PHYTOCHEMICAL SCREENING FOR ANTIMICROBIAL ACTIVITIES OF THE EXTRACTS OF GUAVA (*Psidium guajava* L.) AGAINST ENTERIC BACTERIAL PATHOGENS

MSc THESIS

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Phytochemical Screening for Antimicrobial Activities of the Extracts of Guava (*Psidium guajava* L.) against Enteric Bacterial Pathogens

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I hereby certify that I have read and evaluated this Thesis entitled *Phytochemical Screening for Antimicrobial Activities of the Extracts of Guava (Psidium guajava L.) against Enteric Bacterial Pathogens* prepared under my guidance by *Olyad Erba*. I recommend that it be submitted as fulfilling the thesis requirement

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DEDICATION

I dedicate this thesis to my wife Megartu Huluka for her unforgettable and valuable encouragements in my academic career while I was performing this study and to my daughter Motu Olyad for her love and leisure activity.

STATEMENT OF THE AUTHOR

By my signature below, I declare and affirm that this Thesis is my own work. I have followed all ethical and technical principles of scholarship in the preparation, data collection, data analysis and compilation of this Thesis. Any scholarly matter that is included in the Thesis has been given recognition through citation.

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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
DMSO	Dimethylsulfoxide
EPHI	Ethiopian Public Health Institute
FQs	Fluoroquinolones
GAE	Gallic Acid Equivalent
LSD	Least Significant Difference
MBC	Minimum Bactericidal Concentration
MHA	Mueller Hinton Agar
MIC	Minimum Inhibitory Concentration
MRSA	Methicillin- Resistant *Staphylococcu aureus*
NLSI	National Laboratory Standards Institute
TAE	Tannic Acid Equivalent
TPC	Total Phenolic Content
VRE	Vancomycin-Resistant *Enterococci*
WHO	World Health Organization
ZOI	Zone of Inhibition

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ABSTRACT

Phytochemicals are important as protective and disease fighting compounds. The guava (Psidium guajava L.) is a phytotherapic plant used in folk medicine. It is believed to have active components that help to treat and manage various infectious diseases. The present study was carried out to screen and quantify the major phytochemicals and to evaluate antibacterial activities of guava leaf and bark extracts. Extraction was done by maceration using ethanol solvent. Qualitative analysis of phytochemicals was carried out using standard protocol and phytochemical contents of leaf and bark extracts or powders were determined using spectrophotometric and gravimetric methods. Antibacterial activities of guava leaf and bark extracts were determined by disc diffusion and broth dilution methods. The results of phytochemical analysis revealed the presence of alkaloids, saponins, steroids, tannins and terpenoids and the absence of flavonoids and phlobatannins in both leaf and bark extracts. The concentration of crude alkaloid (121 mg/g), terpenoid (110 mg/g), phenolic (3.92 mg /g) and tannin (3.05 mg/g) constituents were higher in leaf extract than bark extract while that of saponin (82 mg/g) was higher in bark extract than in leaf extract. The results of antibacterial activities revealed that both leaf and bark extracts have inhibitory activities against both gram-positive and gram-negative isolates. Both leaf and bark extracts resulted in the same high mean zone of inhibition (10 mm) against Salmonella Typhi. Staphylococcus aureus was inhibited at the highest value of minimum inhibitory concentration (7.5 mg/g, 5 mg/g) by leaf and bark extracts, respectively. On the basis of the present finding, guava leaf and bark extracts might be good candidates in the search for a natural antimicrobial agent. This study provides scientific understanding for further isolation and identification of the specific active compound of the guava leaf and bark extracts that responsible for the antibacterial activity.

***Keywords**: Antimicrobial activity, disc diffusion, Psidium guajava, Phytochemical analysis, Salmonella Typhi*

1. INTRODUCTION

Plant secondary compounds are organic molecules that found in different parts of the plants. They act as protective and disease fighting compounds, so they are required by humans to help them in preventing or fighting diseases (Begum *et al.,* 2010). The major plant secondary compounds include terpens/terpenoids, phenolics and alkaloids (Taiz and Zeiger, 2006; Rockwood, 2006). They are compounds that are products of secondary metabolism and are known for plant defense mechanisms. Drugs derived from natural products are usually secondary metabolites and their derivatives. Phytochemical screening 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).

Researches 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).

Medicinal plants have played a significant role in ancient traditional systems of medication in many countries. Indigenous herbs are used as remedies against various diseases in the traditional system of medicine or in ethnomedical practices (Aziz *et al*., 2014). Guava (*Psidium guajava* L.) is an important food crop and medicinal plant which has been widely used as food and in folk medicine around the world. Many studies have demonstrated the ability of guava to exhibit hepatoprotective, antimicrobial, cardioprotective, antidiabetic and antioxidant activities supporting its traditional uses (Kangogo *et al*., 2014)

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 resistant human pathogenic organisms has necessitated a search for new antimicrobial substances from other sources including plants (Adekunle and Adekunle, 2009; Bhatia *et al*., 2012).

Enteric bacteria such as *Salmonella, Shigella, Campylobacter* species, *vibrio cholerae* and diarrheogenic *Escherichia coli* are common cause of diarrhea in under-five children (WHO, 2010; Omulo *et al*., 2015). Development of antibiotics 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 faecalis* is one of the *Enterococci* 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. 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 phytotherapic 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).

There is a strong interest for further research for identification and quantification of the bioactive components of guava plant considering variation of phytochemical contents of the guava across cultivars and readily accessibility of the plant to local populace in the tropics (Shiruth *et al.,* 2013); as well as the identification and quantification of the phytochemical constituents of a given medicinal plant are considered as a pre-requisite to the scientific use of the compounds in modern medication. Although guava is cultivated as fruit crop in different parts of Ethiopia, there has been no study conducted on Ethiopian cultivars with respect to their secondary compounds compositions and contents, and their antimicrobial properties. This study was aimed to address this gap.

