PHYTOCHEMICAL ANALYSIS AND EVALUATION FOR ANTIMICROBIAL ACTIVITIES OF THE EXTRACTS OF *Psidium guajava* L. AND *Calpurnia aurea* (Ait.) Benth, AGAINST SELECTED HUMAN BACTERIAL PATHOGENS

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Phytochemical Analysis and Evaluation for Antimicrobial Activities of the Extracts of Guava (*Psidium guajava* L.) and Calpurnia (*Calpurnia aurea)* (Ait.) Benth, against Selected Human Bacterial Pathogens

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I hereby certify that I have read and evaluated this Thesis entitled *Phytochemical Analysis* and *Evaluation for Antimicrobial Activities of the Extracts of Psidium guajava L. and Calpurnia aurea against Selected Human Bacterial Pathogens* prepared under my guidance by *Solomon berhanu*. I recommend that it be submitted as fulfilling the thesis requirement.

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I dedicate this thesis to my family members for their love and care in the success of my life and memorable and valuable encouragements in my academic career while I was acting this study.

STATEMENT OF THE AUTHOR

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ACRONYMS AND ABBREVIATIONS

AAS	Atomic Absorption Spectroscopy
AMR	Antimicrobial Resistance
ANOVA	Analysis of Variance
ATCC	American Type Culture Collection
CDC	Center for Disease Control
CLSI	Clinical Laboratory Standard Institution
CFU	Colony Forming Unity
DMSO	Dimethyl sulfoxide
EBI	Ethiopian Biodiversity Institute
EPHI	Ethiopian Public Health Institute
FQs	Fluoroquinolones
GAE	Gallic Acid Equivalent
GAS	Group A Staphylococcus
LSD	Least Significant Difference
MDR	Multiple Drug Resistance
MHA	Mueller Hinton Agar
MIC	Minimum Inhibitory Concentration
MRSA	Methicillin- Resistant *Staphylococcus aureus*
NA	Nutrient Agar
NLSI	National Laboratory Standards Institute
TAE	Tannic Acid Equivalent
TMP	Traditional Medicinal Plants
TPC	Total Phenolic Content
WHO	World Health Organization
ZOI	Zone of Inhibition

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ABSTRACT

Plants are rich in a wide diversity of secondary metabolites which have been found to exhibit antimicrobial, antioxidant and antitumor activities. Guava (Psidium guajava L., Myrtaceae) and (Calpurnia aurea L. Fabaceae) are known for their traditional medicinal values. These studies were conducted with the objective of analyzing the secondary compounds profile of the two plant species and evaluate their antimicrobial properties on selected human bacterial pathogenes. Extraction was done by maceration using ethanol solvent. Qualitative analysis of phytochemicals was carried out using standard protocols and phytochemicals were quantified from leaf and bark extracts or powders using Spectrophotometric or gravimetric method. Antibacterial activities of leaf and bark extracts were determined by disc diffusion and broth dilution methods. Results of qualitative analysis showed that both plants have tannins, steroids, saponins, terpenoids and alkaloids in their leaves and barks. Whereas flavonoids and phlobatannins were absent in both plants' leaves and barks extracts. There was significant concentration difference between the different types of compounds analyzed from leaves and barks of both plants. Saponins were found to be higher in both P. guajava and C. aurea leaves followed by total terpenoids, alkaloids, total phenols and tannin. In barks of both plants, however, total terpenoids was highest followed by saponins, alkaloids, total phenols and tannins. Each group of compound also varied significantly ($P<0.05$) on different parts of both species. Both plants' bark extracts had significantly higher crude phenolic contents than that of leaf extracts, and both plants' leaf extracts had significantly higher crude tannin contents than that of bark extracts. Whereas both plants' bark extracts had significantly higher saponin content than both plants' leaf extracts. Leaf extract had generally higher contents of quantified phytochemical than bark extracts. Comparison between extracts and control (ethanol with no inhibitory effect (0mm ZOI) showed that all extract types significantly inhibited the growth of all bacterial isolates. However, the isolates showed sensitivity difference to extracts. When compared with commercial antibiotic however, all extracts performed less in inhibiting bacterial growth. Generally the MIC required to inhibit growth ranged from 25-100mg/ml and the MIC required to suppress growth of the isolates varied with bacterial species and extract type. The leaf and bark extracts of each plant also showed different MIC to suppress growth of the tested bacteria. In both plants, leaf extracts showed more antibacterial activities against all tested isolates than bark extracts. Overall, this study showed that both Psidium guajava and Calpurnia aurea have potential compounds that can help manage the growth of these clinical isolate bacterial pathogens and isolation and identification of specific potent compound(s) is required in the future.

Keywords: *Calpurnia aurea*; Disc diffusion; *E. coli* O157:H7, *Psidium guajava*; *Salmonella typhi*

1. INTRODUCTION

Plants are rich in a wide diversity of secondary metabolites which have been found to exhibit antimicrobial, antioxidant, anti-infectious and antitumor activities. The affordability, reliability, availability and low toxicity of bio-chemicals in therapeutic use has made them widespread for application (Akroum *et al*., 2009). A large number of pathogens have developed resistance to multiple antibiotics, because of the mutagenic nature of bacterial DNA, the rapid multiplication of bacterial cells, constant transformation of bacterial cells and therefore be untreatable. Thus, countless studies have been directed on medicinal plants for antimicrobial activities and efficacy (Stace, 1997; Chitia *et al*., 2014).

Medicinal plants are source of important substances for the study of their traditional use through the verification of pharmacological effects and can be a natural composite source that acts as new anti-infectious agents. A number of medicinal plants have been screened for antimicrobial activities in recent years and efforts have been made to identify their active constituents. In spite of recent development in the synthetic drug, chemistry and production of antibiotics, plants still occupy an important role in the modern and traditional systems all over the world (Lin *at el.,* 2002).

Plant secondary compounds are organic molecules that are found in different parts of the plants. They act as protective and disease fighting compounds, so they are required by humans to prevent or fight diseases (Begum *et al.,* 2010). The major plant secondary compounds include terpens/terpenoids, phenolics and alkaloids (Taiz and Zeiger, 2006; Rockwood, 2006). They are products of secondary metabolism and are known in plant defense mechanisms. Drugs derived from natural products are usually secondary metabolites and their derivatives. Phytochemical analysis of different plants have revealed numerous bioactive compounds including alkaloids, tannins, flavonoids, glycosides and saponins. These plant secondary metabolites serve as defense mechanisms against many microorganisms, insects and herbivores (Compean and Ynalvez, 2014).

Research show that most plants of folk medicine are rich in secondary compounds, though the type and amount vary with plant families, species and parts of plants (Compean and Ynalvez,

2014). Variation in secondary compounds may also exist within a species mainly due to plant genotypes, developmental stages and geographical locations, among others (Penuelas and Llusia, 2001). There is a continuous and urgent need to discover new antimicrobial compounds with diverse chemical structures and novel mechanisms of action because there has been an alarming increase in the incidence of new and re-emerging infectious diseases. In recent years, drug resistance to human pathogenic bacteria has been commonly reported from all over the world. In the present scenario, the emergence of multiple drug resistance in human pathogenic organisms has necessitated a search for new antimicrobial substances from other sources including plants1 (Adekunle and Adekunle, 2009; Bhatia *et al*., 2012).

Enteric bacteria such as *Salmonella*, *Shigella*, *Campylobacter* species, *Vibrio cholerae* and diarrheogenic *Escherichia coli* are common causes of diarrhea in under-five children (WHO, 2010; Omulo *et al*., 2015). Development of antibiotic resistance by these enteric bacterial pathogens against easily accessible and commonly prescribed drugs has become a major concern throughout the world, particularly in developing countries of East Africa including Ethiopia (Omulo *et al*., 2015). *Staphylococcus aureus* is also a pathogen of greater concern because of its ability to cause a diverse array of life-threatening infections and its capacity to adapt fast to the different environmental conditions (Bachir and Abouni, 2015). These features have made infections of *S. aureus* increasingly difficult to treat because of the fast rate at which it develops resistance to common antimicrobial agents (Onanuga and Temedie, 2011).

