated with TAMIU.

Once arrived, aluminum pans were used to separate the plant parts, then each of the plant components was thoroughly washed with water and chopped into pieces. Plant components were then sun-dried for 10 days, grounded to course powder with a High-speed Universal Grinder (Huangcheng ®), kept in Ziploc bags and stored at room temperature in the dark.

2.2 Preparation of Plant Extracts

The extraction method from Addo-Mensah et al. (2015) was adopted with a few modifications. Briefly, sequential Soxhlet extractions were performed for each of the dried powdered plant components. The bark, fruits, leaves, and stems were extracted in 3 days successively with 400 mL of petroleum ether (PE), acetone (ACE), and 9:1 ethanol/deionized water (ETOH). Each extraction was initially conducted using 15.0058 g, 50.0279 g, 10.0003 g, and 50.0000 g of the powdered plant components (bark, fruit, leaves, and stems, respectively). PE extractions of the leaves and stems was repeated with a larger amount of powdered material (110.0013 g and 130.0096 g, respectively) because the initial extractions produced an insignificant amount of product.

The solvents were removed from each extract via evaporation using a rotary evaporator (Heidolph Laborota™). The extracts were converted into a powdered state by re-dissolving the extracts in deionized water, freezing them, and placed in a freeze-dryer (FreeZone 2.5 Liter Benchtop System by Labconco) for 3-7 days. Finally, the crude extracts were weighed and stored in the dark at 10^{o}C to prevent photo-degradation.

The plant extraction yield can be extrapolated by using the weight of the original plant material used during the extraction process and the weight of the produced crude extract. The extraction yield was determined for each extraction using the percent yield equation:

$$\text{Extract yield} = ((\text{wt. of extract})/(\text{wt. of original}))*100 \qquad (1).$$

Extracts were then dissolved in dimethyl sulfoxide (DMSO) to produce 100 mg/mL stock solutions. Using DMSO as the solvent, concentrations of 5, 15, 25, 50 mg/mL was prepared from the stock solutions. The prepared extract concentrations and the stock solutions were stored in the dark at 10^{o}C until further use.

2.3 Microorganisms

The *general* trend that gram positive bacteria are more sensitive to plant extract than gram negative bacteria has been well documented (Chatterjee et al. 2007; Karou et al. 2011; Shai et al. 2013; Zongo et al. 2009). In addition, *V. volkensii* (bark, fruit, leaves, roots, and stems) is used to treat and manage venereal diseases (Jeruto et al. 2015; Pascaline et al. 2010). Antimicrobial studies were carried out using gram-negative and gram-positive bacteria, based on availability. The gram-negative bacteria included: *Escherichia coli* strain B (EC), *Salmonella enterica* Subsp. *enteritidis* (SE) and *Shigella flexneri* (SF). Whereas the gram-positive bacteria included: *Enterococcus faecalis* (EF), *Staphylococcus aureus* (SA) and methicillin-resistant *Staphylococcus aureus* (MRSA). Common aetiologic agents of venereal diseases such as

Chlamydia trachomatis, *Neisseria gonorrhea* and *Treponema pallidum*, were unavailable, therefore *E. coli*, *S. flexneri*, *E. faecalis*, and *S. aureus* were used because they are representative of pathogenic organisms associated with venereal diseases (Okoli and Iroegbu, 2004).

2.4 Preparation of Microorganisms

Sterile Mueller-Hinton (MH) agar was prepared according to the manufacturer's instructions (Addo-Mensah et al. 2015). Briefly, 38 g of the agar powder was added to 1000 mL of deionized water and brought to a light boil while stirring, until the agar was a homogeneous solution and placed in the autoclave for sterilization. After sterilization, the agar cooled down to room temperature for approximately 40 min and then added to sterile petri dishes. Once added to the sterile petri dishes, the media remained at room temperature until it solidified. The agar plates were then stored upside down, to ensure the media is moisture-free, at 10^{o}C. Stock bacterial strains (EC, SE, SF, EF, SA, and MRSA) was sub-cultured on Muller-Hinton agar plates, incubated at 30^{o}C for 20 h and stored at 10^{o}C until further use.

2.5 Inoculum Preparation

Sterile nutrient broth was prepared, and 2 mL of the broth was added to sterile tubes. Using sterile tooth picks, a single colony of a microorganism was selected from its respective agar plate culture and the tooth pick containing the colony was transferred into one of the nutrient broth tubes. The broth culture and a tube with only 2 mL of the sterile nutrient broth (negative control) was placed in a water bath shaker and incubated at 37^{o}C for 18 h. After incubation, the sterile nutrient broth was used as a blank for adjusting the turbidity of the actively grown broth culture using a spectrophotometer (VWR UV-3100PC Spectrophotometer). The absorbance of the suspension was adjusted to a level of A = 0.132 ± 0.005 at 625 nm and the

suspension was used later for inoculation onto Mueller-Hinton agar plates. This procedure was repeated for all microorganisms.

2.6 Determination of Antimicrobial Activity

The antimicrobial activity of extracts was determined via disc diffusion method (Addo-Mensah et al. 2015) using Mueller-Hinton agar plates. Briefly, 100 µL of the respective inoculum was added to moisture-free agar plates utilizing sterile L-shaped rods to evenly distribute the inoculum. Individual 6 mm disc filter paper discs were impregnated with 20 µL of the respective previously prepared extract concentrations (5, 15, 25, 50 mg/mL) and applied onto the plates.

To ensure that the solvent (DMSO), which was used for preparing the different extract concentrations, did not inhibit the microbial growth, a single disc was impregnated with 20 µL of the DMSO, which served as the negative control, and applied to the plates. A single vancomycin (30 µg) antibiotic disc was applied to the plates and served as the antibacterial positive control (APC) for the respective microorganism. Vancomycin was chosen based on availability and it is known to inhibit bacteria cell wall biosynthesis by forming complexes with N-acetylmuramic acid and N-acetylglucosamine, subsequently causes transpeptidase reaction to be inhibited (Allen and Nicas, 2003).

After all the discs had been applied to the plates, the plates were incubated at 38^{o}C for 18-20 h. Following incubation, the zone of inhibition (ZOI) was observed. Using a Vernier caliper, the inhibition zone diameter was measured in millimeters for all plates. The mean ZOI in mm ± the standard error of the means (SEM) was recorded. All tests were carried out by sextet and repeated four times to minimize experimental error.

2.7 Experimental Design and Statistical Analysis

Statistical analyses were performed on the response variable, size of the zone of inhibition (ZOI in mm) for each plant extract material (i.e. bark, fruit, leaf, stem). The first was a two-way Analysis of Variance (ANOVA) associated with a 5x4 factorial experiment for each of the six microorganisms (i.e., EC, SE, SF, EF, SA, and MRSA). The first factor was the type of extract (five types, namely; ACE, ETOH, PE, APC, DMSO) and the level of concentration (four levels, namely; 5, 15, 25, and 50) was the other factor.

The second set was a three-way ANOVA, which combined the data across microorganisms, with the addition microorganism as a factor, resulted in the a 6x5x4 factorial experiment. The factors were microorganism (EC, SE, SF, EF, SA, MRSA), type of extract (ACE, ETOH, PE, APC, DMSO) and concentration (5, 15, 25, 50), which translates to 120 treatment combinations in each complete block (Sokal and Rohlf, 2012). The blocks were comprised of the time that each of the four replications was performed (i.e., week 1, week 2, week 3, and week 4). A Duncan's Multiple Range Test (DMRT) was employed for each plant material to identify which of the extract means was or was not significantly different (Sokal and Rohlf, 2012). To compare the 18 interaction means for each plant material, a 95% confidence interval was implemented for each of the microorganism-extract, where overlapping intervals indicated no significant difference. All analysis was carried-out using the general linear model facility of the software Statistical Packages for the Social Sciences version 24.0 (SPSS, Chicago, Illinois).

RESULTS & DISCUSSIONS

3.1 Plant Extract Yield

The extent that biologically active compounds are extracted depends largely on the method of extraction and type of solvent used, their concentration in the plant tissues and the extraction period (Eloff, 1998; Nworgu et al. 2008). Extraction yields for all plant parts were generally low, this supports previous studies on members of the Rubiaceae family, which extraction yields for bark, fruit, leaf, and stems were less than 8% (Aro et al. 2015; Okokli and Iroegbu, 2004). The percent yield of the ACE crude extracts (Table 1) ranged from 0.52% to 2.71% with the leaf extract having the highest percentage yield of 2.71% followed

Table 1: Extraction yields of *V. volkensii* plant components by petroleum ether, acetone, and ethanol solvents

Plant part	Solvent	Wt. of plant material (g)	Wt. of extract (g)	Plant material extracted (%)
Bark	ACE	15.0058	0.1451	0.97±0.012
	ETOH	15.0058	0.4796	3.20±0.012
	PE	15.0058	0.0512	0.34±0.011
Fruit	ACE	50.0279	0.5595	1.12±0.011
	ETOH	50.0279	0.5610	1.12±0.012
	PE	50.0279	0.8355	1.67±0.013
Leaf	ACE	10.0003	0.2706	2.71±0.014
	ETOH	10.0003	0.3885	3.88±0.013
	PE	110.0013	0.3664	0.33±0.004
Stem	ACE	50.0000	0.2595	0.52±0.005
	ETOH	50.0000	0.4198	0.84±0.005
	PE	130.0096	0.0498	0.038±0.006

Plant materials were sequentially extracted with 400 mL of petroleum ether [PE], acetone [ACE], and 9:1 ethanol/deionized water [ETOH] in 3 days successively. Plant material extracted (%) was calculated using equation 1: ((wt. of extract)/(wt. of original plant material))*100; ±: Standard error.

by the fruit and bark extracts (1.12% and 0.97%), respectively. The lowest ACE yield obtained was from the stem extract (0.52%). The percentage yield of the ETOH extracts ranged from 0.84% to 3.88% with the leaf and bark extracts having the highest percentage yield of 3.88% and 3.30%, respectively. The lowest ETOH yield obtained was from the stem and fruit extracts (0.84% and 1.12%), respectively. The percentage yield of the PE extracts ranged from 0.038% to 1.67% with the fruit extract having the highest percentage yield of 1.67% followed by the bark and leaf extracts (0.34% and 0.33%), respectively. The lowest yield obtained was from the stem PE extract (0.038%).

3.2 Antimicrobial Activity of Extracts

Plant extracts in the genus *Vangueria* have been reported to possess antimicrobial properties (Chatterjee et al. 2007; Mahlo et al. 2010; Shai et al. 2013). Disc diffusion assays were conducted to determine if antimicrobial activity was present in the extracts of *V. volkensii*, therefore extracts with the highest measured zones of inhibition for each extract, which was the 50 mg/ml concentrations, was observed. The susceptibility of bacteria to an extract is indicated by an inhibition zone diameter of 9 mm or more around the disc (Akomo et al. 2009; Sarker et al. 2014; Zongo et al. 2009). The sensitivity status of the bacteria is determined by measuring the inhibition zone of diameter to the nearest whole millimeter, which defines the bacteria as resistant (≤9 mm), moderately sensitive (10-11 mm), or sensitive (≥12 mm) to the antibiotic (Sarker et al, 2014).