The general objectives of this study was to screen and quantify the major secondary compounds and to evaluate antibacterial activities of leaf and bark extracts of guava collected from Bate village, Haramaya district, Oromia regional state, Ethiopia.

Specific objectives of the study were to:

- Identify the major secondary compounds of guava leaf and bark
- determine and compare contents of major secondary compounds of guava leaf and bark
- evaluate antibacterial activities of the guava leaf and bark extracts against selected *Staphylococcus aureus*, *Salmonella* Typhi and *Enterococcus faecalis* human pathogens.

2. LITERATURE RIVIEW

2.1. 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). Researches 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 abiotic stress (e.g. UV-ß exposure) and important for the communication of the plants with other organisms (Schafer, 2009).

Secondary metabolites are being the subject for 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 screening 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).

2.1.1. Alkaloids

Alkaloids are naturally occurring chemical compounds containing basic nitrogen atoms. The name alkaloid is derived from the word alkaline and is used to describe any nitrogen containing base. Alkaloids can also be defined as heterocyclic nitrogen compounds that are reported to be useful against infectious diseases. For example, morphine, cordine and berberine were found potentially to be active against trypanosomes and plasmodia. Alkaloids are also reported to have microbiocidal and anti-diarrhoeal effect 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 alkaloid (4.32%) from the aqueous pulp extracts of *Tamarindus indica* and antibacterial activity of the extracts against *Escherichia coli, Staphylococcus aureus* and *Pseudomonas aeruginosa*. Besides, Agbafor *et al*.(2011) found abundant alkaloid in extracts of *Zapoteca portoricensis* leaf and the extracts inhibited the growth of *Escherichia coli, Staphylococcus aureus, Streptococcus pyogenes, Klebsiella pneumonia and Pseudomonas aeruginosa*. Furthermore, Garba and Okeniyi. (2012) reported a high antimicrobial activities of the total alkaloids extracted from *Carica papaya* L., *Calotropis procera* (Ait.)*, Mangifera indica* L., and *Psidium guajava* against *Staphylococcus aureus*, *Streptococci*, *Lactobacillus* spp. and *Candida albicans* at concentration of 0.6 mg/ml.

2.1.2. Terpenoids

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-neoplastics, 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 abiotic stresses (Yadav *et al*., 2014).

Studies conducted on different plant secondary metabolites extraction showed that terpene 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, Abdulhamid *et al*. (2014) found a high concentrations of ethanolic extract bark of *Psidium guajava* L. 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 present 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*.

2.1.3. Phenolics

Phenolics are one of the most ubiquitous groups of secondary metabolites found throughout the plant kingdom. They encompass a 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 phenolics 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 microflora and diminishing their digestive ability. The scent and pigmentation conferred by low-molecular-weight phenylpropanol derivatives attract symbiotic microbes, pollinators and animals that disperse fruits (Bhattacharya *et al*., 2010)

Flavonoids are a type of phenolics. Many flavonoids have been known to have antioxidant, anti-inflammatory and antitumor activity. Agbafor *et al*. (2011) reported the presence in aqueous extract of *Zapoteca portoricensis* (Jacq.) leaf and demonstrated that these secondary metabolites could be partaking 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 agaist *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) Raf 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.

2.2. Biochemical Constituents of Guava

Guava is a rich source of dietary fibers, vitamin A, C, folic acid and various dietary minerals like potassium, copper and manganese. Hassimotto and Genoverse. (2005) reported that a single *P*. guajava fruit contains about four times the amount of vitamin C as an orange.

Okunrobo *et al*. (2010) conducted phytochemical analysis of the powdered leaf of *Psidium guajava* L. showed the presence alkaloids, carbohydrates, reducing sugars, tannins, anthraquinones, terpenoids, flavonoids, glycosides and saponins. In another phytochemical analysis of the *Psidium guajava* leaf, Offor. (2015) reported higher levels of glycosides and saponins, moderate levels of tannins and flavonoids, and relatively low levels of alkaloids, phenols and steroids. Tijjani *et al*. (2014) conducted elemental analysis by Atomic absorption spectroscopy (AAS) revealed that the stem bark contained potassium (4.70mg/l), magnesium (0.11mg/l), Iron (0.23mg/l), zinc (0.70mg/l) , Phosphorus (4.70mg/l) and cadmium (0.25mg/l) minerals. Minerals such as chromium, Lead and sodium were not detected. They also screened phytochemical of the bark and revealed the presences carbohydrate, tannins, flavonoids, terpeniods, saponins, cardiac glycosides and the absence of anthraquinones and alkaloids.

2.3. Antibacterial Activities of Guava Extracts

Guava is a phytotherapic 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).

Bansode and Chavan. (2014) reported the antibacterial activity of guava leaf extracts against nine enteric pathogens tested *Esherichia coli*, *Salmonella* Typhi*, Salmonella paratyphi A, Salmonella paratyphi B, Shigella sonnei, Shigella dysentarie, Enterobactor spp., Citrobactor spp. and Klebsiella spp.* Like wise, Taura *et al*. (2014) conducted antibacterial activity of guava leaf extracts against against *Klebsiella pneumonia*, *Escherichia coli*, *Salmonella spp*.,

Pseudomonas aeruginosa and *Staphylococcus aureus* clinical isolates. The result of sensitivity test indicated that percolation extract at 30°C had activity against *Salmonella spp*., *Staphylococcus aureus* and *P. aeruginosa*; soxhlet extract at 45°C had activity against *Klebsiella spp*., *Staphylococcus aureus* and *P. aeruginosa*; soxhlet extract at 60°C had activity against *P. aeruginosa* and *Staphylococcus aureus* although the activity of all extracts was found much lower than that exhibited by standard antibiotic (ciprofloxacin) used as positive control

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. Fifty gram of powder mixed with 200 ml of methanol and boiled for 48 hr and another 50 g was also boiled in 200 ml of water for 30 min. The extracts were filtered and evaporated to dryness at 40°C. The dried extracts re-dissolved in 20% v/v dimethyl sulfoxide making 40 and 20 mg/ml of the extract concentrations from which 50 µL was taken and used for antibacterial activity tests. The study results showed that the methanolic and aqueous extracts exhibited antibacterial activity against methicillin resistant *Staphylococcus aureus*. The minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) of the aqueous extract ranged from 125 to 500 µg/ml while that of methanolic extract ranged from 62.5 to 250 µg/ml.