Furthermore, *Enterococcus* has traditionally regarded as low grade pathogens, but has emerged as an increasingly important cause of nosocomial infections in the last decade. *Enterococcus faecalis* is one of the *Enterococcus responsible* for human nosocomial infections. The most common nosocomial infections produced by this organism are urinary tract infections, followed by intra-abdominal and pelvic infections (Marsha at el.,2010) .A major reason why this organism survives in hospital environment is the intrinsic resistance to several commonly used antibiotics and perhaps more importantly, their ability to acquire resistance to all currently available antibiotics by receipt of foreign genetic material through the transfer of plasmids and transposons. The emergence of vancomycin-resistant *Enterococci* (VRE) is a cause of concern; as once established, it is very difficult to control (Sood *et al.*, 2008).

Plants are the most naturally effective and cheapest sources of drugs (Prince and Prabakaran, 2011). So, the increasing incidence of pathogenic microorganisms becoming resistant to antibiotics continuously has led to the search for newer, more effective, affordable and readily available sources from local medicinal plants or herbs (Adekunle and Adekunle, 2009). Guava is such a phytotherapeutic plant used in folk medicine. It is believed to have active components that help to treat and manage various infectious diseases. Many parts of this plant have been used in traditional medicine to manage conditions like malaria, gastroenteritis, vomiting, diarrhea, dysentery, wounds, ulcers, toothache, coughs, sore throat, inflamed gums, and a number of other conditions (Biswas *et al*., 2013). Similarly, *Calpurnia aurea* is used for the treatment of amoebic dysentery and diarrhea in animals, killing head lice in humans and ticks in animals, syphilis, diarrhea, leishmaniasis, tapeworm, trachoma, Tinea capitis, wound, scabies, elephantiasis and different swellings (Asres *et al*., 1986). Though there are some previous reports on the medicinal properties of both *P. guajava* and *C. aurea* further study on their phytochemistry and in vitro evaluation of their antimicrobial properties on some selected medically important bacterial pathogenes is necessary. This study is, therefore, conducted with the following objectives.

General Objective:

- To analyze the major secondary compound groups of *Psidium guajava* and *Calpurnia aurea* and to evaluate the antimicrobial activities of their crude extracts against selected human bacterial pathogens.

Specific objectives of the study were to:

- Qualitatively detect the presence of major groups of secondary compounds from leaves and barks of *Psidium guajava* and *Calpurnia aurea* collected from different parts of East Hararge.
- Quantify and compare contents of the major detected secondary compounds from leaves and barks of *Psidium guajava* and *Calpurnia aurea*.
- Determine the antibacterial activities and minimum inhibitory concentration of extracts of both plants on *Escherichia coli O157:H7, Salmonella typhi, Staphylococcus aureus* and *Streptococcus pyogenes.*

2. LITERATURE REIVIEW

2.1. Medicinal plants

Plants are rich in a wide diversity of secondary metabolites which have been found to exhibit antimicrobial, antioxidant, anti-infectious and antitumor activities. In Africa, the use of traditional medicine has persisted over the years and the last few decades have witnessed an upsurge of interest in traditional medicine and other alternative forms of healthcare in the developing and developed countries (Duru *et al.,* 2006). The affordability, reliability, availability and low toxicity of bio-chemicals in therapeutic use has made them widespread for implementation in medical health care sector (Akroum *et al*., 2009). Herbal medicines are obtained from seeds, berries, roots, leaves, barks and flowers of plants for their therapeutic or medicinal value (Seekers, 2009).

Medicinal plants are source of important substance for the study of their traditional use through the verification of pharmacological effects and can be natural composite sources that act as new anti-infectious agents. A number of medicinal plants have been screened for antimicrobial activity in recent years and efforts have been done to identify their active constituents. In spite of recent development in the synthetic drug, chemistry and production of antibiotics, plants still occupy an important role in the modern and traditional systems in all over the world. Due to the indiscriminate use of antibacterial drugs, the microorganisms have developed resistance to many commercial antibiotics (Lin *at el.,* 2002).

An increased number of pathogens have developed resistance to multiple antibiotics (Multiple Drug Resistance), because of the mutagenic nature of bacterial DNA, the rapid multiplication of bacterial cells, constant transformation of bacterial cells and therefore be untreatable. Therefore, investigation of chemical compounds within medicinal plants has become desirable, and countless studies have been directed on medicinal plants for antimicrobial activities and efficacy (Stace, 1997). Many efforts have been made to discover new antimicrobial compounds from various sources such as microorganisms, animals and medicinal plants. Systematic investigation of folk medicine may result in the discovery of novel effective compounds. Therefore, several

medicinal plants have been evaluated for possible antimicrobial activity and get remedy from variety of antimicrobial origin (Shanmuga *et al.,* 2002).

2.1.1. *Psidum guajava*

Psidium guajava, Myrtaceae is known for its nutritional value. It originated in the tropical South America and widely grown in many other areas such India, Bangladesh, Thailand, Brazil, California, Florida, and West Indies. Different parts of it (root, bark, leaves and fruits) are reported for use in folk medicine, and possess many pharmacological properties (Begum, 2002; Mittal *et al*., 2010). Evidences show that the leaves and bark of *P. guajava* possess a long history of medicinal uses (Nwinyi *et al*., 2008; Vyas *et al*., 2010).

The aqueous extract of guava leaves, for example, has been reported to be efficacious in the treatment of various types of gastrointestinal (GIT) disturbances such as diarrhea, inhibition of the peristaltic reflex and gastroenteritis (Lutterodt, 1998). Moreover, the whole part of the plant is used as skin tonic and is used for the treatment of disorders such as dysmenorrheal, miscarriages, uterine bleeding and premature labor. Recent studies on the pharmacological properties of the bark, fruit and leaves depict the plant has antibacterial, hypoglycemic, anti-inflammatory, antipyretic, spasmolytic and central nervous system depressant properties (Begum, 2002). In general, the pharmaceutical studies conducted on *Psidium guajava* indicated the immense potential of this plant in the treatment of conditions such as diarrhea, gastroenteritis and rotavirus enteritis, wounds, acne, dental plaque, malaria, allergies, coughs, diabetes, cardiovascular disorder, degenerative muscular diseases, inflammatory aliments including rheumatism and menstrual pain, liver diseases and cancer (Gutierrez *et al*,.2008).

2.1.2. *Calpurnia aurea*

The *Calpurnia aurea* (Ait.) Benth. (Fabaceae) is a yellow-flowered small tree or shrub (Natal Laburnum) widely distributed in Africa from Cape Province to Eritrea. *Calpurnia aurea* is used for the treatment of amoebic dysentery and diarrhea in animals, killing head lice in humans and ticks in animals, treatment of syphilis, diarrhea, leishmaniasis, tapeworm, trachoma, *Tinea capitis*, wound, scabies, elephantiasis and different swellings occurs in Southern India (Asres *et al*., 1986; Mohammed *et al.,* 2005).

2.2. Plant Secondary Metabolites

Plant secondary compounds are complex chemicals made by plants that are not essential to the life of the plant. These compounds are organic compounds that are variously distributed within the plant kingdom or found only in restricted lineages (Koul, 2008). Research show that most plants of traditional medicine are rich in secondary compounds, though the type and amount vary with plant species. Plants produce a high diversity of natural products or secondary metabolites with a prominent function in the protection against predators and microbial pathogens on the basis of their toxic nature and repellence to herbivores and microbes. Some of secondary metabolites are involved in defense against a biotic stress (e.g. exposure to UV and ß radiation) and important for the communication of the plants with other organisms (Schafer, 2009).

Secondary metabolites are being the subject of many research studies because these compounds exhibit many biological activities. These include anti-microbial, anti-fungal, anti-cancer and anti-inflammatory activities. Drugs derived from natural products are usually secondary metabolites and their derivatives. Phytochemical analysis of different plants has revealed numerous bioactive compounds including alkaloids, tannins, flavonoids, glycosides and saponins. These plant secondary metabolites serve as defense mechanisms against many microorganisms, insects and herbivores (Compean and Ynalvez, 2014).