The negative control (DMSO) in all assays performed did not exhibit any inhibition, thus indicating that the inhibition observed in the assays was attributed to the positive control (APC) or the antibacterial properties exhibited by active components of the extract. All extracts (bark, fruit, leaf and stem) were shown to be less sensitive to the bacteria compared

Table 2: Antimicrobial activity of *V. volkensii* bark extracts

Extract	Concentration (mg/mL)	Mean Zone of Inhibition in mm ± SEM					
		Gram Negative Bacteria			Gram Positive Bacteria		
		EC	SE	SF	EF	SA	MRSA
ACE	5	7.43 ± 0.18	7.98 ± 0.29	8.61 ± 0.35	8.98 ± 0.25	7.41 ± 0.31	8.87 ± 0.17
	15	7.94 ± 0.13	8.55 ± 0.23	8.80 ± 0.39	10.12 ± 0.15	7.80 ± 0.39	9.40 ± 0.23
	25	9.33 ± 0.17	9.80 ± 0.24	9.11 ± 0.40	10.45 ± 0.26	7.79 ± 0.30	9.68 ± 0.32
	50	9.77 ± 0.24	9.84 ± 0.35	9.72 ± 0.46	11.50 ± 0.37	8.27 ± 0.29	10.17 ± 0.41
APC	30	16.09 ± 0.13	19.52 ± 0.40	15.13 ± 0.50	15.53 ± 0.19	17.95 ± 0.14	15.55 ± 0.53
DMSO	−	6.00 ± 0.00	6.00 ± 0.00	6.00 ± 0.00	6.00 ± 0.00	6.00 ± 0.00	6.00 ± 0.00
ETOH	5	7.28 ± 0.01	8.36 ± 0.08	8.43 ± 0.18	8.83 ± 0.58	8.51 ± 0.48	8.59 ± 0.28
	15	7.73 ± 0.02	8.81 ± 0.15	8.94 ± 0.21	9.47 ± 0.28	8.27 ± 0.46	9.17 ± 0.31
	25	8.38 ± 0.15	9.48 ± 0.29	9.28 ± 0.16	10.02 ± 0.14	10.78 ± 1.11	10.14 ± 0.42
	50	8.89 ± 0.26	10.48 ± 0.25	9.54 ± 0.40	10.73 ± 0.09	10.63 ± 0.70	10.94 ± 0.45
APC	30	18.06 ± 0.40	20.20 ± 0.25	15.81 ± 0.65	16.24 ± 0.40	16.42 ± 0.69	17.41 ± 0.54
DMSO	−	6.00 ± 0.00	6.00 ± 0.00	6.00 ± 0.00	6.00 ± 0.00	6.00 ± 0.00	6.00 ± 0.00
PE	5	7.73 ± 0.28	8.58 ± 0.46	9.13 ± 0.21	9.39 ± 0.37	6.87 ± 0.18	7.38 ± 0.20
	15	8.23 ± 0.20	8.81 ± 0.11	9.52 ± 0.14	9.56 ± 0.33	6.90 ± 0.21	9.53 ± 0.23
	25	9.12 ± 0.25	9.33 ± 0.22	10.13 ± 0.12	10.25 ± 0.11	7.55 ± 0.25	10.21 ± 0.17
	50	9.53 ± 0.41	9.84 ± 0.25	10.78 ± 0.09	11.20 ± 0.37	8.21 ± 0.31	10.85 ± 0.34
APC	30	16.45 ± 0.58	20.40 ± 0.27	15.44 ± 0.39	17.83 ± 0.59	18.74 ± 0.42	17.02 ± 0.66
DMSO	−	6.00 ± 0.00	6.00 ± 0.00	6.00 ± 0.00	6.00 ± 0.00	6.00 ± 0.00	6.00 ± 0.00

±: standard error of the means (SEM); ACE: acetone; ETOH: 9:1 ethanol/deionized water; PE: petroleum ether; DMSO: dimethyl sulfoxide (negative control); APC (antibiotic positive control): Vancomycin (30 µg disc); EC: *Escherichia coli* B strain; SE: *Salmonella enterica* Subsp. *enteritidis*; SF: *Shigella flexneri*; EF: *Enterococcus faecalis*; SA: *Staphylococcus aureus*; MRSA: methicillin-resistant *Staphylococcus aureus*.

to the positive control (APC). The observed ZOIs for the APC in all assays ranged from 13.94 to 27.11 mm, indicating that the bacteria were sensitive to APC.

3.2.1 Antimicrobial Activity of Bark Extracts

All bacteria were susceptible to *V. volkensii* bark extracts, except *S. aureus* for ACE and PE extracts and *E. coli* B strain for ETOH extracts (Table 2). The zones of inhibition (mm) ranged from 9.77 to 11.50 mm for ACE extract, 9.54 to 10.94 mmfor ETOH extract, and 9.53 to 11.20 mm for PE extract. The highest zone of inhibitions for the ACE and PE extracts (11.50 and 11.20 mm, respectively) was against *E. faecalis* (Figures 1a and 1b). Whereas, the highest zone of inhibition for ETOH (10.94 mm) was against methicillin-resistant *S. aureus* (Figure 1c).

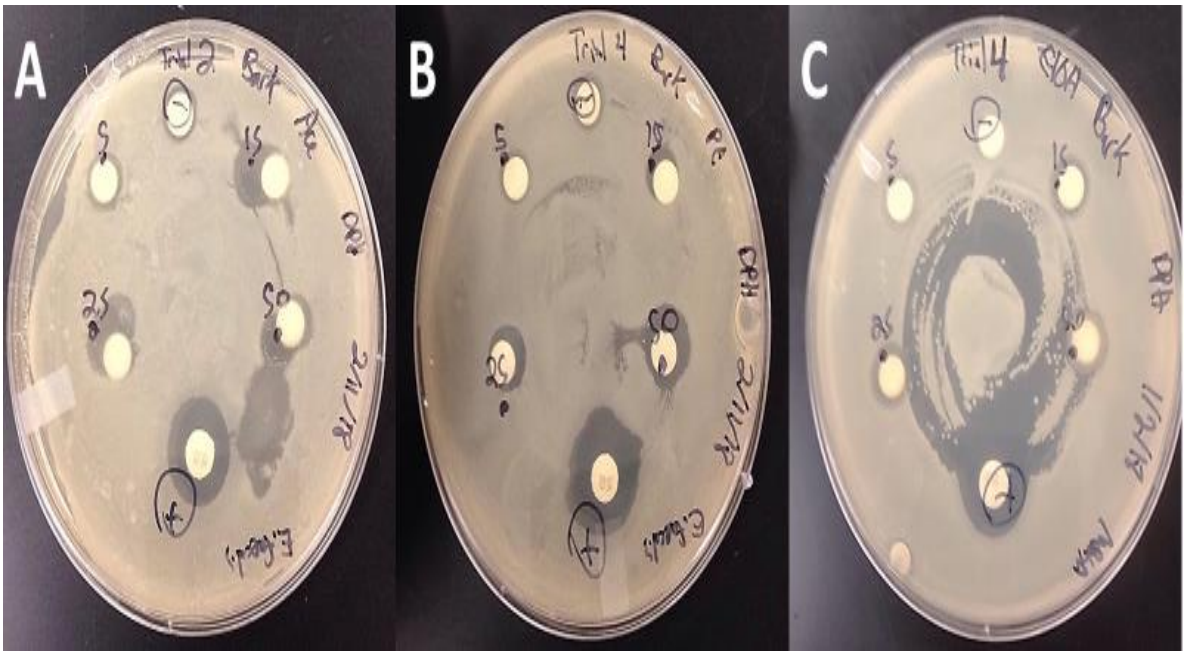

Figure 1: Antimicrobial disc diffusion assay results of *V. volkensii* bark extract.
Note: (a) ACE extract against *E. faecalis* (EF) plated on MH agar (b) PE extract against *E. faecalis* (EF) plated on MH agar (c) ETOH extract against methicillin-resistant *Staphylococcus aureus* (MRSA) plated on MH agar. On plate, 5, 15, 25, 50: extract concentrations (5, 15, 25, 50 mg/ml, respectively), +: APC (Vancomycin), -: negative control (DMSO). Representative of four replications run in sextet.

Staphylococcus aureus was resistant and *E. faecalis* was sensitive to ACE extract. The other four bacteria (*E. coli* B strain, *S. enterica* Subsp. *enterica, S. flexneri* and methicillin-resistant *S. aureus*) were moderately sensitive to ACE extract. All bacteria were moderately sensitive against ETOH and PE extracts, except for *E. coli* B strain and *S. aureus*, which exhibited resistance to ETOH and PE extracts, respectively. The results were comparable to other plant species in the Rubiaceae family. For instance, previous antimicrobial studies conducted on *Vangueria edulis* and *Vangueria infausta* showed bark ETOH and PE extracts against *E. coli* and *S. aureus* of having low antimicrobial activity (Bishay et al. 2012; Mbukwa et al. 2007).

3.2.2 Antimicrobial Activity of Fruit Extracts

All bacteria were susceptible to *V. volkensii* fruit extracts, except *S. aureus* for ETOH extract (Table 3). The zones of inhibition (mm) ranged from 11.07 to 14.01 mm for ACE extract, 8.30 to 12.88 mm for ETOH extract, and 9.65 to 11.61 mm for PE extract. The highest zone of inhibitions for the ETOH and PE extracts (12.88 and 11.61 mm, respectively) were against *S. enterica* Subsp. *enteritidis* (Figures 2a and 2b). Whereas, the highest zone of inhibition for ACE (14.01 mm) was against methicillin-resistant *S. aureus* (Figure 2c). Mahomoodally and Dilmohamed (2016) reported alcoholic and water extracts from *V. madagascariensis* fruit to have good antimicrobial activity against *E. faecalis* and methicillin-resistant *S. aureus*. In the current study, fruit ACE, ETOH and PE extracts against *E. faecalis* exhibited relatively high zones of inhibition (12.00, 11.68, and 11.09 mm, respectively). Likewise, against methicillin-resistant *S. aureus*, relatively high zones of inhibition for these same fruit extracts (ACE, ETOH, and PE), which were 14.01 mm, 12.23 mm and 10.85 mm, respectively.