2.4. Enteric Bacterial Infections

The gastrointestinal tract represents a suitable ecosystem for enteric bacterial pathogens possibly due to the mucous nature and the presence of macro and micro-nutrients on the epithelial cell lining. Some enteric bacterial species can be highly pathogenic when they invade and colonize the digestive tract, thereby causing gastrointestinal disorders which range from diarrhoea, gastroenteritis, shigellosis, salmonellosis, to life threatening consequences (Godstime *et al*., 2014). Enteric bacterial pathogens, the major causes of food borne gastroenteritis in humans, remain important health problems worldwide. Such infections associated with food contamination are the major public health problems especially in developing countries resulting in morbidity, mortality and socioeconomic impacts such as high rates of hospitalizations and high treatment costs (Teshale *et al*., 2015).

Enteric infections, with or without overt diarrhea, have profound effects on intestinal absorption, nutrition, and childhood development as well as on global mortality. Oral rehydration therapy has reduced the number of deaths from dehydration caused by infection with an enteric pathogen, but it has not changed the morbidity caused by such infections (William *et al*., 2008). Among many bacterial species implicated in gastrointestinal diseases (the *enterobacteriaceae* family) include *Clostridium difficile, Salmonella enterica, S. enteritidis, S. Heidelberg, S. typhymurium, Shigella dysenteriae, Klebsiella spp., Enterobacter spp., Vibrios spp., Yersinia pestis, Proteus spp, Bacillus cereus, Helicobacter pylori, Campylobacter coli, Campylobacter jejuni*, enterotoxigenic *Escherichia coli* (Godstime *et al*., 2014).

2.4.1. *Salmonella* Typhi infection

Salmonella enterica, subspecies *enterica*, serovar *typhi* causes Typhoid fever which is a blood infection. The bacterium is commonly referred to as S*almonella typhi* or *Salmonella* Typhi or *S. typhi* to identify it from other members of the bacterial group *Salmonella* (often called "non-typhi *Salmonella*") (CDC, 2013). Humans are the only known reservoir of *Salmonella* Typhi. Infected individuals carry *S.* Typhi in their intestinal tract and bloodstream and periodically shed the bacteria in their stool and less commonly in their urine. Transmission from host to host occurs if food or water contaminated with such fecal matter is ingested. Blood from an infected person also could transmit the bacterium to other people. Following ingestion of contaminated food or water, *S.* Typhi enters the small intestine, multiplies, and spreads into the bloodstream. The resulting infection is systemic. The gall bladder, liver, intestines, and spleen are commonly affected (CDC, 2013; Gopinath *et al*., 2012).

The emergence of antimicrobial resistant salmonella strains traced back to during the late 1980s and early 1990s. In these years, the occurrence of multiple drug-resistant *S.* Typhi and *S.* Paratyphi A strains, i.e. resistant to chloramphenicol, ampicillin and co-trimoxazole at a time, led to the use of fluoroquinolones (FQs) for the treatment of enteric fever (Crumb *et al*., 2004). The widespread use of FQs resulted in increased rate of *Salmonella enterica* strains with reduced susceptibility to these drugs (Molbak *et al*., 2002).

2.4.2. *Staphylococcus aureus* infection

Staphylococcus aureus is a major human pathogen that causes a wide range of clinical infections. It is a leading cause of bacteremia and infective endocarditis as well as osteoarticular, skin and soft tissue, pleuropulmonary and device-related infections (Bachir and Abouni**,** 2015; Tong *et al*., 2015). Among the predominant bacteria involved in food-borne diseases, *Staphylococcus aureus* is also the leading cause of gastroenteritis resulting from the consumption of enterotoxins-contaminated food. Symptom-onset is abrupt and the disease may be severe enough to warrant hospitalization, but is usually self-limiting and does not require specific therapy (Bachir and Abouni, 2015**)**.

The past two decades have witnessed two clear shifts in the epidemiology of *S. aureus* infections: first, a growing number of health care associated infections, particularly seen in infective endocarditis and prosthetic device infections, and second, an epidemic of community-associated skin and soft tissue infections driven by strains with certain virulence factors and resistance to ß-lactam antibiotics (Tong *et al*., 2015). It is 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. aureu* increasingly difficult to treat because of the fast rate at which it develops resistance to common antimicrobial agents. Multiple antibiotics resistance is a major health concern in the treatment of staphylococcal infections, especially infections of methicillin-resistant *S. aureus* (MRSA) which occurs due to the extensive use of antimicrobial agents, coupled with the transmission of an appreciable proportion of the organism by person-to-person contacts. Multiple antibiotic resistant *S. aureus* is one of the common causes of severe nosocomial infections, and the gastrointestinal tract is an important source of its transmission (Onanuga and Temedie, 2011).

2.4.3. *Enterococcus faecalis* infection

*Enterococc*i are traditionally regarded as low grade pathogens, have emerged as an increasingly important cause of nosocomial infections in the last decade. Although about a dozen *Enterococcus* species have been identified, only two are responsible for the majority of human

infections, *i.e*., *Enterococcus faecalis* and *E. faecium*. The most common nosocomial infections produced by these organisms are urinary tract infections (associated with instrumentation and antimicrobial resistance), followed by intra-abdominal and pelvic infections. They also cause surgical wound infections, bacteraemia, endocarditis, neonatal sepsis and rarely meningitis (Sood *et al*., 2008).

A major reason why these organisms survive in hospital environment is the intrinsic resistance to several commonly used antibiotics. The emergence of vancomycin-resistant *Enterococci* (VRE) is a cause of concern, as once established, it is very difficult to control. Moreover, there can be transfer of resistant gene from *Enterococci* to *Staphylococcus aureus* thereby posing a threat to the patient safety and also challenges for the treating physicians (Sood *et al*., 2008).