2.2.1. Alkaloids

The term alkaloids (alkali – like) was commonly used to designed basic heterocyclic nitrogenous compounds of plant origin that are physiological active. They are class of naturally occurring organic nitrogen-containing bases. Alkaloids have diverse and important physiological effects on humans and other animals. Well-known alkaloids include morphine, strychnine, quinine, ephedrine, and nicotine. Alkaloids are bases and usually form colorless crystalline solids with a bitter taste. They have a wide range of effects and are used as medicines and poisons. For example, morphine, cordine and berberine were found potentially to be active against trypanosomes and plasmodia. Alkaloids are also reported to have micro-biocidal and ant diarrheal effects due to their effect on transit time of small intestine and their ability to intercalate with microbial deoxyribonucleic acid (Garba and Okeniyi, 2012). Abukakar *et al.* (2008) reported the highest concentration of alkaloid (4.32%) from the aqueous pulp extracts of

Tamarindusindica and confirmed their antibacterial activity of against *Escherichia coli, Staphylococcus aureus* and *Pseudomonas aeruginosa*. Besides, Agbafor *et al*. (2011) found abundant alkaloids in extracts of Zapotecaportoricens were leaf and the extracts inhibited the growth of *Escherichia coli, Staphylococcus aureus, Streptococcus pyogenes, Klebsiella pneumoniae and Pseudomonas aeruginosa*. Furthermore, Garba and Okeniyi. (2012) reported high antimicrobial activities of the total alkaloids extracted from *Carica papaya* L. *Calotropis procera* (Ait.) *Mangifera indica* L .and *Psidium guajava and Calpurnia aurea* against *Staphylococcus aureus, Lactobacillus* spp. and *Candida albicans* at concentration of 0.6 mg/ml.

2.2.2. Terpenoids

The terpenoids, sometimes called isoprenoids, are a large and diverse class of naturally occurring organic chemicals derived from five-carbon isoprene units assembled and modified in thousands of ways. Most are multicyclic structures that differ from one another not only in functional groups but also in their basic carbon skeletons. These compounds can be found in all living things, and are the largest group of natural products. About 60% of known natural products are terpenoids (Valarmathy *at el*., 2010).

Terpenoids, which constitute the most abundant and structurally diverse group of plant secondary metabolites, play an important role in plant-insect, plant-pathogen, and plant-plant interactions. Monoterpenes and sesquiterpenes are the majority of volatile compounds released from plants after herbivore damage, attracting arthropods that prey on or parasitize herbivores, then avoiding further damage (Cheng *et al*., 2007). Plant terpenoids are used extensively for their aromatic qualities. They play a role in traditional herbal remedies and are under investigation for anti-bacterial, anti-neoplastic, and other pharmaceutical functions. Plants do not only accumulate terpenes for herbivore defense, but also emit volatile blends in response to herbivory and many other biotic and a biotic stresses (Yadav *et al*., 2014).

Studies conducted on different plant secondary metabolites extraction showed that terpenes was found in the extracts at different concentration level and that the extracts had antimicrobial properties. Munyendo *et al.* (2011) reported the existence of terpenoids in abundant numbers in leaf and root extracts of *Ocimum gratissimum* L and the extracts had antibacterial activities against methicillin-resistant *Staphylococcus aureus* (MRSA) and *Pseudomonas aeruginosa*.

Besides, Abdul amid *et al*. (2014) found some high concentration of terpenoids in bark ethanolic extract of *Psidium guajava*, *Calpurnia aurea* and the extracts exhibited anti-*Streptococcus faecalis,* anti-*Staphylococcus aureus,* anti-*Bacillus subtilis,* anti-*salmonella spp.* and anti-*Escherichia coli* activity. Sibi *et al*. (2012) also reported a high concentration of terpenoids in the ethanolic leaf extract of *Morinda citifolia* L. and that this extract was found to have anti-*Pseudomonas aeruginosa* and anti-*Staphylococcus epidermidis activities*.

2.2.3. Phenolics

Phenols, sometimes called phenolics, are a class of chemical compounds consisting of a hydroxyl(—OH) group bonded directly to an aromatic hydrocarbon group. The simplest of the class is phenol, which is also called carbolic acid C6H5OH. Phenolic compounds are classified as simple phenols or polyphenols based on the number of phenol units in the molecule (Khoddami *et al, 2013).*

Phenolics are one of the most ubiquitous groups of secondary metabolites found throughout the plant kingdom. They encompass very large and diverse group of aromatic compounds characterized by a benzene ring and one or more hydroxyl groups. Several internal and external factors, including trauma, wounding, drought and pathogen attack, affect the synthesis and accumulation of phenolics. Furthermore, the biosynthesis of phenolic in chloroplasts and their accumulation in vacuoles are enhanced on exposure to light (Bhattacharya *et al*., 2010). Phenolics serve a dual function of both repelling and attracting different organisms in the plant's surroundings. For example, simple phenolic acids, complex tannins and phenolic resins on the plant surface deter birds by interacting with the gut micro flora and diminishing their digestive ability. The scent and pigmentation conferred by low molecular weight phenyl propanol derivatives attract symbiotic microbes, pollinators and animals that disperse fruits (Bhattacharya *et al*., 2010).

Flavonoids are a type of phenolics, and many flavonoids have been known to have antioxidant, anti-inflammatory and antitumor activity. Agbafor *et al*. 2011 reported the presence in aqueous extract of *Zapotecaportoricens* (Jacq.) leaf and demonstrated that these secondary metabolites could be taking part in the anti-*Pseudomonas aeruginosa* activity. Likewise, Sibi *et al*. (2012) found that ethanolic leaf and root extracts of *Morinda citifolia* L. had a high concentration of

flavonoids and the ethanolic root extract exhibited antimicrobial activity against *P. aeruginosa and Staphylococcus epidermidis*. Tannins are polymeric phenolic substances found in nearly all plant parts. Mariita *et al*. (2011) reported that methanolic extract of *Scandoxus multiflorus* (Martyn) had high concentrations of tannins and exhibited antibacterial activity against *Mycobacterium fortuitum, Staphylococcus aureus and Salmonella* Typhi. In addition, they found methanolic extract of *Acacia nilotica* (L.) Delile exhibited anti *S. aureus* and ant-*Pseudomonas aeruginosa* activities.

2.3. Antibacterial Activities of Guava and Calpurnia Extracts

P. guajava and *C. aurea* are phytotherapic plant used in folk medicine. They are believed to have active components that help to treat and manage various infectious diseases. Many parts of these plants have been used in traditional medicine to manage conditions like malaria, gastroenteritis, vomiting, diarrhea, dysentery, wounds, ulcers, toothache, coughs, sore throat, inflamed gums, killing head lice in humans, ticks in animals, syphilis, tape worm, trachoma, elephantiasis, Tinea capitis and a number of other conditions (Biswas *et al*., 2013 ; Tadeg *et al*.,2005)

Bansode and Chavan (2014) and Fadipe *et al*, (2013) reported the antibacterial activity of guava and Calpurnia leaf extracts against nine enteric pathogens tested *Escherichia coli*, *Salmonella* Typhi, *Salmonella paratyphi A, Salmonella paratyphi B, Shigella sonnies, Shigella dysentarie, Enterobactor spp. Citrobactor spp. and Klebsiella spp.* Likewise, Taura *et al*. (2014); Havagiray *et al*. (2004) and Saravanan *et al.* (2010)reported antibacterial activity of guava and Calpurnia leaf extracts against *Klebsiella pneumonia*, *Escherichia coli*, *Salmonella spp*., *Pseudomonas aeruginosa, Streptococcus Pyogenes s*and *Staphylococcus aureus* clinical isolates. Esimone *et al*. (2012) studied antibacterial activity of the water and methanolic extracts of guava stem bark against eight methicillin resistant *Staphylococcus aureus* (MRSA) isolates.

2.4. Antibiotic resistance

For several decades antimicrobial resistance (AMR) has been a growing threat to the effective treatment of an ever-increasing range of infections caused by bacteria, parasites, viruses and fungi. Antimicrobial resistance results in reduced efficacy of anti-bacterial, anti-parasitic, anti-viral and anti-fungal drugs, making the treatment of patients difficult, costly, or even impossible.

The development of AMR is a natural phenomenon in microorganisms and is accelerated by the selective pressure exerted by use and misuse of antimicrobial agents in humans and animals. The current lack of new antimicrobials on the horizon to replace those that become ineffective brings added urgency to the need to protect the efficacy of existing drugs (WHO, 2014).