Table 3: Antimicrobial activity of *V. volkensii* fruit extracts

Extract	Concentration (mg/mL)	Mean Zone of Inhibition in mm ± SEM					
		Gram Negative Bacteria			Gram Positive Bacteria		
		EC	SE	SF	EF	SA	MRSA
ACE	5	9.39 ± 0.32	10.46 ± 0.24	8.66 ± 0.23	9.47 ± 0.36	7.99 ± 0.24	10.04 ± 0.74
	15	9.67 ± 0.32	11.29 ± 0.25	9.32 ± 0.23	10.50 ± 0.36	9.68 ± 0.27	11.53 ± 0.87
	25	10.14 ± 0.35	12.17 ± 0.45	11.41 ± 0.79	11.10 ± 0.31	11.26 ± 0.52	12.52 ± 1.10
	50	11.07 ± 0.54	13.61 ± 0.67	12.67 ± 1.07	12.00 ± 0.46	12.47 ± 0.38	14.01 ± 1.50
APC	30	15.06 ± 0.38	23.68 ± 0.29	27.11 ± 2.95	19.37 ± 0.31	25.05 ± 0.76	26.65 ± 0.34
DMSO	−	6.00 ± 0.00	6.00 ± 0.00	6.00 ± 0.00	6.00 ± 0.00	6.00 ± 0.00	6.00 ± 0.00
ETOH	5	8.73 ± 0.26	8.98 ± 0.23	8.03 ± 0.33	9.22 ± 0.44	6.61 ± 0.13	8.83 ± 0.17
	15	9.37 ± 0.18	10.15 ± 0.14	8.77 ± 0.30	9.92 ± 0.17	7.08 ± 0.19	10.18 ± 0.42
	25	9.96 ± 0.30	10.67 ± 0.16	9.38 ± 0.25	10.43 ± 0.24	7.65 ± 0.26	11.04 ± 0.64
	50	10.82 ± 0.32	12.88 ± 0.55	10.51 ± 0.18	11.68 ± 0.23	8.30 ± 0.34	12.23 ± 0.46
APC	30	14.19 ± 0.20	23.79 ± 0.26	20.53 ± 4.24	18.82 ± 0.55	20.75 ± 1.58	24.30 ± 0.75
DMSO	−	6.00 ± 0.00	6.00 ± 0.00	6.00 ± 0.00	6.00 ± 0.00	6.00 ± 0.00	6.00 ± 0.00
PE	5	8.96 ± 0.08	8.50 ± 0.11	7.71 ± 0.17	9.02 ± 0.36	6.75 ± 0.12	7.49 ± 0.78
	15	9.39 ± 0.21	10.13 ± 0.07	8.42 ± 0.21	9.84 ± 0.07	7.69 ± 0.29	8.93 ± 0.43
	25	9.70 ± 0.28	10.76 ± 0.12	9.07 ± 0.24	10.32 ± 0.13	8.44 ± 0.33	10.20 ± 0.53
	50	10.28 ± 0.17	11.61 ± 0.44	9.87 ± 0.31	11.09 ± 0.26	9.65 ± 0.09	10.85 ± 0.58
APC	30	13.94 ± 0.14	23.47 ± 0.82	22.86 ± 1.81	19.89 ± 0.10	24.79 ± 0.23	25.59 ± 0.93
DMSO	−	6.00 ± 0.00	6.00 ± 0.00	6.00 ± 0.00	6.00 ± 0.00	6.00 ± 0.00	6.00 ± 0.00

±: standard error of the means (SEM); ACE: acetone; ETOH: 9:1 ethanol/deionized water; PE: petroleum ether; DMSO: dimethyl sulfoxide (negative control); APC (antibiotic positive control): Vancomycin (30 µg disc); EC: *Escherichia coli* B strain; SE: *Salmonella enterica* Subsp. *enteritidis*; SF: *Shigella flexneri*; EF: *Enterococcus faecalis*; SA: *Staphylococcus aureus*; MRSA: methicillin-resistant *Staphylococcus aureus*.

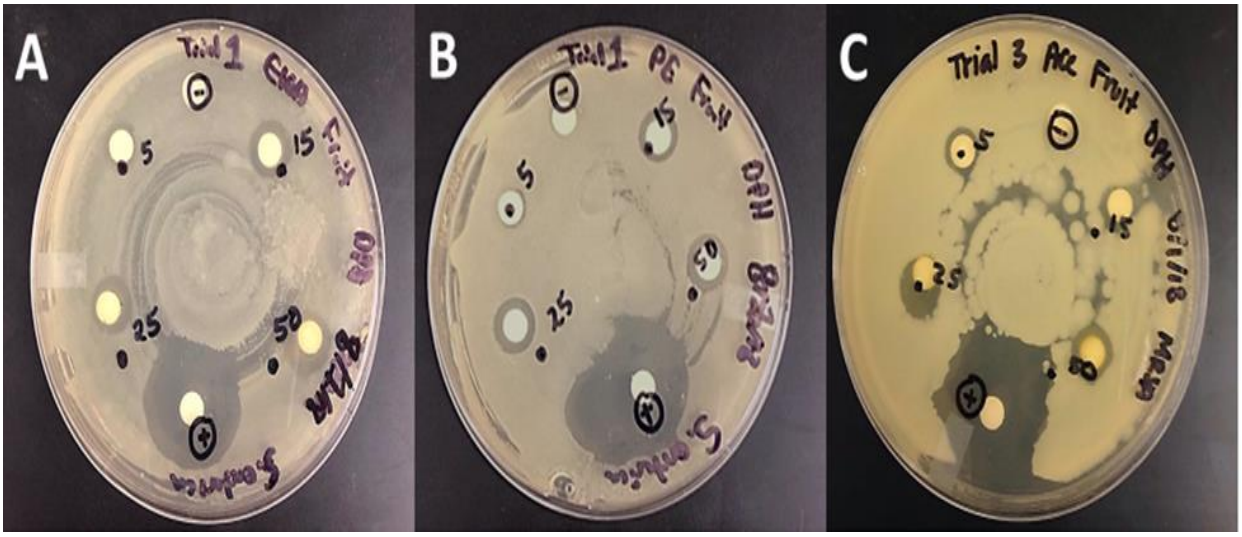

Figure 2: Antimicrobial disc diffusion assay results of *V. volkensii* fruit extract.
Note: (a) ETOH extract against *S. enterica* Subsp. *enteritidis* (SE) plated on MH agar (b) PE extract against *S. enterica* Subsp. *enteritidis* (SE) plated on MH agar (c) ACE extract against methicillin-resistant *Staphylococcus aureus* (MRSA) plated on MH agar. On plate, 5, 15, 25, 50: extract concentrations (5, 15, 25, 50 mg/ml, respectively), +: APC (Vancomycin), -: negative control (DMSO). Representative of four replications run in sextet.

All bacteria were either sensitive or moderately sensitive to the fruit extracts, except for *S. aureus*, which was resistant to ETOH extract. All bacteria, except for *E. coli* B strain, were sensitive to ACE extract. *E. coli* B strain and *S. flexneri* were moderately sensitive against ETOH extracts, whereas *S. enterica* Subsp. *enteritidis*, *E. faecalis* and methicillin-resistant *S. aureus* were sensitive to ETOH extract. *S. enterica* Subsp. *enteritidis* was sensitive to PE and the other five bacteria were moderately sensitive to PE.

In Ramalingum and Mahomoodally (2014), fruit alcoholic extracts from *V. madagascariensis* when tested against *S. aureus* exhibited very poor antimicrobial activity, also they did not report the its zone of inhibition due to being very low. Similarly, in the current study, the zone of inhibition for fruit ETOH extract against *S. aureus* was also very low (8.30

Table 4: Antimicrobial activity of *V. volkensii* leaf extracts

Extract	Concentration (mg/mL)	Mean Zone of Inhibition in mm ± SEM					
		Gram Negative Bacteria			Gram Positive Bacteria		
		EC	SE	SF	EF	SA	MRSA
ACE	5	7.75 ± 0.14	8.19 ± 0.27	8.47 ± 0.08	9.40 ± 0.40	7.46 ± 0.23	10.22 ± 0.14
	15	8.35 ± 0.14	8.93 ± 0.39	8.84 ± 0.12	10.41 ± 0.37	7.70 ± 0.30	10.99 ± 0.33
	25	9.65 ± 0.10	9.42 ± 0.37	9.69 ± 0.21	10.55 ± 0.40	8.44 ± 0.39	11.14 ± 0.55
	50	10.17 ± 0.13	10.48 ± 0.28	10.18 ± 0.18	11.24 ± 0.49	8.48 ± 0.20	11.56 ± 0.56
APC	30	16.61 ± 0.35	20.17 ± 0.14	16.07 ± 0.97	18.87 ± 0.50	17.90 ± 0.26	18.92 ± 0.81
DMSO	−	6.00 ± 0.00	6.00 ± 0.00	6.00 ± 0.00	6.00 ± 0.00	6.00 ± 0.00	6.00 ± 0.00
ETOH	5	7.93 ± 0.21	8.50 ± 0.46	8.74 ± 0.14	9.61 ± 0.47	8.61 ± 0.40	9.40 ± 0.34
	15	9.01 ± 0.26	8.90 ± 0.37	9.60 ± 0.19	10.90 ± 0.30	9.47 ± 0.18	10.09 ± 0.24
	25	9.64 ± 0.32	9.59 ± 0.50	10.56 ± 0.34	11.55 ± 0.41	9.31 ± 0.71	10.65 ± 0.44
	50	10.97 ± 0.14	10.68 ± 0.80	11.48 ± 0.72	12.21 ± 0.11	12.34 ± 1.94	11.55 ± 0.36
APC	30	17.78 ± 0.55	20.08 ± 0.23	15.94 ± 0.49	17.79 ± 0.53	17.61 ± 0.90	16.64 ± 0.45
DMSO	−	6.00 ± 0.00	6.00 ± 0.00	6.00 ± 0.00	6.00 ± 0.00	6.00 ± 0.00	6.00 ± 0.00
PE	5	8.98 ± 0.32	9.44 ± 0.18	8.36 ± 0.35	9.74 ± 0.27	6.83 ± 0.12	9.66 ± 0.53
	15	9.41 ± 0.26	10.13 ± 0.31	8.96 ± 0.17	10.06 ± 0.27	7.21 ± 0.18	10.62 ± 0.79
	25	9.89 ± 0.25	10.94 ± 0.19	9.59 ± 0.09	10.67 ± 0.38	7.45 ± 0.17	11.73 ± 1.43
	50	11.25 ± 0.55	11.60 ± 0.44	10.37 ± 0.16	11.70 ± 0.35	7.55 ± 0.38	12.50 ± 1.62
APC	30	14.72 ± 0.82	23.19 ± 1.54	28.64 ± 2.74	18.94 ± 0.54	18.92 ± 0.17	25.51 ± 0.23
DMSO	−	6.00 ± 0.00	6.00 ± 0.00	6.00 ± 0.00	6.00 ± 0.00	6.00 ± 0.00	6.00 ± 0.00

±: standard error of the means (SEM); ACE: acetone; ETOH: 9:1 ethanol/deionized water; PE: petroleum ether; DMSO: dimethyl sulfoxide (negative control); APC (antibiotic positive control): Vancomycin (30 µg disc); EC: *Escherichia coli* B strain; SE: *Salmonel enterica* Subsp. *enteritidis*; SF: *Shigella flexneri*; EF: *Enterococcus faecalis*; SA: *Staphylococcus aureus*; MRSA: methicillin-resistant *Staphylococcus aureus*.