2.5. 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, Amoxacillin, 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 enterica sub-species arizonae and 4 (12.1%) were Salmonella group A. Salmonella isolates were highly resistant to Ampicillin (93.9%) followed by Augmentin (75.8%) and Trimethoprim/Sulfamethoxazole (48.5%). However, the isolates showed high susceptibility to Ciprofloxacin and Norfloxacin (93.9% each) followed by Gentamicin (87.9%). Likewise, the Salmonella isolates showed 90.9% of multidrug-resistance. *Salmonella enterica* nine` subspecies arizonae were the dominant strains of Salmonella isolated from children with acute diarrhea in that study (Yemane *et al*., 2014).

3. MATERIALS AND METHODS

3.1. Study Area

The plant samples were collected from Bate Village which is found near to the Haramaya University. The experimental study was conducted in the laboratories of the Department of Biology at Haramaya University. Phytochemical analysis was carried out in General Laboratory while anti-microbial activity tests were carried out in Microbiology Laboratory, Haramaya University main campus is located at about 510 km East of Addis Ababa, between Dire Dawa and Harar towns. Geographically, it is located at 09.0^0N and 42.0^0 E and at an altitude of 1950 meters above sea level.

3.2. Plant Material and Extract Preparation

Fully mature and healthy *Psidium guajava* leaves and barks were collected randomlyfrom Bate village Haramaya District, East Hararghe, Ethiopia. They were collected into plastic bags with appropriate labeling. The collected leaves and barks were authenticated at the botany laboratory and Herbarium of Haramaya University. Then, they were washed under running tap water to remove dusts and were dried under shade at room temperature for 15 days. Thereafter, they were grounded to fine powder using electric miller (IKA A11 basic). The dry powders obtained were stored in air tight plastic containers and kept in refrigerator at 4ºC until they were used for qualitative analysis and extraction.

Modified method of Shah and Yadav (2015) was used for extraction. Briefly, the dried powders were extracted by maceration using 97% ethanol as solvent. Eighty grams of powder and 400 ml of ethanol were added to 500 ml capacity Erlenmeyer flasks and mixed by shaking. Then, the flasks were wrapped with aluminum foil to avoid evaporation and were shaken on a platform shaker for 3 days at room temperature. Some of the resulting extracts were concentrated by heating on a hot plate at about 30 to 40ºC for 30 min and were subsequently used for qualitative analysis. The rest were evaporated to dryness at room temperature for four days and preserved at 4ºC until used for qualitative and quantitative analysis, as well as for anti-bacterial activity tests

3.3. Analysis of Phytochemicals

3.3.1. Qualitative Analysis of Major Secondary Metabolites

Qualitative analysis of major secondary metabolites tannins, phlobatannins, saponins, flavonoids, steroids, terpenoids and alkaloids of the guava was carried out on the concentrated and solidified ethanolic extract and on the powdered specimen using standard procedures as described below.

Test for tannins: One 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 five 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: Zero point five gram of each solidified extract 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 saponins: One 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 Erlenmeyer flask. After cooling, the following tests were carried out according to Ajayi *et al*. (2011).

Foam test: Two point five ml of filtrate was added to a test tube and diluted to 10 ml with distilled water. It was shaken vigorously for 2 minutes. Formation of froth confirmed the presence of saponin in the filtrate.

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

Test for flavonoids: Two 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 steroids (Lieberman-Burchard's Test): Two 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, 2 ml of concentrated sulphuric acid was added from the side of the test tube. The change of red colour through blue to green indicated the presence of steroids (Gayathri and Kiruba, 2014).

Test for terpenoids (Salkowski test): Five 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 interphase confirmed positive results for the presence of terpenoids (Biswas *et al*., 2013).

Test for alkaloids**:** Two ml of 1% HCl was added to 6 ml of each concentrated ethanolic extract in different test tubes. Each mixture was heated for 2 min in a water bath while stirring continuously. It was then cooled and filtered. The resulting filtrate was tested with Mayer's Reagent for the presence of alkaloids as described by Adachukwu *et al*. (2013). One ml of the filtrate was added to 0.5 ml of Mayer's reagent. Formation of cream yellow precipitate indicated the presence of alkaloids.

3.3.2. Quantitative Analysis of Major Secondary Metabolites

The preserved powder and solidified extracts of the guava were used for standard quantitative estimation of the major secondary metabolites. All experiments were done in triplicate.

Determination of total phenolics content: Spectrophotometric method was used to quantify total phenol content in the guava leaf and bark extracts as described by Cavalcanti de Amorim *et al*. (2012). Brefily, 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, 0.2, 0.3, 0.4 and 0.5 ml volumes of stock solution were pipetted and transferred into separate pint flasks. Five hundred µl 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 obtained 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 the 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 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: Five hundred mg of casein was transferred into each a 25 ml capacity Erlenmeyer flasks. Then, 0.5 ml stock solution of the extracts (prepared for determination of total phenol contents) and 5 ml of distilled water were added in to the flask. After two hours, the extracts were filtered and these filtrates were used to determine non-tannic

phenols using a procedure similar to total phenol determination. The tannin content will be calculated as:

$$\text{Tannin} = \text{TPC} - \text{non-tannin phenol}$$

Determination of alkaloid content using the Harborne (1973) method: Three gram of the powder was weighed and added into a 50 ml capacity Erlenmayer flask. Then, 20 ml of 10% acetic acid in ethanol was added into the flask which was soon covered and the solution was allowed to stand for 4 hrs. Next, the solution was filtered 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 10% 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 saponin content: Saponin content was determined by Obadoni and Ochuko (2001). Fifteen ml of 20% aqueous ethanol was added into a conical flask containing 15 g 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 separatory funnel and 5 ml of diethyl ether was then added and the resulting mixture was shaken gently. The bottom aqueous layer was then drained. 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 resulting solution was shaken gently. 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 to at 60°C to constant weight and they were then measured; the saponin content was calculated as mg per grams of the sample extract used.