Drug resistance is a large and growing problem in infections that account for most of Africa's disease burden, including malaria, tuberculosis (TB), HIV infection, and respiratory and diarrheal diseases. Many bacterial and parasitic diseases could, until recently, be treated with inexpensive antimicrobial agents, but treatment has been made more expensive and less successful by the emergence and spread of resistant organisms (Arrow, 2004).

Bacteria can be intrinsically resistant to certain antibiotics but can also acquire resistance to antibiotics via mutations in chromosomal genes and by horizontal gene transfer. Several factors contribute to resistance by pathogens causing gastroenteritis in the setting of a developing country like Ethiopia. These include frequent overuse, misuse and factors related to the potency and quality of antimicrobials and the distribution of resistant strains (Sharma *et al*., 2005).

Retrospective studies conducted in Ethiopia show that the prevalence of *Salmonella* (5.3% to 15.4%) and *Shigella* (5% to7.5%) was high with antibiotic resistance pattern ranging from 0% in case of ciprofloxacin and nalidixic acid to 100% in case of Ampicillin (Getachew *et al*., 2014). According to study done in Jimma health center from total of 260 diarrheal sample, 129 (49.6%) were positive for intestinal parasite, *Shigella* and *Salmonella* species. *Shigella* species showed 100 % resistances to Ampicillin, Amoxicillin, and Cotrimoxazole. All *Salmonella* isolates were resistant against Amoxicillin. All *Shigella* and *Salmonella* species were susceptible to Ceftriaxone, Ciprofloxacin and Gentamycin (Getenet *et al*., 2014). Another study done in Bahir Dar town, Ethiopia showed that from the total 422 stool samples, 33 (7.8%) showed positive results for *Salmonella* species. From the 33 Salmonella isolates, 29 (87.9%) were *Salmonella* enteric a sub-species arizonae and 4 (12.1%) were *Salmonella* group A. *Salmonella* isolates were highly resistant to Ampicillin (93.9%) followed by Augment in (75.8%) and Trimethoprim/ Sulfamethoxazole (48.5%). However, the isolates showed high susceptibility to Ciprofloxacin and Norfloxacin (93.9% each) followed by Gentamycin (87.9%). Likewise, the *Salmonella* isolates showed 90.9% of multidrug-resistance (Yemane *et al*., 2014).

3. MATERIALS AND METHODS

3.1. Description of the study Area

The leaves and barks of *Psidium guajava* and *Calpurnia aurea* plant samples were collected from Karsa woreda and Gandamudde Village, Haramaya District, which is found near Haramaya University. The experimental study was conducted in Haramaya University, Department of Biology Laboratories. Phytochemical analysis and extraction was carried out in General Laboratory while antimicrobial activity tests were carried out in Microbiology Laboratory, Haramaya University is found at about 510 km East of Addis Ababa, and latitude of 09.0^0N and longitude of 42.0^0 E with an altitude of 1950 meters above sea level. The study site is situated in sem-arid tropical belt of Eastern Ethiopia and is characterized as sub humid type of climate with an average rain fall of 810.7mm. The mean annual maximum temperature was 24.7^oc and monthly values range from 23.1^oC - 25.7^oc and the mean annual minimum temperature was 11.4^oc and monthly values range between 4.6^oC and 14.4^oC Ethiopian National Meteorological Agency, (Wogayehu, 2005).

3.2. Plant Material and Extract Preparation

Fresh leaves and barks of *Psidium guajava* (Zaytuna) and *Calpurnia aurea* (Digitta) were collected from Karsa and Haramaya Districts. And was authenticated at the Botany Laboratory Herbarium of Haramaya University. Samples were washed under tap water and air-dried under laboratory condition and the powders were stored in refrigerator until use for extraction for qualitative and quantitative determination and antimicrobial bioassay. Extraction was made by macerating 80 gm of the powder in 400 ml of 97% ethanol in 500 ml in Erlenmeyer flasks. The solvent-powder mixture was shaken on a rotary shaker for 4 days being wrapped with aluminum foil to avoid evaporation at room temperature. Some of the obtained extracts were concentrated by heating on a hot plate at about 30 to 40ºC for 30 min and were consequently used for qualitative analysis. The rest were evaporated to dryness at room temperature for seven days and preserved at 4ºC until used for qualitative and quantitative analysis, as well as for anti-bacterial activity tests (Taura *et al*., 2014; Shah and Yadav, 2015).

3.3. Analysis of Chemicals from Psidium guajava and Calpurnia aurea

3.3.1. Qualitative Analysis of Major Secondary Metabolites

Qualitative analysis of tannins, phlobatannins, steroids, terpenoids, saponins, flavonoids and alkaloids of the *P. guajava* and *C. aurea* was carried out using the concentrated and solidified ethanolic extract or the powdered specimen using standard procedures as described below.

Test for tannins: 1 gram of each powdered sample was separately added into 20 ml of distilled water in test tubes. Then, the mixtures were boiled in water bath for 7 minutes and were filtered while hot using filter paper into Erlenmeyer flasks. After cooling, 1 ml of the filtrate was diluted to 5 ml solution using distilled water and then a few drops (2-3) of 10% ferric chloride were added to it. Formation of bluish-black or brownish-green precipitate indicated the presence of tannins (Ajayi *et al*., 2011).

Test for phlobatannins: Solidified extracts of 0.5 g was placed into separate test tubes and mixed with 20 ml of distilled water. The mixtures were boiled in water bath for 10 min. After cooling, the mixture was separately filtered through a Whatman No 1 filter paper. Thereafter, 2 ml of 1% aqueous hydrochloric acid was added to each mixture and shaken to develop red precipitate that indicates the presence of phlobatannins (Shaik *et al*., 2011).

Test for steroids (Lieberman-Bur chard Test): 2 ml chloroform and 10 drops of acetic acid were placed in a test tube. Then, 0.5 ml of concentrated ethanolic extract was added to the test tube and mixed with the solvent. Next, 1.5 ml of concentrated sulphuric acid was added from the side of the test tube. The change of red color through blue to green indicated the presence of steroids (Gayathri and Kiruba, 2014).

Test for terpenoids (Salkowski test): 5 ml of each concentrated ethanolic extract was mixed with 2 ml of chloroform in separate test tubes, and then 2 ml of concentrated sulfuric acid was added carefully and shaken gently to form a layer. A reddish brown coloration of the inter-phase confirmed positive results for the presence of terpenoids (Biswas *et al*., 2013).

Test for saponins: 1 gram of each powdered sample was placed into separate test tubes and mixed with 10 ml of distilled water. Then, the mixtures were boiled in a water bath for 10 min

and were filtered, while hot in to 50 ml Erlenmeyer flask. After cooling, the following tests were carried out according to Ajayi *et al*. (2011).

Foam test: The filtrate 2.5 ml was added to a test tube and diluted to 10 ml with distilled water. It was then shaken vigorously for 2-3 minutes. Formation of foam or froth confirmed the presence of saponins in the filtrate.

Emulsion test: 2 drops of olive oil were added to the frothing and the mixture was shaken vigorously for 1-3 minutes. Formation of a fairly stable emulsion indicated the presence of saponins.

Test for flavonoids: 2 ml of each of the concentrated ethanolic extract was added into different test tubes. Then, 4 drops of 10% NaOH solution were added and heated in water bath for 10 min. The intensity of yellow color which became colorless on addition of 10 drops of 1% hydrochloric acid showed the presence of flavonoids (Adachukwu *et al*., 2013).

Test for alkaloids: 1.5 ml of 1% HCl was added to 4.5 ml of each concentrated ethanolic extract in different test tubes. Each mixture was heated for 2 min in a water bath while stirring heated and shaked continuously. It was then cooled and filtered. The resulting filtrate was tested with Dragendorffs reagent for the presence of alkaloids as described by Adachukwu *et al*. 2013. 1ml of the filtrate was added to 0.4 ml of Dragendorffs reagent. Formation of cream yellow precipitate indicated the presence of alkaloids.

3.3.2. Quantitative Determination of Major Secondary Metabolites

The stored powder and solidified extracts of the *Psidium guajava* and *Calpurnia aurea* were used for standard quantitative estimation of the major secondary metabolites. All experimental analysis was done in triplicates.