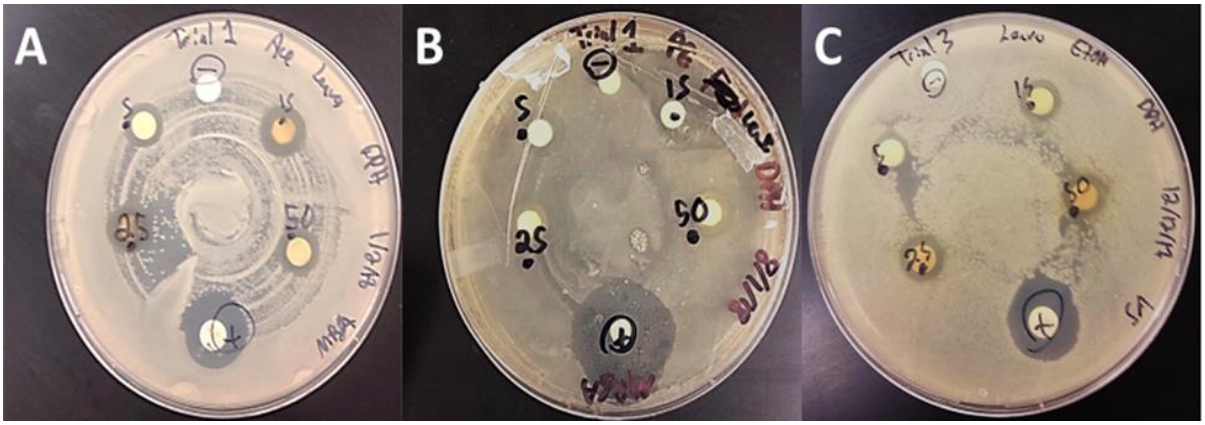

Figure 3: Antimicrobial disc diffusion assay results of *V. volkensii* leaf extract.
Note: (a) ACE extract against methicillin-resistant *Staphylococcus aureus* (MRSA) plated on MH agar (b) PE extract against methicillin-resistant *Staphylococcus aureus* (MRSA) plated on MH agar (c) ETOH extract against *S. aureus* (SA) plated on MH agar. On plate, 5, 15, 25, 50: extract concentrations (5, 15, 25, 50 mg/ml, respectively), +: APC (Vancomycin), -: negative control (DMSO). Representative of four replications run in sextet.

mm). In addition, the very poor antimicrobial activity that they reported, supports the notion that *S. aureus* was resistant to the fruit ETOH extract reported in the current study

3.2.3 Antimicrobial Activity of Leaf Extracts

All bacteria were susceptible to *V. volkensii* leaf extracts, except *S. aureus* for ACE and PE extracts (Table 4). The zones of inhibition (mm) ranged from 8.48 to 11.56 mm for ACE extract, 10.68 to 12.34 mm for ETOH extract, and 7.55 to 12.50 mm for PE extract. The highest zone of inhibitions for the ACE and PE extracts (11.56 and 12.50 mm, respectively) was against methicillin-resistant *S. aureus* (Figures 3a and 3b). Whereas, the highest zone of inhibition for ETOH (12.34 mm) was against *S. aureus* (Figure 3c). Likewise, leaf ETOH extracts against S. aureus have frequently been reported to exhibit high antimicrobial activity in many Rubiaceae species, for instance; *Mitrogyna rubrostipulata, Pavetta ternifolia, Rubia cordifolia, Vangueria edulis* and *Vangueria infausta* (Bishay et al. 2012; Shia et al. 2013; Vlietinck et al. 1995).

All bacteria were moderately sensitive to ACE extract, except for *S. aureus* and methicillin-resistant *S. aureus*, which were resistant and sensitive, respectively, to ACE extract. The gram-negative bacteria (*E. coli* B strain, *S. enterica* Subsp. *enteritidis*, *S. flexneri*) were moderately sensitive to ETOH extract, while the gram-positive bacteria *E. faecalis*, *S. aureus*, methicillin-resistant *S. aureus*) were sensitive to ETOH extract. *S. aureus* was the only bacteria deemed resistant to PE extract, the others were deemed either moderately sensitive (*E. coli* B strain, *S. flexneri*) or sensitive (*S. enterica* Subsp. *enteritidis*, *E. faecalis*, methicillin-resistant *S. aureus*) to PE extract.

Shia et al. (2013) studied the antibacterial activity of sixteen plant species from South Africa, including *V. infausta*. The *V. infausta* leaf ETOH extract, exhibited the highest antibacterial activity compared to the other fifteen plant species. *V. infausta* leaf ETOH extract against *E. faecalis*, *S. aureus*, *E. coli* and *Salmonella* sp., in which, it was reported that the leaf ETOH extract were highly active against all bacteria. In the present study, the zones of inhibition for leaf ETOH extract against *E. faecalis* (11.48 mm), *S. aureus* (12.34 mm), *E. coli* (10.97mm) and *S. enterica* Subsp. *enteritidis* (10.68 mm) was relatively high, indicating good antimicrobial activity. Thus, results were comparable to the results of the study conducted by Shia et al. (2013).

3.2.4 Antimicrobial Activity of Stem Extracts

All bacteria were susceptible to *V. volkensii* stem extracts (ACE and ETOH), except *S. aureus* for ACE (Table 5). The zones of inhibition (mm) ranged from 8.02 to 11.38 mm for ACE extract and 10.00 to 11.36 mm for ETOH extract. The highest zone of inhibition for ACE extract (11.38 mm) was against *E. faecalis* and for ETOH extract (11.36 mm) was against methicillin-resistant *S. aureus* (Figure 4a and 4b).Recall, the antimicrobial activity of *V. volkensii*

Table 5: Antimicrobial activity of *V. volkensii* stem extracts

Extract	Concentration (mg/mL)	Mean Zone of Inhibition in mm ± SEM					
		Gram Negative Bacteria			Gram Positive Bacteria		
		EC	SE	SF	EF	SA	MRSA
ACE	5	7.33 ± 0.16	8.63 ± 0.11	8.46 ± 0.43	9.34 ± 0.35	7.03 ± 0.14	7.80 ± 0.30
	15	8.17 ± 0.36	8.65 ± 0.09	9.38 ± 0.51	9.70 ± 0.29	7.50 ± 0.16	9.29 ± 0.27
	25	9.27 ± 0.38	9.47 ± 0.26	10.02 ± 0.39	10.54 ± 0.25	7.65 ± 0.33	10.05 ± 0.18
	50	9.28 ± 0.55	10.46 ± 0.31	10.66 ± 0.40	11.38 ± 0.05	8.02 ± 0.30	10.84 ± 0.30
APC	30	17.05 ± 0.78	20.42 ± 0.31	16.08 ± 0.69	16.56 ± 0.84	18.89 ± 0.35	17.10 ± 0.33
DMSO	−	6.00 ± 0.00	6.00 ± 0.00	6.00 ± 0.00	6.00 ± 0.00	6.00 ± 0.00	6.00 ± 0.00
ETOH	5	7.58 ± 0.17	8.06 ± 0.21	8.36 ± 0.21	8.50 ± 0.16	7.80 ± 0.28	8.38 ± 0.36
	15	8.35 ± 0.20	9.02 ± 0.45	9.49 ± 0.39	9.27 ± 0.18	8.98 ± 0.35	9.63 ± 0.22
	25	9.39 ± 0.30	10.20 ± 0.42	9.52 ± 0.05	9.85 ± 0.21	10.10 ± 0.72	10.07 ± 0.26
	50	10.21 ± 0.41	10.52 ± 0.56	10.00 ± 0.38	10.66 ± 0.30	11.15 ± 0.82	11.36 ± 0.59
APC	30	18.39 ± 0.96	20.63 ± 0.27	15.00 ± 0.37	16.38 ± 0.54	16.44 ± 0.13	18.25 ± 0.30
DMSO	−	6.00 ± 0.00	6.00 ± 0.00	6.00 ± 0.00	6.00 ± 0.00	6.00 ± 0.00	6.00 ± 0.00
PE	5	∗	∗	∗	∗	6.79 ± 0.10	∗
	15	∗	∗	∗	∗	7.27 ± 0.20	∗
	25	∗	∗	∗	∗	7.93 ± 0.26	∗
	50	∗	∗	∗	∗	8.19 ± 0.36	∗
APC	30	∗	∗	∗	∗	18.32 ± 0.29	∗
DMSO	−	∗	∗	∗	∗	6.00 ± 0.00	∗

∗ Not observed, due to lack of production of extract

±: standard error of the means (SEM); ACE: acetone; ETOH: 9:1 ethanol/deionized water; PE: petroleum ether; DMSO: dimethyl sulfoxide (negative control); APC (antibiotic positive control): Vancomycin (30 µg disc); EC: *Escherichia coli* B strain; SE: *Salmonella enterica* Subsp. *enteritidis*; SF: *Shigella flexneri*; EF: *Enterococcus faecalis*; SA: *Staphylococcus aureus*; MRSA: methicillin-resistant *Staphylococcus aureus*.

stem PE extract was only conducted on *S. aureus* due to the lack of crude PE extract produced (table 1). Therefore, *S. aureus* was not susceptible (8.19 mm) to PE. Stem ETOH extract against all microorganisms exhibited relatively high zones of inhibition. Using the agar diffusion method, Vlietinck et al. (1995) reported stem ETOH extract of *Mitragyna rubrostipulata*, which is a Rubiaceae species, to have high activity against *S. aureus*, which supports the findings in the current study.

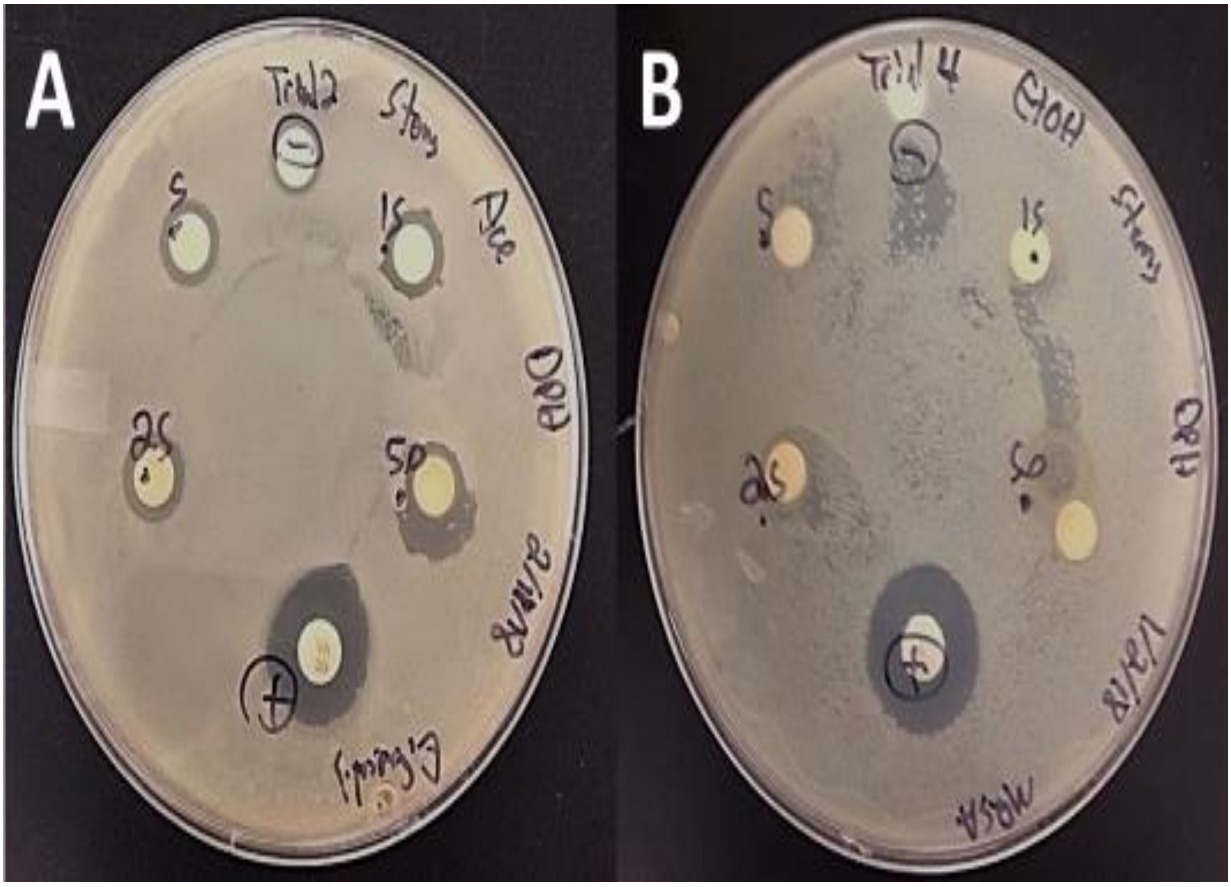

Figure 4: Antimicrobial disc diffusion assay results of *V. volkensii* stem extract.
Note: (a) ACE extract against *E. faecalis* (EF) plated on MH agar (b) ETOH extract against methicillin-resistant *Staphylococcus aureus* (MRSA) plated on MH agar. On plate, 5, 15, 25, 50: extract concentrations (5, 15, 25, 50 mg/ml, respectively), +: APC (Vancomycin), -: negative control (DMSO). Representative of four replications run in sextet.