Determination of terpenoid content: Two 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. The mass of dried ether extract, considered as crude terpenoid, was measured and its content was calculated as mg per grams of the sample powder used (Ferguson, 1956).

3.4. Anti-bacterial Assay

3.4.1. Collection of Test Organisms

Enteric bacterial pathogens of human clinical isolate were obtained from Ethiopian Public Health Institute (EPHI) Addis Ababa, Ethiopia. The isolates include one gram-negative pathogen (*Salmonella* Typhi) and two gram-positive pathogens (*Entrococcus faecalis* and *Staphylococcus aureus*).

3.4.2. Sub- culturing and Standardization of Inoculum

Each of the enteric bacterial pathogens obtained from EPHI 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 the 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.

3.4.3. Antibacterial Activity Test

Preparation of the test extracts solution: A stock solution of the extract (200mg/ml) was prepared by reconstituting 0.4 g of each of the dried extracts in 2 ml of ethanol and was used for disc diffusion method of anti-bacterial activities. Another stock solution of extract (20 mg/ml) was

prepared by reconstituting 100 mg of each of the dried extracts in 5 ml of ethanol and from the stock the solution of different concentrations were prepared making 2-fold serial dilutions as follows: five sterile test tubes were arranged on a test tube rack and 1 ml of sterile distilled water was dispensed into them. From the stock solution, 1 ml was transferred into the first test tube and subsequently successive serial dilutions of the extract were carried out. The resultant concentrations in the test tubes were 10, 5, 2.5, 1.25, 0.625 mg/ml (Esimone *et al.*, 2012). They were used, along with the stock, for determination of minimum inhibitory concentration.

Preparation of antibiotic impregnated discs: Whatman No.1 filter paper discs of 6 mm diameter were punched out with the aid of paper punch and were placed in petriplate. They were then sterilized by autoclaving at 121°C for 15 min. After that, the discs were cooled and they were 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)

Following this step, the impregnated discs were dispensed 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 sealed 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 were recorded. For each selected enteric bacterial isolate, the experiment was carried out in parallel and with three replications (Thompson *et al*., 2011; Biswas *et al*., 2013)

3.4.5. Determination of Minimum Inhibitory Concentration

Two ml of nutrient broth was added into six 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 24 hrs. After which, they were observed for absence or presence of visible growth. The lowest concentration without visible growth of organisms was regarded as the MIC. The experiment was carried out for each organism in duplicates (Taura *et al*., 2012).

3.5. Method of Data Analysis

Statistical Package for Social Science, Version 20 (SPSS; Chicago, IL, USA), was used to analyze the data. The data recorded from determination of phytochemical contents in leaf and bark extracts were analyzed by independent sample T-test for comparison while that recorded from antibacterial disc diffusion tests were analyzed using One-way analysis of variance (ANOVA). The significance of output of these statistical tools was considered at $p< 0.05$.

4. RESULTS

4.1. Qualitative Phytochemical Analysis

The results of the qualitative phytochemical analysis revealed the presence of alkaloids, saponins, steroids, tannins and terpenoids both in leaf and bark extracts. Whereas, flavonoids and phlobatannins were absent in both of the extracts. (Table 1).

Table 1: Results of the phytochemical screening from ethanolic extracts of guava leaf and bark

Screened phytochemical	Guava leaf	Guava bark
Alkaloid	+	+
Flavonoid	-	-
Saponin	+	+
Steroid	+	+
Tannin	+	+
Terpenoid	+	+
Phlobatannin	-	-

(+) indicates the presence of the phytochemical while (-) indicates the absence of the phytochemical

4.2. Quantitative phytochemical determination

The result of quantitative determination of phytochemicals was summarized in table 2. As can be seen from the table, Crude alkaloid (121 mg/g, 115.33 mg/g), terpenoid (110 mg/g, 103 mg/g), phenolic (3.92 mg/g, 3.01 mg/g) and tannin contents (3.05 mg/g, 2.14 mg/g) were found to be higher in leaf extract than in bark extract respectively. Crude saponin content (82 mg/g) of leaf extract was found to be higher than crude saponin (24.4) of bark extract.

Table 2: Quantity of phytochemicals in ethanolic extracts of guava leaf and bark

Amount of Phytochemical (in mg/g of crude extract or powder)	Guava leaf	Guava bark
Crude alkaloid	121 ± 11.72^{A}	115.33 ± 17.67^{A}
Crude terpenoid	110 ± 7.64^{A}	103 ± 4.41^{A}
Crude phenolic	3.92 ± 0.09^{A}	3.01 ± 0.07^{B}
Crude saponin	24.4 ± 2.33^{A}	82 ± 6.66^{B}
Crude tannin	3.05 ± 0.05^{A}	2.14 ± 0.08^{B}

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 $P<0.05$.

4.3. Antibacterial Activity test

Determination of the inhibition zones by means of the disc diffusion method showed that leaf and bark extracts at concentration of 2 mg/disc showed inhibitory effect on all tested clinical isolates. (Table 3).

Table 3: The antibacterial activities of the leaf and bark extracts of guava on clinical isolates of pathogenic bacteria

Anti-microbial agent	Concentration	Zone of inhibition in mm on clinical isolates		
		E. faecalis	*S. aureus*	*S.* Typhi
Leaf extract	2 mg/disc	5 ± 2.52^{Aa}	9 ± 0.58^{Ab}	10 ± 0.76^{Ab}
Bark extract	2 mg/disc	5.3 ± 2.68^{Aa}	7.2 ± 0.17^{Aa}	10 ± 0.76^{Aa}
Ciprofloxacin	5 µg/disc	24.5 ± 0.87^{Ba}	39.7 ± 1.42^{Bb}	25.2 ± 0.18^{Ba}

The values are Mean ± Standard error of mean (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 superscript compares between means in row, and means with similar small letters show no significant difference, whereas means with different small letters show significant difference at $P<0.05$.