Determination of total phenolic content: Spectrophotometric method was used to quantify total phenol content in the *Psidium guajava* and *Calpurnia aurea* leaf and bark extracts as described by Cavalcanti de Amorim *et al*. (2012). Briefly, stock solution of tannic acid (0.1 mg/ml, w/v) was prepared by dissolving 10 mg of tannic acid in 100 ml of 80% ethanol. Then, 0.10, 0.20, 0.30, 0.40 and 0.50 ml volumes of stock solution were pipetted and transferred into separate pint

flasks. Then 500µl of 10% Folin-ciocalteu solution was added to each of the pint flasks and mixed homogeneously with the resulting solution for 10 seconds. Then, they were allowed to stand for 5 minutes. Thereafter, 1 ml of 7.5% sodium carbonate was mixed homogeneously with the resulting solution for 30 seconds. Next, the final volume was adjusted to 10 ml with distilled water in order to obtain the final standard tannic acid concentration of 1, 2, 3, 4 and 5 µg/ml. These standard reaction mixtures were allowed to stand for 30 minutes after which their absorptions were measured at 760 nm using distilled water as a blank. Calibration curve was constructed from resulting data.

Stock solution of extracts (1 mg/ml, w/v) was prepared by dissolving 10 mg of the solidified extract in 10 ml of 80% ethanol. Then, 500 µl stock of the extract was transferred to test tube. Next, 500 µl of the Folin-Ciocalteu solution and 1 ml of 7.5% sodium carbonate solution were added in the test tube. The final volume was adjusted to 10 ml by adding 8 ml of distilled water. The sample solutions were kept at room temperature for 30 minutes and their absorptions were measured at 760 nm using distilled water as a blank. Total phenolic content (TPC) was calculated as Tannic acid equivalent (TAE) by using the formula described by Mohamed *et al*. (2011).

TPC = C*V/M

Where TPC is the total phenolic content in mg/g of the extracts as Tannic Acid Equivalent (TAE), C is the concentration of tannic acid established from the calibration curve in mg/ml, V is the volume of the extract solution in ml and M is the weight of the extract used in grams.

Determination of tannin content using the Van Burden and Robison (1981) method Powder (0.5 gm) was shaken with 10 ml of 2M HCL in a test tube for 5 minutes. The contents were then transferred into 100 ml capacity Erlenmeyer flasks and made up to 50 ml and then filtered. Take to 5 ml of the filtrate in a test tube, 3 ml of 0.1 M $FeCl_3$ in 0.1 M and 3 ml of 0.008 M potassium Ferro cyanide were added within 10 minutes of addition, the absorbance was read at 720 nm. In this experiment, tannic acid was used to prepare a standard calibration curve for quantification.

Determination of alkaloid content using the Harborne (1973) method 3 gram of the powder was weighed and added into a 50 ml capacity Erlenmeyer flask. Then, 20 ml of 10% acetic acid in ethanol was added into the flask which was soon covered with cotton and the solution was allowed to stand for 4 hrs. Next, the solution was filtered by Whatman No 1 filter paper and

concentrated ammonium hydroxide was added drop wise to the filtrate until the formation of precipitate was stopped. The whole solution was then allowed to settle the precipitate. Then, precipitate was collected, washed with dilute ammonium hydroxide and then filtered. The obtained residue was dried and weighed. Alkaloid content was calculated as mg per grams of the sample powder used.

Determination of saponins content: Saponins content was determined by Obadoni and Ochuko (2001). 15 ml of 20% aqueous ethanol was added into a conical flask containing 3 gram of the extract. It was then heated over a hot water bath for 4 hr. with continuous stirring at about 55°C. After filtering the mixture, the residue was re-extracted with another 30 ml of 20% ethanol. The resulting filtrates were combined and reduced to 10 ml over water bath at about 90°C. Then, it was transferred into a 250 ml capacity separator funnel and 5 ml of diethyl ether was then added and the resulting mixture was shaken vigorously. The bottom aqueous layer was then recovered. The top diethyl ether layer was washed using small quantities of water twice and aqueous layer was drained accordingly and combined. Thereafter, 15 ml n-butanol was added to the combined aqueous layer solution and the obtaining solution was shaken vigorously. The solution was washed twice with 2.5 ml of 5% aqueous sodium chloride followed by discarding bottom aqueous layer while top n-butanol layer was transferred to pre-weighed Petri-plate and heated in a water bath for evaporation. Then, samples were dried in the oven at 60°C to constant weight and they were then measured. The Saponins content was calculated as mg per grams of the sample extract used.

Determination of terpenoids content: 2 grams of powder soaked in 50 ml of 97% ethanol for 24 hr. The extracts were filtered using Whatman No.1 filter paper. The filtrate was added into separating funnel followed by addition 50 ml of petroleum ether. The resulting mixture was shaken and allowed to stay for 5 minutes for layer formation. Then, the bottom layer was drained and discarded while top petroleum ether was collected and concentrated to dryness using rotary evaporator at 40°C stay for 18 hrs. The mass of dried ether extract, considered as crude terpenoids, was measured and its content was calculated as mg per grams of the sample powder used (Ferguson, 1956).

3.4. Antibacterial Assay

3.4.1. Preparation of different concentrations of the crude extract

The stock solution (200 mg/ml) was prepared by reconstituting 4 grams of each of the dried extracts in 20 ml of ethanol. Different concentrations (25 mg/ml, 50 mg/ml, 75 mg/ml, 100 mg/ml and 125 mg/ml) of each of the extracts were prepared from their respective stocks. For preparing the 25 mg/ml, 50 mg/ml, 75 mg/ml, 100 mg/ml and 125 mg/ml) concentrations, 1.25 ml, 2.5 ml, 3.75 ml, 5 ml and 6.25 ml of the different stock solutions of the extracts were transferred respectively to separate 10 ml volumetric flasks. The flasks were filled up to 10 ml mark with ethanol (Alabi *et al*., 2012; Dougahri 2008).

3.4.2. Collection of Human Bacterial Pathogens

For anti-microbial bioassay, selected Human Bacterial Pathogens of clinical isolates were obtained from Ethiopian Public Health Institute (EPHI) and Ethiopian Biodiversity Institute (EBI) Addis Ababa, Ethiopia. The isolates included two gram-negative pathogens namely *Escherichia coli* O157:H7 (EBI) and *Salmonella Typhi* and two gram-positive namely *Staphylococcus aureus* and *Streptococcus pyogenes*.

3.4.3. Culture media and Inoculum preparation

Each of the human bacterial pathogens obtained from EPHI and EBDI was cultured on separate nutrient agar plate and incubated for 24 hr. at 37°C to obtain colonies. Two to three colonies formed on the plate were picked up with a sterile inoculating loop and transferred into a test tube containing sterile normal saline and vortexed thoroughly. This was repeated until the turbidity of each bacterial suspension matched the turbidity of 0.5 McFarland Standards as described by the Clinical Laboratory Standards Institute (CLSI, 2012). The resulting suspension was then used as inoculum for the test pathogen used in the antibacterial susceptibility test.

For anti-microbial bioassay, Whatman No.1 filter paper discs of 6 mm diameter were punched out with the aid of paper punch and were placed in Petri plate. They were then sterilized by autoclaving at 121°C for 15 min. After that, the discs were cooled and impregnated with 0.01 ml of the prepared test solutions of each extract and ethanol (Taura *et al*., 2014).

Inoculation of Mueller Hinton Agar (MHA) plates: Within 15 minutes after adjusting the turbidity of the suspension of inoculum, a sterile cotton swab was dipped into the adjusted suspension and rotated several times by pressing firmly on the inside wall of the tube above the fluid level. This removed excess fluid from the swab. Then, the dried surface of Mueller-Hinton Agar plate was inoculated by streaking using the swab three times over the entire surface and rotating the MHA plates approximately 60° each time to ensure an even distribution of the inoculum. Then, the MHA plates were left open for three to five minutes to allow for any excess surface moisture to be absorbed (CLSI, 2012).