Escherichia coli strain B and *S. aureus* were resistant to ACE extract while the other four bacteria were moderately resistant to ACE extract. All bacteria were moderately sensitive to ETOH extracts. *Staphylococcus aureus*, which was the only bacteria tested against PE extract, was resistant to PE extract.

Overall, nearly all extracts (bark, fruit, leaf and stem) exhibited broad spectrum activity against both gram-positive and gram-negative bacteria. However, *E. coli* B strain (gram negative) and *S. aureus* (gram positive) bacteria was resistant to some extracts. Those extracts against *E. coli* B strain were the bark ETOH and stem ACE and those extracts against *S. aureus* were the bark, leaf and stem ACE and PE extracts and the fruit ETOH extract. The fruit and leaf extracts (ACE, ETOH, PE) exhibited the most antimicrobial potential via all microorganisms exhibited sensitivity against extracts, except for *S. aureus*, which was resistant to the fruit ETOH extract and the leaf ACE and PE extracts. The general trend is that gram positive bacteria are more sensitive to plant extracts than gram negative bacteria has been well documented (Chatterjee et al. 2007; Karou et al. 2011; Shai et al. 2013; Zongo et al. 2009). The cell wall biochemical composition of gram-positive bacteria (outer peptidoglycan layer) and gram-negative bacteria (outer phospholipidic membrane) may result in susceptibility of gram-positive bacteria, compared to gram-negative bacteria (Karou et al. 2011). However, the results of this study were not consistent with the general trend (Tables 2-5).

3.3 Inhibitory Activity of Extracts for Each Microorganism

Members of the Rubiaceae family contain inhibitory properties (Akomo et al., 2009); Aro et al., 2015; Shai et al., 2013). The inhibitory activity of *V. volkensii* extracts were examined via Analysis of Variance (ANOVA) and Duncan's Multiple Range Tests (DMRT). The mean ZOIs of the 50 mg/ml concentrations of extracts were used for DMRTs. All analyses showed

Table 6: Comparison of bark extract mean zones of inhibition for each microorganism

	Mean zone of inhibition (mm)					
	Gram Negative Bacteria			Gram Positive Bacteria		
Extracts	EC	SE	SF	EF	SA	MRSA
DMSO	6.0000±0.00[a]	6.0000±0.00[a]	6.0000±0.00[a]	6.0000±0.00[a]	6.0000±0.00[a]	6.0000±0.00[a]
ACE	8.6175±0.36[b]	9.0431±0.55[b]	9.0619±0.81[b]	10.2650±0.51[b]	7.8181±0.65[b]	9.5331±0.57[b]
ETOH	8.0744±0.22[b]	9.2850±0.38[b]	9.0500±0.48[b]	9.7631±0.55[b]	9.5481±1.38[c]	9.7119±0.74[b]
PE	8.6556±0.57[b]	9.1394±0.52[b]	9.8906±0.29[c]	10.0994±0.59[b]	7.3844±0.48[b]	9.4956±0.48[b]
APC	16.8717±0.37[c]	20.0417±0.31[c]	15.4650±0.51[d]	16.5358±0.38[c]	17.7050±0.42[d]	16.6592±0.58[c]

In a column, means with the same letter are not significantly different at the 0.05 level by Duncan's Multiple Range Test. ±: standard error of means (SEM); ACE: acetone; ETOH: 9:1 ethanol/deionized water; PE: petroleum ether; DMSO: dimethyl sulfoxide (negative control); APC: Vancomycin (antibiotic positive control); EC: *Escherichia coli* B strain; SE: *Salmonella enterica* Subsp. *enteritidis*; SF: *Shigella flexneri*; EF: *Enterococcus faecalis*; SA: *Staphylococcus aureus*; MRSA: methicillin-resistant *Staphylococcus aureus*.

significant ($p<0.05$) interactions of mean ZOIs between microorganisms and extracts. In addition, all analyses indicated a significant difference ($p<0.05$) of mean ZOIs among microorganisms and among concentrations. Analysis of variance was repeated for all extracts (bark, fruit, leaf, stem) by removing the mean ZOIs of DMSO and APC to determine if any significant difference ($p<0.05$) initially observed, was due to these controls, which did not change the significance ($p<0.05$).

3.3.1 Inhibitory Activity of Bark Extracts for Each Microorganism

All extracts (ACE, ETOH, PE) for *V. volkensii* bark exhibited a significant difference ($p<0.05$) of ZOI means from DMSO and APC when tested against six bacterial strains (Table 6).

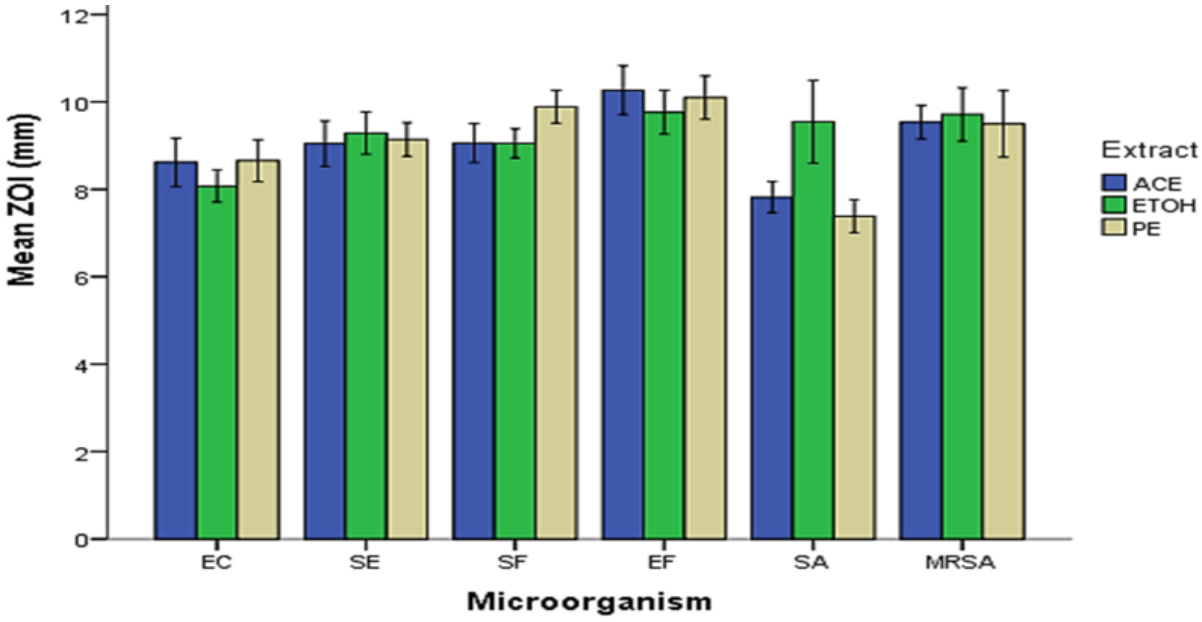

Figure 5: *V. volkensii* bark microorganism-extract combinations with their 95% confidence intervals.
Note: Negative and positive control not depicted due to significantly different ZOI means compared to extracts. ACE: acetone; ETOH: 9:1 ethanol/deionized water; PE: petroleum ether; EC: *Escherichia coli* B strain; SE: *Salmonella enterica* Subsp. *enteritidis*; SF: *Shigella flexneri*; EF: *Enterococcus faecalis*; SA: *Staphylococcus aureus*; MRSA: methicillin-resistant *Staphylococcus aureus*. Mean values are from four replications run in sextet.

When tested against *E. coli* B strain, *S. enterica* Subsp. *enteritidis*, *E. faecalis*, and methicillin-resistant *S. aureus*, extracts did not exhibit a significant difference ($p<0.05$) of mean ZOIs from each other. Mean ZOIs of extracts against *E. coli* B strain, *S. enterica* Subsp. *enteritidis*, *E. faecalis*, and methicillin-resistant *S. aureus* ranged from 8.0744 to 8.6556 mm, 9.0431 to 9.2850 mm, 9.7631 to 10.2650 mm and 9.4956 to 9.7119 mm, respectively. Against *S. flexneri*, PE extract had a mean ZOI of 9.8906 mm and it was significantly higher ($p<0.05$) than ACE and ETOH extracts which had ZOI means of 9.0619 mm and 9.0500 mm, respectively. Likewise, ETOH extract against *S. aureus* had a mean ZOI of 9.5481 mm and it was significantly higher ($p<0.05$) than ACE and PE extracts with mean ZOIs of 7.8181 mm and 7.3844 mm, respectively (Table 6 and Figure 5). Analysis of variance of the mean ZOIs for these extract-microorganism interactions indicate a significant difference of $p<0.001$ (Table 7).

Table 7: ANOVA for bark extract zones of inhibition

Sources of variation	DF	SS	MS	F
Block	3	1.602	0.534	1.045
Extract (EX)	2	1.682	0.841	1.645
Microorganism (MI)	5	79.684	15.937	31.174***
Concentration (CO)	3	137.025	45.675	89.344***
EX×CO	6	1.271	0.212	0.414
MI×CO	15	7.797	0.520	1.017
MI×EX	10	54.064	5.406	10.575***
MI×EX×CO	30	19.388	0.646	1.264
Error	345	176.373	0.511	

**, ***Denote significance at the 0.01 and 0.001 level, respectively

3.3.2 Inhibitory Activity of Fruit Extracts for Each Microorganism

All extracts (ACE, ETOH, PE) for *V. volkensii* fruit exhibited a significant difference

Table 8: Comparison of fruit extract mean zones of inhibition for each microorganism

	Mean zone of inhibition (mm)					
	Gram Negative Bacteria			Gram Positive Bacteria		
Extracts	EC	SE	SF	EF	SA	MRSA
DMSO	6.0000±0.00[a]	6.0000±0.00[a]	6.0000±0.00[a]	6.0000±0.00[a]	6.0000±0.00[a]	6.0000±0.00[a]
ACE	10.0700±0.77[b]	11.8838±0.81[c]	10.5138±1.15[b]	10.7681±0.75[b]	10.3519±0.70[c]	12.0263±2.11[c]
ETOH	9.7225±0.53[b]	10.6725±0.54[b,c]	9.1756±0.53[b]	10.3119±0.54[b]	7.4119±0.46[b]	10.5719±0.85[b,c]
PE	9.5813±0.37[b]	10.2506±0.38[b]	8.7681±0.46[b]	10.0688±0.41[b]	8.1356±0.42[b]	9.3681±1.16[b]
APC	14.3992±0.24[c]	23.6492±0.46[d]	23.5017±0.78[c]	19.3583±0.32[c]	23.5283±0.86[d]	25.5150±0.67[d]

In a column, means with the same letter are not significantly different at the 0.05 level by Duncan's Multiple Range Test. ±: standard error of means (SEM); ACE: acetone; ETOH: 9:1 ethanol/deionized water; PE: petroleum ether; DMSO: dimethyl sulfoxide (negative control); APC: Vancomycin (antibiotic positive control); EC: *Escherichia coli* B strain; SE: *Salmonella enterica* Subsp. *enteritidis*; SF: *Shigella flexneri*; EF: *Enterococcus faecalis*; SA: *Staphylococcus aureus*; MRSA: methicillin-resistant *Staphylococcus aureus*.