The results of minimum inhibitory concentrations (MICs) of the extract were represented in Table 4. As it can be seen from the table, the lowest MIC of both leaf and bark extracts observed against *Salmonella* Typhi and highest MIC against *Staphylococcus aureus*.

Table 4: The minimum inhibitory concentrations of the leaf and bark extracts (0.1 ml) of guava against the three clinical isolates

Extract	MIC values for clinical isolates		
	E. faecalis	*S. aureus*	*S.* Typhi
Leaf extract	5.00 mg/ml	7.500 mg/ml	3.750 mg/ml
Bark extract	2.500 mg/ml	5.000 mg/ml	1.250 mg/ml

5. DISCUSSION

5.1. Qualitative and Quantitative Phytochemical Analysis

Modern pharmacy prefers single ingredients on the grounds that dosage can be more easily quantified. One of such efforts includes detailed analysis of phytochemical constituents of such plants (Obaineh and Shadrach, 2013). In the present study, a preliminary qualitative phytochemical analysis was carried out to identify the major secondary metabolites such as tannin, phlobatannins, saponins, flavonoids, steroids, terpenoids and alkaloids present in the ethanol macerated leaf and bark extracts of *guajava*. In addition, quantitative determination of crude alkaloid, terpenoid, phenolic and tannin contents of leaf and bark extracts were carried out.

The results of qualitative analysis revealed the presence of alkaloids, saponins, steroids, tannins and terpenoids both in leaf and bark extracts, but the results showed the absence of flavonoids and phlobatannins in both of the extracts. The quantitive analysis showed that the difference in contents of crude alkaloid (121 mg/g, 115.33 mg/g) and terpenoid (110 mg/g, 103 mg/g) of leaf and bark extracts, respectively, were found to be insignificant ($p>0.05$). While he difference in contents of phenolic (3.92 mg/g, 3.01 mg/g TAE) and tannin contents (3.05 mg/g, 2.14 mg/g TAE) of leaf and bark extracts, respectively,were found to significant ($p<0.05$). Crude saponin content (82 mg/g) of bark extract was found to be significantly higher than crude saponin content (24.4 mg/g) of bark extract ($p>0.05$). Leaf extract had generally higher contents of quantified phytochemicals than bark extract. 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. Supporting this, 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, while Asghari *et al*. (2014) reported that alkaloids were mainly located in bark and root in June, but the leaf were major storage site of alkaloids in July.

Previously, different researchers have done phytochemical screening of *guajava* leaf ethanol macerated extracts. Similar to present study, Taura *et al*. (2014) found alkaloids, saponins and tannins, but they did not get flavonoids in the extracts. Biswas *et al*. (2013) reported the presence

of tannin, flavonoids and terpenoids, but absence of saponins. Gayathri and Kiruba (2014) detected the presence of terpenoids and total phenolics, while at the same they showed absence of alkaloids, flavonoids and sterols in ethanol extracts. Some researchers also reported the presence of some screened phytochemicals from guava bark ethanol extracts. Okunrobo *et al*. (2012) detected alkaloids, flavonoids, tannins and saponins. Abdulhamid *et al*. (2014) also detected alkaloids, flavonoids, saponins, tannins, steroids and terpenoids. 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. Tijjani *et al*. (2014) also found tannins, flavonoids, terpeniods and saponins, but not alkaloid in extract prepared using soxhlet apparatus.

Results of the current quantitative study have been compared with previous studies conducted under similar standard method for quantitative determination of phytochemical of macerated leaf and barks. Similar to this study, comparative study conducted by Obaineh and Shadrach (2013) showed higher crude alkaloid (0.60 mg/g), saponin (0.02 mg/g), tannin (11.54mg/g) and phenol (1.67 mg/g) contents in ethanolic leaf extract than crude alkaloid (0.07 mg/g), saponin (0.01 mg/g), tannin (3.85mg/g) and phenol (0.45 mg/g) contents in ethanolic bark extract. Unlike this study, the result showed a higher crude saponin content in ethanolic leaf extract than ethanolic bark extract.

Compared to phytochemical content of present study, Zahidah *et al*. (2013) reported lower crude phenol (3.69 mg/g GAE) content in aqueous leaf extract. whereas, Ibe *et al*. (2014) reported higher contents of crude total phenol(111.86 GAE in mg/g) and tannins(142.93 TAE in mg/g) in methanolc extracts of stem bark. The difference of the phenol contents may be related to the constituent of phenolic compounds in the extracts that could extracted best in water or methanol or ethanol. Charis *et al*, (undated) reported a higher extractability of phenolic compounds: hydroxytyrosol, tyrosol, oleuropein, caffeic, *p*-hydroxybenzoic, *p*-hydroxyphenyl acetic, protocatechuic and rosmarinic acid in ethanol at room temperature. Other phenolic acids like vanillic, ferulic, sinapic and syringic possessed a higher extractability for methanol, while gallic, cinnamic and coumaric were showed higher extractabily in water. Rahababa *et al*. (2010) reported that methanol extraction at 60°C found to be the best for extracting phenolic compounds

while distilled water extraction at 60°C conditions found to be the best for extracting anthocyanin.

In general, comparison of the current study with some that have been previously conducted showed that phytochemical composition and content from the same plant species and plant part varies. The variation was may be due to the difference in plant material and extract preparation process such as, extraction methods, percent and volumes of the solvent used, temperature and time period used for extraction, or concentration or drying of extracts. These factors were supported by previous study. Accordingly, Agbafer *et al*. (2011) reported that a successful recovery of biologically active compound from plant material is largely dependent on the type of solvent used in the extraction procedure. Wendakoon *et al*. (2012) revealed that bioactive components and contents of plant extracts depend on the concentration of ethanol, time period and temperature used in the extraction process.