The impregnated discs were then placed onto the surface of the inoculated agar plate using sterile forceps. Each disc was pressed down to ensure complete contact with the agar surface. The discs were distributed evenly so they were no closer than 24 mm from center to center (CLSI, 2012). Commercial ciprofloxacin discs (5 μg) were used as positive control and the pure solvent (ethanol) impregnated discs were used as negative control.

The MHA plates were then closed with Parafilm and incubated at 37°C for 24 hrs. After incubation, the diameters of the zone of inhibition around each disc were measured to the nearest millimeter along two axes (i.e. 90° to each other) by using transparent ruler and the means of the two readings we rerecorded. For each selected human bacterial isolate, the experiment was carried out in parallel and with three replications (Thompson *et al*., 2011; Biswas *et al*., 2013).

3.4.4. Determination of Minimum Inhibitory Concentration

The Minimum Inhibitory Concentration (MIC) of each extract was determined by using increased concentrations of the extracts. Therefore, 2 ml of nutrient broth was added into each of the five test tubes and 0.1 ml of the prepared concentration of each extract was mixed with the nutrient broth. Thereafter, standardized inoculum of 0.1 ml of the test pathogen was dispensed into the test tube containing the suspension of nutrient broth and the extract. Then, all test tubes were properly corked and incubated at 37°C for 24hrs. After which, they were observed for absence or presence of visible growth. The lowest concentration without visible growth of organisms was recorded as the MIC. The experiment was carried out for each organism in duplicates (Taura *et al*., 2012; Alabi *et al,* .2012).

3.5. Method of Data Analysis

Statistical Package for Social Sciences, Version 16.0 (SPSS; Chicago, IL, USA), was used to analyze the data. Antimicrobial activities of extracts and quantity of secondary compounds were analyzed using One-way Analysis of Variance (ANOVA) and one sample T- test. The mean values were considered statistically significant at $p< 0.05$.

4. RESULTS AND DISCUSSION

4.1. Qualitative Analysis of Phytochemicals of *P. guajava and C. aurea*

In the current study a qualitative phytochemical analysis from leaves and barks of *Psidium guajava* and *Calpurnia aurea* revealed that of the major secondary compound groups tested, saponins, steroids, alkaloids, tannins and terpenoids were found both in leaf and barks of both plant. However, flavonoids and phlobatannins were absent in both plants (Table 1). Previously, different researchers have analyzed phytochemical profiles of *P. guajava* and *C. aurea* leaf ethanol extracts. For example, similar to the current study, Taura *et al*. (2014) found alkaloids, saponins and tannins in ethanol leaf extract of *P. guajava*, but did not get flavonoids. Biswas *et al*. (2013) reported the presence of tannin, flavonoids and terpenoids, but absence of saponins in ethanolic leaf extract of *P. guajava*. Umer *et al.* (2013) and Letha *et al,* 2009 reported the presence of alkaloids, tannins, flavonoids and saponins, but absence of terpenoids and steroids in 80% methanol extract of *C. aurea* leaves. Gayathri and Kiruba (2014) recognized the occurrence of terpenoids and total phenolics, while at the same time the absence of alkaloids; flavonoids and saponins in ethanolic extract of *P. guajava leaves.* Sofowora, 1984 reported the presence of tannin, alkaloids, flavonoids and saponins, but absence of steroids in ethanol extracts of *C. aurea leaves.* And chemical investigations of *Calpurnia aurea* have resulted in the isolation of a series of alkaloids, phenolic compounds, flavonoids, flavonols, which also found in the genus *Calpurnia* (Adedapo *et al*., 2008); but have not got saponins, steroids, terpenoids and tannin which are presented in this study.

Some researchers also reported the presence of some secondary compounds from ethanolic extracts of *P. guajava and C .aurea.* Okunrobo *et al*. (2012) found alkaloids, flavonoids, tannins and saponins from stem bark ethanol extracts of *P. guajava*. The presence of alkaloids, flavonoids, phenols, saponins, steroids and tannin in *P. guajava* and *C. aurea* bark were reported by Osuagwu *et al* (2007), Iniaghe *et al.* (2009) and Omoyeni and Aluko (2010). Abdulhamid *et al*. (2014) also detected alkaloids, flavonoids, saponins, tannins, steroids and terpenoids from stem bark extract of *P. guajava* macerated in (90%) ethanol. Aziz *et al*. (2014) detected flavonoids, tannins, saponins and total phenol in extract prepared using soxhlet apparatus, but they could not detect alkaloids in the extract *P. guajava* bark. Tijjani *et al*. (2014) also found

tannins, flavonoids; terpenoids, saponins, cardiac glycosides, but not alkaloid in extracts prepared using soxhlet apparatus from *P. guajava* bark.

No standardized extraction protocol has been developed for preparation of herbal extracts, but 20-95% of ethanol-water mixture is frequently used by the herbal medicine industry to prepare ethanolic extracts (Ganora, 2008). Therefore, ethanol is widely used to obtain crude extracts of phytochemical from plant materials in the herbal medicine industry for therapeutic applications. Due to the variation in composition of active compounds, a given plant may require different concentrations of ethanol to achieve maximum recovery of bioactive components.

Comparison of the recent study with some that have been previously conducted show that phytochemical composition of the extracts from the same species and plant part under the same solvent varies in one or more compounds, suggesting that extract composition may vary depending on many factors such as extraction methods, or percent and volumes of the solvent used, or temperature and time period used for extraction, or concentration or drying of extracts, as well as variety (cultivar) used, plants' age, genotype, physiological and environmental conditions (Wendakoon *et al*., 2012; Obaineh and Shadrach, 2013).

Though most plants of folk medicine are rich in secondary compounds, the type and amount vary with plant families, species and parts of plants (Compean and Ynalvez, 2014; (Bishnu *et al*, 2009). Even variation in secondary compounds may also exist within species mainly due to plant genotypes, developmental stages and geographical locations (Penuelas and Llusia, 2001). Apart from a biotic factor, biotic factors also affect plant chemistry. For example, biotic influences such as the presence or absence of natural enemies, competitors or mutualists often change plant secondary metabolites profiles and net production by inducing defensive responses which could occur locally at the site of attack or infection, or can occur systemically at other sites (Moore *et al*., 2014).

Table 1: Major secondary compounds report of leaf and bark ethanolic extracts of *P. guajava* and *C. aurea*

Plant parts	Phytochemicals						
	Tannin	Phlobatannins	Steroid	Saponin	Flavonoids	Alkaloid	Terpenoids
P. guajava leaf	+	-	+	+	-	+	+
P. guajava bark	+	-	+	+	-	+	+
C. aurea leaf	+	-	+	+	-	+	+
C. aurea bark	+	-	+	+	-	+	+

P= Psidium, C= Calpurnia (+) indicates the presence of the phytochemical while (−) indicates the absence of the phytochemical.

4.2. Quantitative Analysis of Phytochemical in *P. guajava* and *C. aurea*

The results of the quantitative analysis of phytochemicals are presented in Table 2 below. There was significant difference between the different types of compounds analyzed from leaves and barks of both plants. Saponins were found to be higher in both *P. guajava* and *C. aurea* leaves followed by total terpenoids, alkaloids, total phenols and tannin (Table2). In barks of both plants, however, total terpenoids was highest followed by saponins, alkaloids, total phenols and tannins. Each group of compound also varied significantly ($P<0.05$) between different parts of both species (Table 2).

Both plants' bark extracts had significantly higher crude phenolic contents than that of leaf extracts, and both plants' leaf extracts had significantly higher crude tannin contents than that of bark extracts ($P<0.05$). Whereas both plants' bark extracts had significantly higher saponin content than both plants' leaf extracts ($P<0.05$). Leaf extract had generally higher contents of

quantified phytochemical than bark extracts. This may be due to the difference among plant parts in their roles in physiology and survival of the plant in different region and season, which influence the accumulation of secondary metabolites. In support of this result, Agati *et al*. (2013) reported that plant leaves regulate the antioxidant system by synthesizing phenolic compounds to act as absorbers of surplus radiation in the epidermal layers.