($p<0.05$) of ZOI means from DMSO and APC when tested against the six bacterial strains (Table 8). When tested against *E. coli* B strain, *S. enterica* Subsp. *enteritidis* and *E. faecalis*, extracts did not exhibit a significant difference ($p<0.05$) in mean ZOIs from each other. Mean ZOIs of extracts

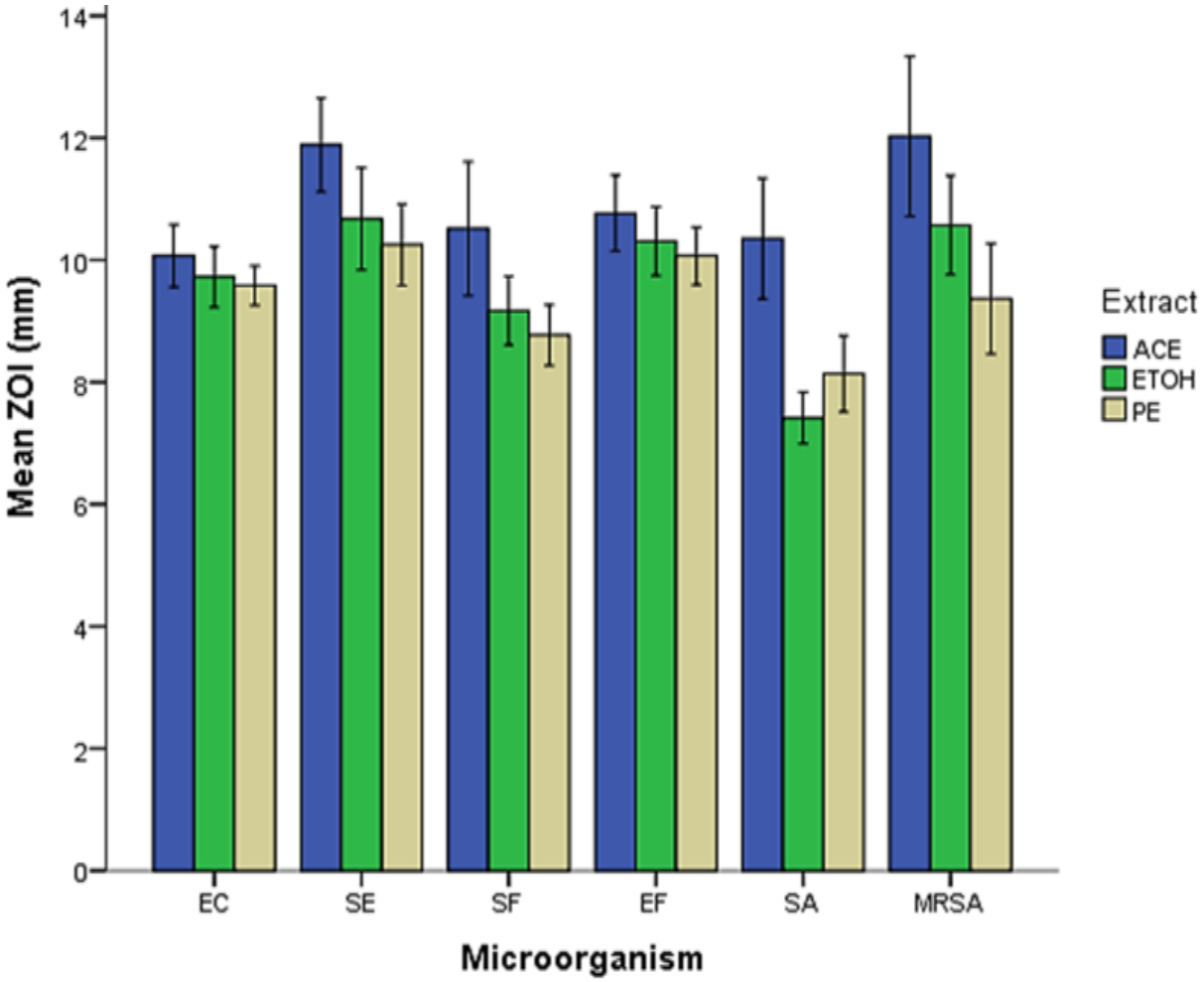

Figure 6: *V. volkensii* fruit microorganism-extract combinations with their 95% confidence intervals.
Note: Negative and positive control not depicted due to significantly different ZOI means compared to extracts. ACE: acetone; ETOH: 9:1 ethanol/deionized water; PE: petroleum ether; EC: *Escherichia coli* B strain; SE: *Salmonella enterica* Subsp. *enteritidis*; SF: *Shigella flexneri*; EF: *Enterococcus faecalis*; SA: *Staphylococcus aureus*; MRSA: methicillin-resistant *Staphylococcus aureus*. Mean values are from four replications run in sextet.

and 8.1356 mm, respectively. When extracts were tested against *S. enterica* Subsp. *enteritidis* against *E. coli* B strain, *S. enterica* Subsp. *enteritidis* and *E. faecalis* ranged from 9.5813 to 10.0700 mm, 8.7681 to 10.5138 mm, and 10.0688 to 10.7681 mm, respectively. Against *S. aureus*, the ACE extract had a mean ZOI of 10.3519 mm and it was significantly different ($p<0.05$) from the ETOH and PE extracts which exhibited mean ZOIs of 7.4119 mmand methicillin-resistant *S. aureus*, the ACE extracts had mean ZOIs of 11.8838 mm and 12.0263 mm, respectively, and were both significantly different ($p<0.05$) from their respective PE extracts (10.2506 mm and 9.3681 mm, respectively). Interestingly, the ETOH extracts against *S. enterica* Subsp. *enteritidis* and methicillin-resistant *S. aureus* had mean ZOIs of 10.6725 mm and 10.5719 mm, respectively, which was not significantly different ($p<0.05$) from their respective

Table 9: ANOVA for fruit extract zones of inhibition

Sources of variation	DF	SS	MS	F
Block	3	12.418	4.139	1.930
Extract (EX)	2	135.138	67.569	31.508***
Microorganism (MI)	5	290.256	58.051	27.070***
Concentration (CO)	3	310.024	103.341	48.188***
EX×CO	6	7.030	1.172	0.546
MI×CO	15	17.973	1.198	0.559
MI×EX	10	52.388	5.239	2.443**
MI×EX×CO	30	14.168	0.472	0.220
Error	345	739.860	2.145	

**, ***Denote significance at the 0.01 and 0.001 level, respectively

Table 10: Comparison of leaf extract mean zones of inhibition for each microorganism

	Mean zone of inhibition (mm)					
	Gram Negative Bacteria			Gram Positive Bacteria		
Extracts	EC	SE	SF	EF	SA	MRSA
DMSO	6.0000±0.00[a]	6.0000±0.00[a]	6.0000±0.00[a]	6.0000±0.00[a]	6.0000±0.00[a]	6.0000±0.00[a]
ACE	8.9838±0.26[b]	9.2563±0.66[b]	9.2975±0.30[b]	10.4044±0.83[b]	8.0188±0.56[b]	10.9794±0.79[b]
ETOH	9.3894±0.47[b]	9.4169±1.06[b]	10.0975±0.70[b]	11.0706±0.65[b]	9.9338±1.62[c]	10.4269±0.70[b]
PE	9.8838±0.68[b]	10.5319±0.57[c]	9.3206±0.38[b]	10.5438±0.64[b]	7.2644±0.43[b]	11.1281±2.18[b]
APC	16.3683±0.57[c]	21.1483±0.64[d]	20.2192±0.94[c]	18.5367±0.84[c]	18.1458±0.44[d]	20.3583±0.50[c]

In a column, means with the same letter are not significantly different at the 0.05 level by Duncan's Multiple Range Test. ±: standard error of means (SEM); ACE: acetone; ETOH: 9:1 ethanol/deionized water; PE: petroleum ether; DMSO: dimethyl sulfoxide (negative control); APC: Vancomycin (antibiotic positive control); EC: *Escherichia coli* B strain; SE: *Salmonella enterica* Subsp. *enteritidis*; SF: *Shigella flexneri*; EF: *Enterococcus faecalis*; SA: *Staphylococcus aureus*; MRSA: methicillin-resistant *Staphylococcus aureus*.

ACE and PE extracts (Table 8 and Figure 6). Analysis of variance of the mean ZOIs for these extract-microorganism interactions indicate a significant difference of $p<0.01$ (Table 9)

3.3.3 Inhibitory Activity of Leaf Extracts for Each Microorganism

All extracts (ACE, ETOH, PE) for *V. volkensii* leaf exhibited a significant difference ($p<0.05$) of ZOI means from DMSO and APC when tested against the six bacterial strains (Table 10). When tested against *E. coli* B strain, *S. flexneri*, *E. faecalis*, and methicillin-resistant *S. aureus*, extracts did not exhibit a significant difference ($p<0.05$) of mean ZOIs from each other. Mean ZOIs of extracts against *E. coli* B strain, *S. flexneri*, *E. faecalis*, and methicillin-resistant *S. aureus* ranged from 8.9838 to 9.8838 mm, 9.2975 to 10.0975 mm, 10.4044 to 11.0706 mm and 10.4269 to 11.1281 mm, respectively. Petroleum ether extract exhibited a mean ZOI of 10.5319 mm when tested against *S. enterica* Subsp. *enteritidis*, which was not significantly different

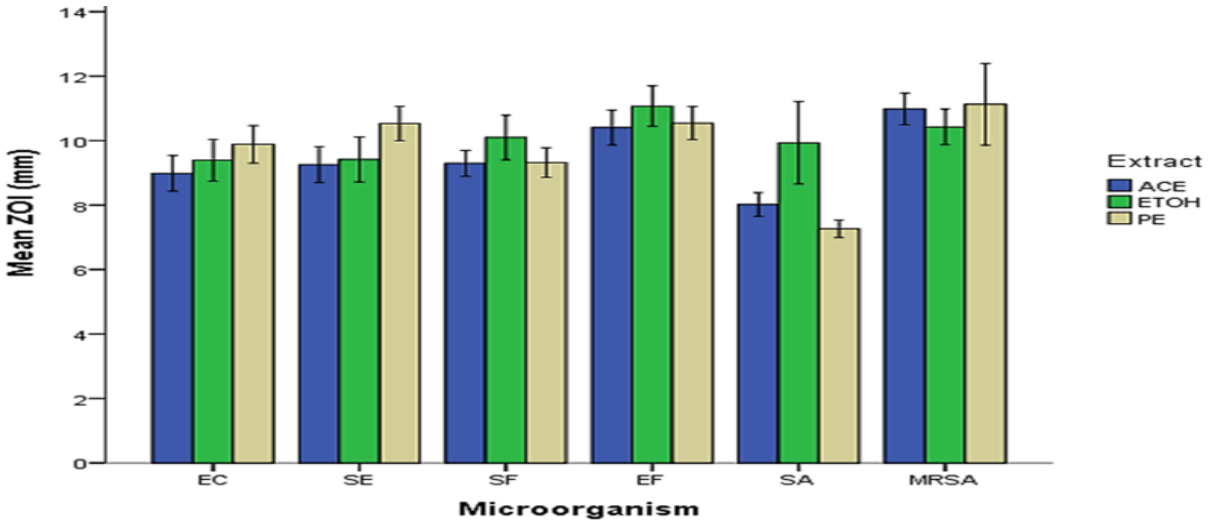

Figure 7: *V. volkensii* leaf microorganism-extract combinations with their 95% confidence intervals.
Note: Negative and positive control not depicted due to significantly different ZOI means compared to extracts. ACE: acetone; ETOH: 9:1 ethanol/deionized water; PE: petroleum ether; EC: *Escherichia coli* B strain; SE: *Salmonella enterica* Subsp. *enteritidis*; SF: *Shigella flexneri*; EF: *Enterococcus faecalis*; SA: *Staphylococcus aureus*; MRSA: methicillin-resistant *Staphylococcus aureus*. Mean values are from four replications run in sextet.