Another factors that contributed to the variation of phytochemical composition and content of plants extracts are related to biological and environmental factors as well as biochemical, physiological, ecological, and evolutionary processes. Supporting these factors contributions to the variations, Compean and Ynalvez (2014) reported that the type and amount of secondary compounds vary with plant species and parts of plants. Penuelas and Llusia (2001) studied that variation in secondary compounds may also exist within species mainly due to plant genotypes, developmental stages and geographical locations. Moore *et al*. (2014) reviewed that the type and amounts of many plant secondary metabolites differ due to variations in resources for growth such as nutrients, light and water, and biotic influences such as the presence or absence of enemies, competitors or mutualists and that the most important mechanism in diversifying secondary metabolites is whole-genome and local-gene duplication.

5.2. Antibacterial Activity test

Antibacterial Activity tests were determined by disc diffusion and broth dilution of MIC test. Turnidge *et al*. (2003) explained that potency of any newly reported antibacterial preparation can be quantified and compared with those already known by determining its MIC value. Acharya *et al*. (2012) reported that disc diffusion test is the routine procedure for susceptibility testing as determining MIC is painstaking and rarely available in resource poor laboratories. In this study, the disc diffusion method was first used, and once the extracts were found to have antibacterial activity, broth dilution was used to determine Minimum Inhibitiory Concentration of extracts

Determination of the inhibition zones by means of the disc diffusion method showed that leaf and bark extracts at concentration of 2 mg/disc showed inhibitory effect on all tested clinical isolates. Leaf extract was the most effective inhibitor against *Salmonella* Typhi (10 mm) while it had the least effective antibacterial activity against *Enterecoccus faecalis* (5 mm). There was no significant difference among the inhibitory effects of leaf extract against *Staphylococcus aureus* and *S.* Typhi ($p>0.05$). Similarly, bark extract was the most effective inhibitor against *S*. Typhi (10 mm) while it had the least inhibitory effect against *E. faecalis* (5.33 mm). The inhibitory activities of bark extract against all tested clinical isolates were not statistically different from each other ($p>0.05$).

Leaf extract resulted in slightly larger mean zone of inhibition against *Staphylococcus aureus* (9 mm) than the bark extract did (7.2 mm). Bark extract made slightly larger mean zone of inhibition against *Enterococcus faecalis* (5.3 mm) than leaf extract did (5 mm). The antibacterial activities of leaf and bark extracts against all tested isolates were not significantly different from one another ($p > 0.05$). Standard antibiotic (ciprofloxacin) was used as a positive control and caused significantly the highest zone of inhibition against all tested clinical isolates compared with those of both extracts ($p < 0.05$). The solvent (97% ethanol) used as a negative control had no anti-bacterial activity against all tested isolates.

The extrinsic parameters like pH of the medium, time period and temperature of incubation, concentration of plant extracts and size of inoculums were not pose much error as they were

fixed and standardized during experiment. But, intrinsic factors such as medicinal plant parts' phytochemical constituent, concentration, solubility and diffusing property might be responsible for variability in diffusion of extract which in turn result in variation of ZOI. Prasai *et al*. (2008), Bhatia *et al*. (2012) and Choudhury *et al*. (2012) reported that disc diffusion depends on measuring the diameter of zone of inhibition (ZOI) formed on the petri-plate and that extrinsic and intrinsic parameters mainly affect the ZOI

The Minimum inhibitory concentrations (MICs) of the extract were determined by preparing double serial dilutions. The ethanol extracts were dried and re-dissolved in mixture of ethanol and water in order to prepare the extracts' double dilutons ranging from 20 mg/ml-0.625 mg/ml. Wendakoon *et al*. (2012) explained that broth dilution method requires preparing various dilutions of the compound under test in a suitable solvent, extracts have to be dried and re-dissolved, and since water frequently doesn't dissolve the intermediate polarity or non-polar components of a dried extract, water miscible solvents such as methanol, ethanol or DMSO has to be used as an alternative in serial dilution assay.

The result of MIC of leaf and bark extracts varied against different test clinical isolates. Lowest MIC of both leaf and bark extracts observed against *Salmonella* Typhi. This result of the broth dilution method agreed with the results obtained from the disc diffusion method in which *Salmonella* Typhi was the most susceptible to the extracts at 2 mg/disc. Both leaf and bark extracts had highest MIC against *Staphylococcus aureus*. This result of broth dilution method disagreed with the results obtained from the disc diffusion method in which *Enterococcus faecalis* was the least susceptible to the extracts at 2 mg/disc.

Interestingly, broth method showed that bark extract had antibacterial activities at lower concentration against all tested isolates than that of leaf extracts had against the tested isolates. This may be due to difference in individual concentrations of constituents of phytochemicals that made up overall tested concentration of leaf and bark extracts and susceptibility of the isolates to the extracts constituent in a concentration dependent pattern. In line with this, Garba and Okeniyi (2012) reported that *Staphylococcus aureus*, *Streptococci* and *Lactobacillus spp*. were susceptile to total alkaloid extracted from *Psidium guajava* leaf differently in concentration dependant

pattern. The extracts inhibited tested micro-organisms including *S. aureus* differently in concentration dependent pattern were also reported by Abukakar *et al*. (2008) and Agbafor *et al*. (2011).

The detected phytochemicals and the quantity of alkaloids, saponins, tannins and terpenoids both in leaf and bark extracts of guava has medical implications. Therefore, they may be responsible for the observed antibacterial activities of leaf and bark extracts. Supporting this, tannin was reported to have antibacterial activity (Mariita *et al*., 2011). Alkaloid was also reported to have microbicidal and anti-diarrhoeal 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). According to study conducted by Soetan *et al*. (2006), crude saponin extract found to have inhibitory effects on *Staphylococcus aureus,* a gram-positive organism. Maatalah *et al*. (2012) showed also that the saponin extract had antimicrobial activity against *Escherichia coli* (ATCC 25922), *S. aureus* (ATCC 6538), *Klebsiella pneumonia*, *Bacillus subtilis* (ATCC 6633), *Pseudomonas aeruginosa* (ATCC 14028) and *Candida albicans*. Terpenoid have also been found to be potential agents in inhibiting pathogenic bacteria (Munyendo *et al*., 2011; Sibi *et al*., 2012; Abdulhamid *et al*., 2014).