Outcomes of the current study have been compared with previous studies conducted under similar standard method for quantitative determination of phytochemical of macerated leaf and barks of both plants. For example Table 2, Obaineh and Shadrach (2013) found significantly lower contents of crude alkaloids, saponins, phenols, tannins in both 97% ethanol leaf and bark extracts of *P. guajava.* Offor (2015) also reported significantly lower contents of crude tannin, alkaloid, saponin and phenol in leaf extract of *P. guajava.* Similarly, Zahidah *et al*. (2013) reported significantly lower crude phenol content in aqueous leaf extract of *P. guajava*. Ibe *et al*. (2014) reported nearly the same contents of crude alkaloid in methanolic extracts of stem bark of *P. guajava*. However, they found significantly higher contents of crude saponins, phenol and tannins.

Despite the existence of genetic control, gene expression, and genotypes, the amount of secondary metabolites present in a given plant may be influenced by biological and environmental factors as well as biochemical, physiological, ecological, and evolutionary processes. Specifically, factors that most affect the occurrence and content of plant secondary metabolites are seasonality, circadian rhythm, plant development, phenology, temperature, altitude, water availability, UV radiation, nutrients, pollution, mechanical stimuli, and attacks by herbivores or pathogens (Chua *et al*., 2015).

Genes and biosynthetic pathways are also underlying causes of plant secondary metabolites variation. Most plant secondary metabolites originate from a small group of precursor compounds, which eventually become modified into diverse end-products in well regulated metabolic pathways, but also subjected to mutation. The most important mechanism in diversifying secondary metabolites is whole genome and local gene duplication (Moore *et al*., 2014).

Table 2: The contents of major phytochemicals (mg/g of crude extract or powder) in ethanolic extracts of leaves and barks of *P. guajava* and *C. aurea.*

Phytochemicals	**Guava leaf**	**Guava bark**	**Calpurnia leaf**	**Calpurnia bark**
Crude alkaloid	30 ± 5.7^{Ab}	63 ± 24^{Aab}	46 ± 13^{Abc}	17 ± 6.6^{Aab}
Crude Saponin	217 ± 44^{Aa}	120 ± 40^{Aab}	360 ± 149^{Aac}	140 ± 40^{Aac}
Crude Terpenoids	66 ± 120^{Ab}	167 ± 84^{Aab}	66 ± 120^{Abc}	170 ± 60^{Bbc}
Crude Phenol	2.7 ± 4.9^{Ab}	3 ± 0.2^{Aac}	2.7 ± 0.63^{Abc}	3.8 ± 0.13^{Aab}
Crude tannin	0.96 ± 0.09^{Ab}	0.44 ± 0.09^{Bac}	0.74 ± 0.07^{Abc}	0.27 ± 0.06^{Bab}

The values are Mean ± Standard error of mean (n=3). Capital letter superscript compares between means in row, and means with similar capital letters represent no significant difference, whereas means with different capital letters are significantly different at $P<0.05$. Small letter superscript compares between means in column, and means with similar small letters show no significant difference, whereas means with different small letters show significant difference at $P<0.05$.

4.3. Test for Antibacterial Activities

The antimicrobial activities of ethanolic extracts (200 mg/ml) of *Psidium guajava* and *Calpurnia aurea* on four different human bacterial pathogens are shown in Table 3. Comparison between extracts and control (ethanol with no inhibitory effect [0mm ZOI]) showed that all extract types significantly inhibited the growth of all bacterial isolates ($P<0.05$). However, the isolates showed sensitivity difference to extracts. For example, guava leaf extract showed the highest zone of inhibitor against *Staphylococcus aureus* (10.33 mm) while calpurnia leaf extract showed the largest zone of inhibition against *Escherichia coli* O157:H7 (9 mm) and the least antibacterial activity against *Streptococcus pyogenes* (7.66 mm). Similarly, guava and calpurnia bark extracts had the highest inhibitory activities against *Staphylococcus aureus, Salmonella Typhi* (10 mm and 8.33 mm) while on the other hand they had the least inhibitory activities against *Streptococcus pyogenes and Staphylococcus aureus* (7.66 mm and 7.16 mm), respectively. No significant difference was observed between all extract types in inhibiting *E.coli O157:H7,* and *Streptococcus pyogenes* ($P<0.05$).

However, Calpurnia leaf extract had least inhibitory effect against *Salmonella Typhi* compared to leaf and bark extracts of guava. Similarly, Calpurnia leaf and bark extracts performed less than Guava leaf and bark extracts to inhibit the growth of *Staphylococcus aureus.* When compared with commercial antibiotic, however, all extracts performed less in inhibiting bacterial growth. Generally, the MIC required inhibiting growth ranged from 25-100mg/ml and the MIC required suppressing growth of the isolates varied with bacterial species and extract type. The leaf and bark extracts of each plant also showed different MIC to suppress growth of the tested bacteria. Broth assay showed that in both plants, leaf extracts showed more antibacterial activities against all tested isolates than bark plant extracts.

Previous studies also showed the antibacterial activities of leaf and bark extracts of guava and Calpurnia. For example, using disc diffusion method, Zahidah *et al.* (2013) reported nearly the same inhibition zone of 100 mg/ml of aqueous leaf extract against *Staphylococcus aureus* present study result. Taura *et al*. (2014) also used disc diffusion method and reported nearly similar inhibition zone of 480 µg/disc solution of dimethyl sulfoxide (DMSO) of dried percolated ethanolic leaf extract against *S. aureus,* but significantly smaller against *Salmonella*

Typhi in present study result. Besides, they used broth dilution method and found lower minimum inhibitory concentration (MIC) of 0.250 mg/ml of the extract solution against *Salmonella Typhi* and MIC of >1mg/ml against *Staphylococcus aureus*. Sanches *et al*. (2005) also used broth dilution method and found significantly smaller MIC of ethanol extracts of leaf and stem bark against *Salmonella aureus*. Besides, Choudhury *et al*. (2012) reported significantly smaller inhibition zone of 2 mg/ml of methanol leaf extract against *Salmonella typhi*, but no inhibition zone against *Staphylococcus aureus* using well diffusion method. They also found lower MIC (2 mg/ml) of the extract solution against *Salmonella Typhi* and MIC of >4 mg/ml against *S. aureus* using broth dilution method. In addition, Esimone *et al*. (2012) found lower range MIC of methanol stem bark extracts against Methicillin Resistant *Staphylococcus aureus* (MRSA) human and animal isolates using agar well diffusion method.

Comparative study of antibacterial activities of *P. guajava* L. leaf and bark extracts against gram-positives and gram-negatives were previously conducted. Some those studies were reviewed and most of their results showed that gram-positives were more susceptible than gram-negatives opposing the results of present study. In line with this, Sanches *et al*. (2005) found that ethanolic leaf and stem bark extracts were more effective against gram-positives than gram-negatives. Abdulhamid *et al*. (2014) also reported that ethanolic leaf extract had more effective antibacterial activity against gram-positives than gram-negatives.

Furthermore, Biswas *et al*. (2013) found that ethanolic leaf extract had inhibitory activity against gram-positives only and Zahidah *et al.* (2013) also reported that the aqueous leaf extract revealed antimicrobial activity against gram-positives only and no antimicrobial against gram-negatives. On the other hand, Goncalves *et al*. (2008) reported that methanol leaf extract had more effective antimicrobial activities against gram-negatives than gram-positives supporting present study.

Presence and quantity of alkaloids, saponins, tannins and terpenoids both in leaf and bark extracts of guava and calpurnia have medical implications and therefore they may be responsible for the observed antibacterial activities in both plant leaf and bark extracts. Tannin stimulates phagocytic cells, host-mediated tumor activities, and other anti-infective actions (Haslam, 1996). It were found to have antibacterial activity (Mariita *et al*., 2011). It forms complex with proteins through hydrogen bonding, covalent bond formation and hydrophobic interactions (Haslam, 1996; Stern *et al*., 1996). Thus, its antimicrobial action is related to its ability to inactivate

microbial adhesions, enzymes and cell envelope transport proteins (Upadhyay *et al*., 2014). Alkaloid was also reported to have microbicidal and anti-diarrheal effect due to their effect on transit time in the small intestine and their ability to intercalate with microbial deoxyribonucleic acid (Garb and Okeniyi, 2012).