Table 11: ANOVA for leaf extract zones of inhibition

Sources of variation	DF	SS	MS	F
Block	3	9.266	3.089	1.043
Extract (EX)	2	15.370	7.685	2.594
Microorganism (MI)	5	207.153	41.431	13.985***
Concentration (CO)	3	183.016	61.005	20.592***
EX×CO	6	7.160	1.193	0.403
MI×CO	15	4.128	0.275	0.093
MI×EX	10	82.129	8.213	2.772**
MI×EX×CO	30	17.454	0.582	0.196
Error	345	1022.094	2.963	

**, ***Denote significance at the 0.01 and 0.001 level, respectively

($p<0.05$) from the ACE and ETOH extracts which exhibited mean ZOIs of 9.2563 mm and 9.4169 mm. Likewise, the ETOH against *S. aureus* had a mean ZOI of 9.9338 mm and it was not significantly different ($p<0.05$) from the mean ZOIs of ACE and PE, which were 8.0188 mm and 7.2644 mm (Table 10 and Figure 7). Analysis of variance of the mean ZOIs for these extract-microorganism interactions indicate a significant difference of $p<0.01$ (Table 11).

3.3.4 Inhibitory Activity of Stem Extracts for Each Microorganism

As stated previous, *V. volkensii* stem PE extracts were only tested against *S. aureus*, because the lack of crude stem PE extract (Table 1). All extracts (ACE, ETOH, PE) for *V. volkensii* stem exhibited a significant difference ($p<0.05$) of ZOI means from DMSO and APC when tested against the six bacterial strains (Table 12). Acetone and ethanol extracts tested against *E. coli* B strain, *S. enterica* Subsp. *enteritidis*, *S. flexneri*, *E. faecalis* and methicillin-resistant *S. aureus* exhibited mean ZOIs that were not significantly different ($p<0.05$) from each other for each respective microorganism. The mean ZOIs for ACE extracts were 8.5138, 9.3025, 9.6350, 10.2438, and 9.4938 mm for *E. coli* B strain, *S. enterica* Subsp. *enteritidis*, *S. flexneri*, *E.*

Table 12: Comparison of stem extract mean zones of inhibition for each microorganism

	Mean zone of inhibition (mm)					
	Gram Negative Bacteria			Gram Positive Bacteria		
Extracts	EC	SE	SF	EF	SA	MRSA
DMSO	6.0000±0.00[a]	6.0000±0.00[a]	6.0000±0.00[a]	6.0000±0.00[a]	6.0000±0.00[a]	6.0000±0.00[a]
ACE	8.5138±0.73[b]	9.3025±0.39[b]	9.6350±0.86[b]	10.2438±0.47[b]	7.5525±0.46[b]	9.4938±0.52[b]
ETOH	8.8850±0.54[b]	9.4531±0.83[b]	9.3444±0.52[b]	9.5725±0.43[b]	9.5075±1.08[c]	9.8613±0.71[b]
PE	*	*	*	*	7.5475±047[b]	*
APC	17.7250±0.87[c]	20.5275±0.19[c]	15.5450±0.53[c]	16.4713±0.69[c]	17.8858±0.26[d]	17.6763±0.32[c]

*Not observed, due to lack of production of extract
In a column, means with the same letter are not significantly different at the 0.05 level by Duncan's Multiple Range Test. ±: standard error of means (SEM); ACE: acetone; ETOH: 9:1 ethanol/deionized water deionized water; PE: petroleum ether; DMSO: dimethyl sulfoxide (negative control); APC: Vancomycin (antibiotic positive control); EC: *Escherichia coli* B strain; SE: *Salmonella enterica* Subsp. *enteritidis*; SF: *Shigella flexneri*; EF: *Enterococcus faecalis*; SA: *Staphylococcus aureus*; MRSA: methicillin-resistant *Staphylococcus aureus*.

faecalis and methicillin-resistant *S. aureus*, respectively. Whereas, the observed ZOI means of ETOH for *E. coli* B strain, *S. enterica* Subsp. *enteritidis*, *S. flexneri*, *E. faecalis* and methicillin-resistant *S. aureus* were 8.8850, 9.4531, 9.3444, 9.5725 and 9.8613 mm, respectively. Against *S. aureus*, ETOH extract exhibited a mean ZOI of 9.5075 mm and it was significantly different ($p<0.05$) from the mean ZOIs of ACE and PE, which were 7.5525 mm and 7.5475 mm, respectively (Table 12 and Figure 8). Analysis of variance of the mean ZOIs for these extract-microorganism interactions indicate a significant difference of $p<0.001$ (Table 13).

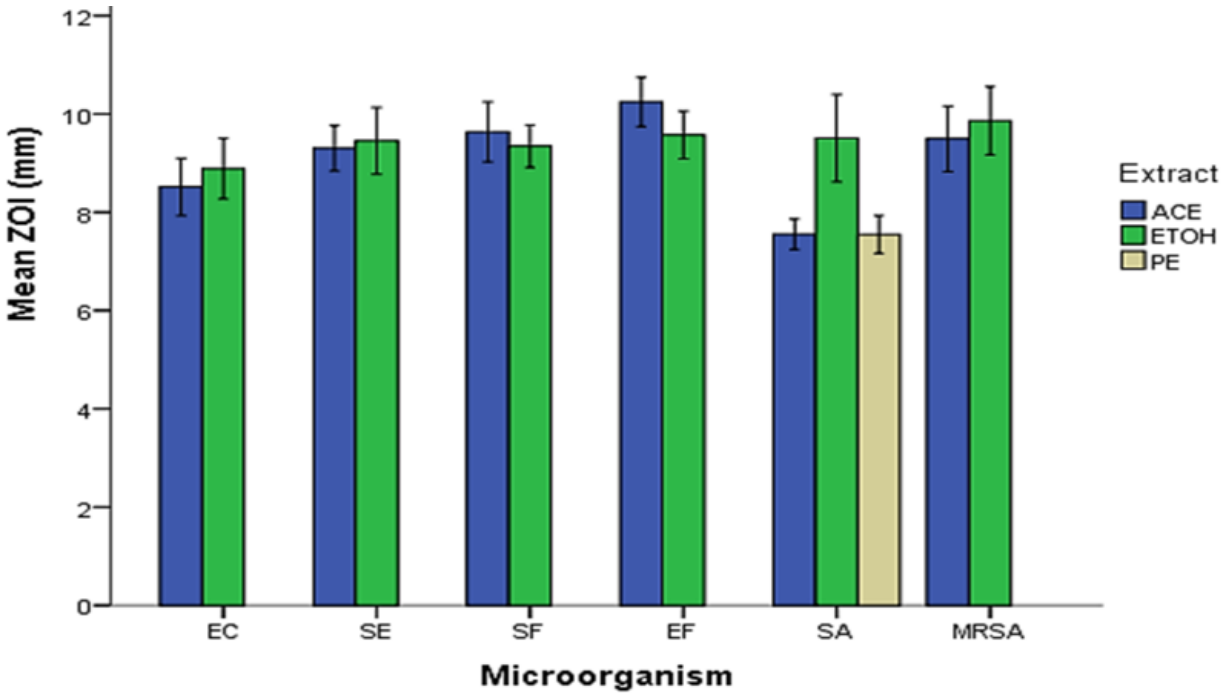

Figure 8: *V. volkensii* stem microorganism-extract combinations with their 95% confidence intervals.
Note: Negative and positive control not depicted due to significantly different ZOI means compared to extracts. ACE: acetone; ETOH: 9:1 ethanol/deionized water; PE: petroleum ether; EC: *Escherichia coli* B strain; SE: *Salmonella enterica* Subsp. *enteritidis*; SF: *Shigella flexneri*; EF: *Enterococcus faecalis*; SA: *Staphylococcus aureus*; MRSA: methicillin-resistant *Staphylococcus aureus*. PE extracts against all microorganism, except SA, are not depicted due to lack of crude PE extract. Mean values are from four replications run in sextet.

Analyses of variance for all extracts (bark, fruit, leaf, stem) indicated a significant difference ($p<0.001$) among microorganisms (Tables 7, 9, 11, and 13). This was expected due to choosing gram-positive and gram-negative bacteria, and different microorganism possess different mechanisms for resistance against antibiotic (Jacoby and Archer, 1991). In addition, the ANOVA for all extracts (bark, fruit, leaf, and stem) showed a significant difference ($p<0.001$) among concentrations. This was also expected because of using a high range of concentrations (5, 15, 25, 50 mg/ml) from one another.

Table 13: ANOVA for stem extract zones of inhibition

Sources of variation	DF	SS	MS	F
Block	3	2.633	0.878	1.559
Extract (EX)	2	15.022	7.511	13.345***
Microorganism (MI)	5	40.610	8.122	14.431***
Concentration (CO)	3	97.686	32.562	57.854***
EX×CO	6	2.527	0.421	0.748
MI×CO	15	5.314	0.354	0.629
MI×EX	5	32.496	6.499	11.547***
MI×EX×CO	15	8.732	0.582	1.034
Error	245	137.893	0.563	

**, ***Denote significance at the 0.01 and 0.001 level, respectively

Additionally, the ANOVA results for the fruit and stem extracts showed that the mean ZOIs among extracts (ACE, ETOH, PE) were significantly different ($p<0.001$) from one another for the *V. volkensii* fruit and stem extracts (Tables 9 and 13). Whereas a significant difference ($p<0.05$) of mean ZOIs was not indicated among extracts (ACE, ETOH, PE) of *V. volkensii* bark and leaf extracts (Tables 7 and 11). The ACE extract for *V. volkensii* fruit had a mean ZOI of

10.9346 mm that was significantly ($p<0.05$) higher than the mean ZOIs of ETOH and PE extracts which exhibited mean ZOIs of 9.6425 mm and 9.3613 mm, respectively (Figure A1). The ACE and ETOH extracts of *V. volkensii* stems exhibited mean ZOIs of 9.1217 mm and 9.4354 mm, respectively, which were significantly ($p<0.05$) higher than the mean ZOI of 7.5450 mm for the PE extract (Figure A2).