Table 3. The antimicrobial activities of the ethanolic extracts of the leaves and barks of both *P. guajava* and *C. aurea.*

	Zone of Inhibition (in mm) on clinical isolates			
Anti microbial agents	***E.coli* O157:H7**	***Salmonella typhi***	***Staphylococcus aureus***	***Streptococcus pyogenes***
Guava leaf extract	9.2 ± 0.7^{Ba}	8 ± 1^{Bb}	10.3 ± 0.6^{Ba}	7.7 ± 0.6^{Bb}
Guava bark extract	7.8 ± 1.3^{Bb}	8 ± 1^{Bb}	10 ± 1^{Ba}	7.7 ± 1^{Bb}
Calpurnia leaf extract	9 ± 1^{Ba}	6.7 ± 0.3^{Cb}	7.5 ± 1.3^{Ca}	8.4 ± 1.2^{Ba}
Calpurnia bark extract	8 ± 1^{Ba}	8.4 ± 1.2^{Ba}	7.2 ± 0.8^{Ca}	8 ± 1^{Ba}
Ciprofloxacin	18.4 ± 0.5^{Ab}	16.7 ± 2.5^{Ab}	14.7 ± 2.5^{Ab}	27 ± 2.6^{Aa}

The values are shown as Mean ± Standard deviations (n=4). Capital letter superscript compares between means in column, and means with similar capital letters represent no significant difference, whereas means with different capital letters are significantly different at $P<0.05$. Small letter superscripts compare between means in row, and means with similar small letters show no significant difference, whereas means with different small letters show significant difference at $P<0.05$.

Table 4. Minimum Inhibitory Concentrations (MIC) of leaf and bark ethanolic extracts of *P. guajava* and *C. aurea*

	MIC (mg/ml) Values for clinical isolates			
Bacterial strain	***G. leaf extract***	***G. bark extract***	***C. leaf extract***	***C. bark extract***
Escherichia coli O157:H7	25mg/ml	50mg/ml	50 mg/ml	37.5 mg/ml
Salmonella Typhi	100 mg/ml	62.5mg/ml	50 mg/ml	25 mg/ml
Staphylococcus aureus	100 mg/ml	75 mg/ml	25mg/ml	62.5 mg/ml
Streptococcus pyogenes	62.5 mg/ml	37.5 mg/ml	25mg/ml	25 mg/ml

Note: G = Guava, C = Calpurnia, MIC = Minimum inhibitory concentration.

5. SUMMARY, CONCLUSION AND RECOMMENDATION

5.1. Summary and conclusion

Plants are rich in a wide diversity of secondary metabolites which have been found to exhibit antimicrobial, antioxidant, anti-infectious and antitumor activities. The affordability, reliability, availability and low toxicity of bio-chemicals in therapeutic use has made them widespread for implementation in medical health care sector.

These studies was conducted with the objective of screening and quantifying major secondary compounds from leaves and barks of *P. gujava* and *C. aurea* and evaluate their extracts against selected human pathogenic bacteria. Qualitative and quantitative chemical analyses were done using powders or extracts obtained in ethanol solvent. Antimicrobial assay was done by disc diffusion method by applying 0.1ml of 200mg/ml extract. Minimum inhibitory concentration (MIC) was also done by broth dilution methods.

Results of qualitative chemical analysis showed that both plant species possess tannins, steroids, saponins, terpenoids and alkaloids, whereas flavonoids and phlobatannins were absent. Quantitative determination of these compounds showed that there was significant difference in their concentration between plant species and plant organs. Each extract type had varying degree of growth inhibition to the four clinical bacterial isolates, i.e., *Escherichia coli* O157:H7, *Salmonella Typhi*, *Staphylococcus aureus* and *Streptococcus pyogenes*. Likewise, each bacterial species showed varying sensitivity to the different extract types. Compared to the commercial antibiotic (Ciprofloxacin**),** all extract types had lower inhibitory effect, but had higher inhibitory effect when compared to the negative control (ethanol).

Generally, the MIC required inhibiting growth ranged from 25-100mg/ml and the MIC required suppressing growth of the isolates varied with bacterial species and extract type. The leaf and bark extracts of each plant also showed different MIC to suppress growth of the tested bacteria. Broth assay showed that in both plants, leaf extracts showed more antibacterial activities against all tested isolates than bark plant extracts. Overall, this study showed that both *Psidium guajava* and *Calpurnia aurea* have potential compounds that can help manage the growth of these clinical isolate bacterial pathogens.

5.2. Recommendations

Based on these findings the following recommendations are forwarded

- Isolation and identification of the specific bioactive compounds responsible for the antibacterial activities should be carried out,
- Assessment of *in vivo* antibacterial activity against human bacterial pathogens using models animal should be conducted.
- The communities should be encouraged to grow guava and *Calpurnia* to develop the practice of using the leaf and bark extracts as medicinal purpose.
- Further study should be carried out on phytochemicals analysis and evaluation of antimicrobial activities of *P. guajava* and *C. aurea.*

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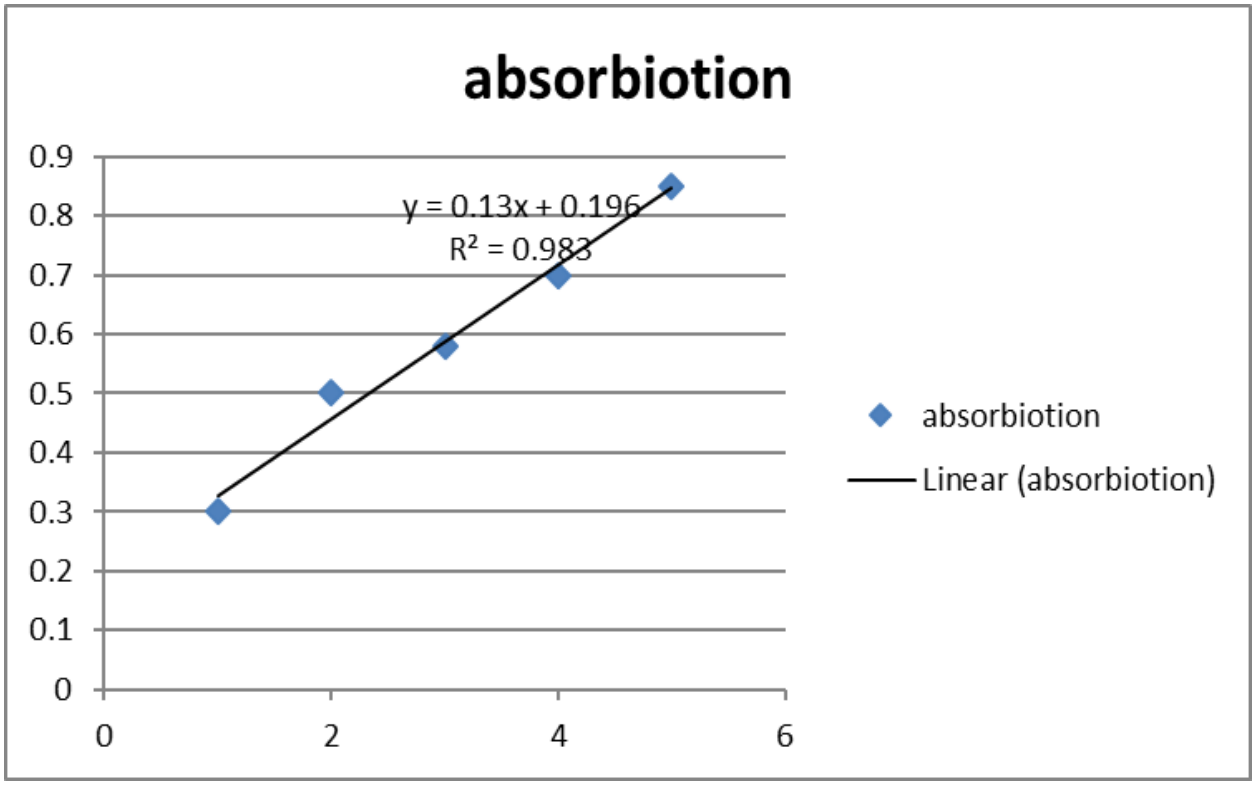
7. APPENDICES

Appendix Figure

Concentration vs. absorption



Appendix figure 1 Standard curve of tannic acid (µg/ml)