Overall, all extracts (bark, fruit, leaf and stem) had mean ZOIs that were significantly ($p<0.05$) higher than DMSO (negative control) and significantly ($p<0.05$) lower than Vancomycin (antibiotic positive control). *V. volkensii* fruit extracts prepared from ACE showed the most antimicrobial activity, measured by mean zones of inhibition, against five of the six bacteria tested; *E. coli* B strain (10.0700 mm), *S. enterica* Subsp. *enteritidis* (11.8838 mm), *S. flexneri* (10.5138 mm), *S. aureus* (10.3519 mm) and methicillin-resistant *S. aureus* (12.0263 mm), and against *E. faecalis*, leaf extract prepared from ETOH exhibited the most activity (11.0706 mm). Methicillin-resistant *S. aureus* had the highest zone of inhibition (12.0263 mm) among all extracts and microorganisms tested (Tables 6, 8, 10, 12). These results support the notion that acetone or alcohol are the solvents of choice for extracting antibacterial compounds from Rubiaceae (Addo-Mensah et al. 2015; Eloff, 1998; Karou et al. 2011). This is primarily because acetone, and alcohol to an extent, has the capability of extracting both polar and nonpolar components, thus, a greater number of components will be extracted (Eloff, 1998; Mahlo et al. 2010).

3.4 Antimicrobial Compounds and Resistance

Microorganisms develop antimicrobial resistance via natural resistance, genetic mutations, and/or horizontal gene transfer (Okwori et al., 2008). The mechanisms by which resistance is accomplished via altering the antibiotic action includes; modifying the antimicrobial

target to alter its chemical composition or destroying it, decreasing the permeability of the antimicrobial, activating efflux pumps, and modifying target sites (Jacoby and Archer, 1991; Munita and Arias, 2016).

Plant-derived antimicrobial compounds can have a variety of action mechanisms depending on the plant, the class compounds and the cellular properties of the target microorganism. Phytochemical screening of *Vangueria* species have shown the presence of several active antimicrobial compounds. The bark, fruit, leaves, and stems of *Canthium multiflorum*, *Morinda lucida*, *V. infausta* and *V. madagascarensis* were reported to contain an abundance of flavonoids and tannins, and few amounts of alkaloids and cardiac glycosides (Chatterjee et al. 2011; Mahomoodally and Dilmohamed, 2016; Ramalingum and Mahomoodally, 2014). The presence of flavonoids and tannins may have contributed to *S. enterica*, *S. flexneri*, *E. faecalis*, *S. aureus* and methicillin-resistant *S. aureus* being sensitive to the *V. volkensii* fruit and leaf extracts seen in the present study (Tables 3-4, 8 and 10). Flavonoids are known to form irreversible complexes with bacterial membranes and extracellular and soluble proteins, resulting in the inhibition of cytoplasmic membrane function, nucleic acid synthesis and energy metabolism (Savoia, 2012). Whereas, tannins cause the inactivation of bacterial adhesions, membrane-bound proteins, and enzymes via hydrogen bonding or act as an iron-chelating agent, thereby causing their inactivation (Savoia, 2012). Previous studies report that *V. volkensii* is most often used to treat and manage venereal diseases (Jeruto et al. 2015; Pascaline et al. 2010). Even though the disc diffusion assay was not performed on common aetiologic agents of venereal diseases such as *Chlamydia trachomatis*, *Neisseria gonorrhea* and *Treponema pallidum*, the bacteria that were tested (i.e. *E. coli*, *S. flexneri*, *E. faecalis*, *S. aureus*) are representative of pathogenic organisms associated with venereal diseases (Okoli and Iroegbu,

2004). Thus, the antimicrobial activity exhibited by the extracts against these bacteria, to some extent, justifies the reported use of *V. volkensii* to treat venereal diseases (Tables 2-5, 6, 8, 10, and 12).

Staphylococcus species have been reported as the most commonly emerging resistant bacterial species and infections are very difficult to treat due to their great pathogenic potential (Mthethwa et al., 2014). In the present study, *S. aureus* was shown to be resistant to the ACE and PE extracts of *V. volkensii* bark, leaves, and stems (Tables 2, 4-5). The resistance mechanism of *S. aureus* against *V. volkensii* is unknown. It is possible that *S. aureus* targeted the *V. volkensii* antimicrobial compounds to alter the chemical composition or destroying it, or by enzyme modification, further research needs to be allocated to determine the exact mechanism.

This study confirmed that *V. volkensii* bark, fruit, leaf and stems, do in fact possess antimicrobial properties, however, the extent as to which are present, and quantity is unknown. Therefore, phytochemical analysis should be conducted on *V. volkensii*. Additionally, since each extract tested contain a repertoire of compounds (i.e., alkaloids, flavonoids, tannins, etc.), antibacterial activity observed could be due to synergy of these compounds. Future studies should be carried out to determine the synergist activity of the antimicrobial compounds. Since the ANOVAs showed that the mean ZOI of all extracts was significant ($p<0.05$), then studies can be conducted to test the antimicrobial sensitivity of a combination of extracts.

CONCLUSION

In conclusion, the disc diffusion assay revealed potential antimicrobial capabilities by *V. volkensii*. The negative control showed no inhibition, which indicated that the inhibition observed in the assays was attributed to the antibiotic positive control and the active components of the extracts. In general, all extracts exhibited broad spectrum activity against both gram-positive and gram-negative bacteria. Most microorganisms were sensitive to all extracts, except for *E. coli* B strain and *S. aureus*, which were resistant to some extracts. More specifically, *E. coli* B strain was resistant to bark ETOH and stem ACE extracts, while *S. aureus* was resistant to bark, leaf and stem ACE and PE extracts, and the fruit ETOH extract.

The ACE extract for *V. volkensii* fruit mean ZOI was significantly ($p<0.05$) higher than the mean ZOIs of ETOH and PE extracts, and the mean ZOIs of ACE and ETOH extracts of *V. volkensii* stems were significantly ($p<0.05$) higher than the mean ZOI of the PE extract. The comparison of extract means for each microorganism revealed fruit extracts prepared from ACE had the most inhibitory activity against all microorganisms, except *E. faecalis*, which the leaf extracts prepared from ETOH exhibited the most inhibitory activity.

The fruit ACE extracts and the leaf ETOH extract may be the most promising extracts to explore for potential use as an antimicrobial drug. Therefore, future studies should be allocated for isolating the active antimicrobial compounds of *V. volkensii* fruits and leaves.

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APPENDIX

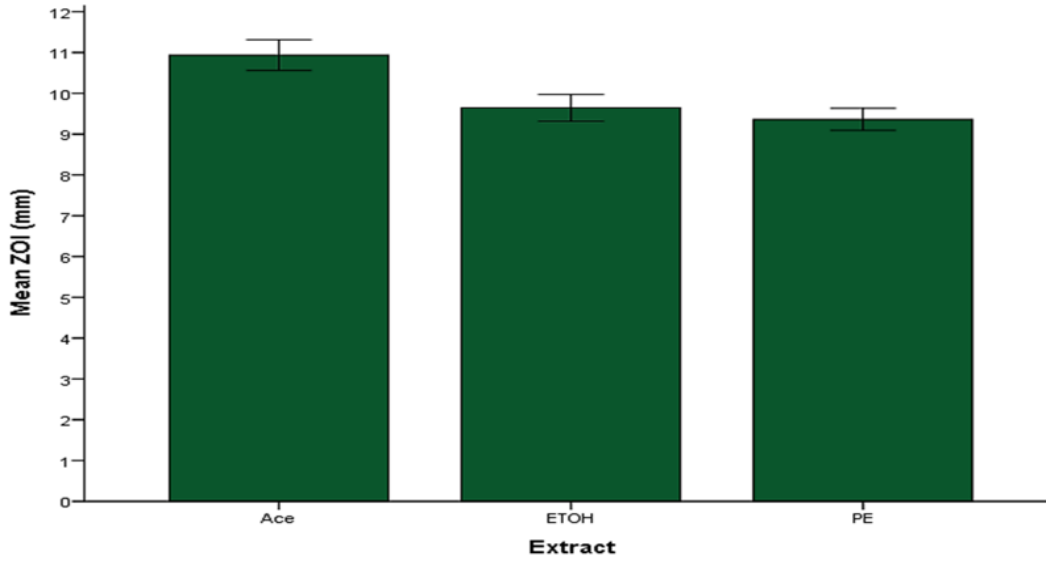

Figure A1: *V. volkensii* fruit extract ZOI means with their 95% confidence intervals.
Note: Negative and positive control not depicted due to significantly different ZOI means compared to extracts. ACE: acetone; ETOH: 9:1 ethanol/deionized water; PE: petroleum ether. Mean values are from four replications run in sextet.

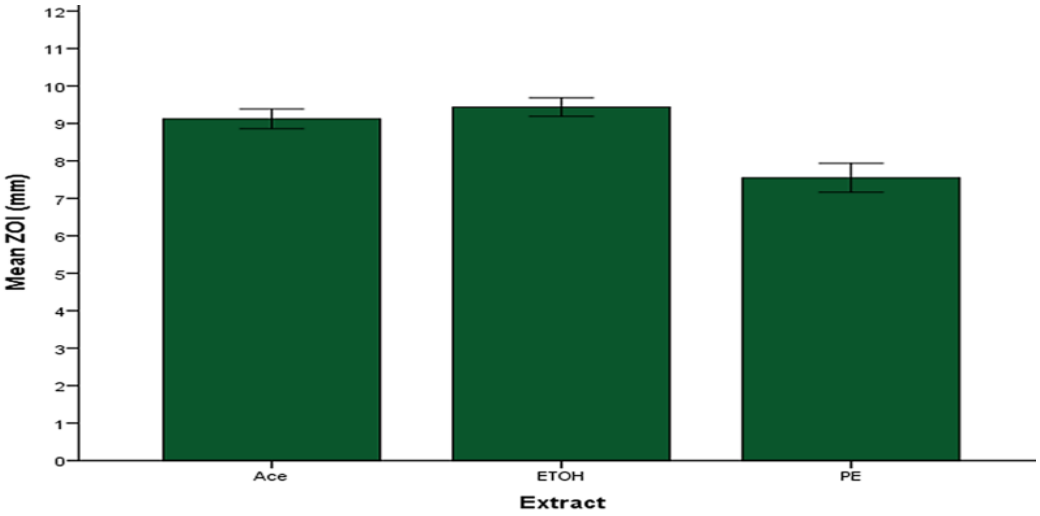

Figure A2: *V. volkensii* stem extract ZOI means with their 95% confidence intervals.
Note: Negative and positive control not depicted due to significantly different ZOI means compared to extracts. ACE: acetone; ETOH: 9:1 ethanol/deionized water; PE: petroleum ether. Mean values are from four replications run in sextet.

VITA

Name:	Douglas Parnell Holland
Address:	7614 Laguna Del Mar Ct., Apt. 623, Laredo, TX 78041
Email Address:	douglasholland@dusty.tamiu.edu
Education:	A.A., Western Texas College, 2010 B.S., Biology-Biochemistry, University of Arkansas at Monticello, 2015 Major Field of Specialization: Biology