Previous studies showed the antibacterial activities of leaf and bark extracts of guava. The results of these studies were compared with the present study. Using disc diffusion method, Zahidah *et al.* (2013) reported nearly the same inhibition zone (10.5 mm) of 100 mg/ml of aqueous leaf extract against *Staphylococcus aureus*. Taura *et al*. (2014) also used disc diffusion method and reported nearly similar inhibition zone (8 mm of) 480 µg/disc solution ethanolic leaf extract against *S. aureus*, but smaller inhibition zone (8 mm) against *Salmonella* Typhi. 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 smaller MIC (0.125 for both) of ethanolic extracts of leaf and stem bark against *Staphylococcus aureus*.

Comparative study of antibacterial activities of *Psidium guajava* leaf and bark extracts against gram-positives and gram-negatives were previously conducted. Opposing the result of this study,

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. However, Goncalves *et al*. (2008) reported that methanolic leaf extract had more effective antimicrobical activity against gram-negatives than gram-positives supporting present study.

The antibacterial activity of guava extracts against a gram-negative (*S*.Typhi) in the present study is may be due to highest crude alkaloids, terpenoid and saponin contents of the extract. Plant extracts with the highest concentration of these phytochemicals being active in inhibiting gram-negative bacteria were previously reported (Abukakar *et al*., 2008; Agbafor *et al*., 2011; Garb and Okeniyi, 2012; Abdulhamid *et al*., 2014). Usually, gram-negative bacteria are resistant to plant extract beacause they have cell wall having effective permeability barrier, a thin lipopolysaccharide exterior membrane which restrict penetration of extruding plant extract (Biswas *et al*., 2013). This outer membrane is may be affected by terpenoid which was speculated to involve membrane disruption by the lipophilic compounds (Mendoza *et al*., 1997) and saponin that was believed to complex with sterols in the cell membrane leading to pore formation (Mert-Turk, 2006). Membrane disruption may expose the bacteria to alkaloid which was believed to intercalates with microbial deoxyribonucleic acid (Garb and Okeniyi, 2012), tannin and steroid, and other phytochemicals of the extract kown to have antibacterial activity. But, this hyphothesis need further researchs to be supported and concluded to be scientific principle.

5. SUMMARY AND CONCLUSIONS

Guava is widely used as source of nutrition and in some cases in traditional medicine. It is believed to have active components that help to treat and manage various infectious diseases. Therefore, this study was aimed at screening the active secondary compounds known to have anti-bacterial activities from guava leaf and bark extracts, quantification of some of detected active secondary compounds and assaying of anti-bacterial activity of the crude ethanolic extracts.

Fully mature and healthy guava leaf and bark was collected, washed, dried and ground. Then, the obtained dry powder was extracted by maceration using ethanol as solvent. The phytochemical screening was performed following standard procedure and quantity of screened crude alkaloids, terpenoids and saponin was determined by following standard procedure of gravimetric method. Besides, the screened out tannin and total phenolics content in the bark and leaf extracts were determined using a techniques of spectrophotometeric method. Anti-bacterial activities of the extracts against three enteric bacterial pathogens, *Salmonella typhi*, *Entrococcus faecalis* and *Staphylococcus aureus*, was determined using disc diffusion and broth dilution methods.

The results of qualitative phytochemical screening of guava leaf and bark extracts revealed the presence alkaloids, saponins, steroids, tannins and terpenoids both in leaf and bark extracts. Whereas, flavonoids and phlobatannins were absent in both extracts. Quantitative analysis revealed that generally leaf extracts had the highest concentration of phytohemicals. Concentration crude of alkaloid (121 mg/g), terpenoid (110 mg/g), phenolics (3.92 mg/g) and tannin (3.05 mg/g) were found in larger in leaf extract than bark extract. There was no significant difference between the concentrations of crude alkaloid and terpenoids found in both extracts. Crude saponin concentration (82 mg/g) was found significantly higher in bark extracts than leaf extract. The result of antibacterial activity test showed that *guajava* leaf and bark extracts shows different degree of inhibition against different isolates except for *Salmonella* Typhi. Both leaf and bark extracts exhibited the most effective antibacterial activity against *Salmonella* Typhi. Antibacterial activities of both extracts were significantly less than that of ciprofloxacin used as positive control.

The results of present study evidenced that studied guava leaf and bark extracts have the ability to inhibit the growth of both the gram-positves and negatives enteric pathogens with its composition and abundant content of bioactive secondary metabolites. Many evidences which confirmed the identified phytochemicals to be bioactive gathered in earlier studies. Presence of these medicinally important constituents in the studied extracts might be responsible for the observed antibacterial activity. Eventhough, antibacterial activities of both extracts were significantly less than that of ciprofloxacin, activity of the extracts was broad and encouraging. Therefore, the guava leaf and bark ethanolic extracts could be seen as a good source for useful drugs and an alternative medicine in the treatment of enteric disorders.

Depending the scope and results of the present study, the following recommendations were forwarded.

- Guava leaf ethanolic extract can be a potential raw material for extraction and purification of therapeutic alkaloids and terpenoids.
- Guava bark ethanolic extract can be a potential raw material for extraction and purification of therapeutic saponins.
- Guava leaf and bark ethanolic extracts could be used for treatment of enteric infections caused by *Salmonella* Typhi.
- Determination of steroid content and minimum bactericidal concentration (MBC) should be conducted.
- Evaluation of *In vivo* antibacterial activity against enteric pathogen using experimental animal should be conducted.
- Isolation and identification of the specific bioactive compound responsible for the antibacterial activity should be carried out.
- The communities should be encouraged to grow guava and to develop habit of using the leaf and bark extracts as nutriceuticals.

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7. APPENDICES

7.1. Appendix Figure

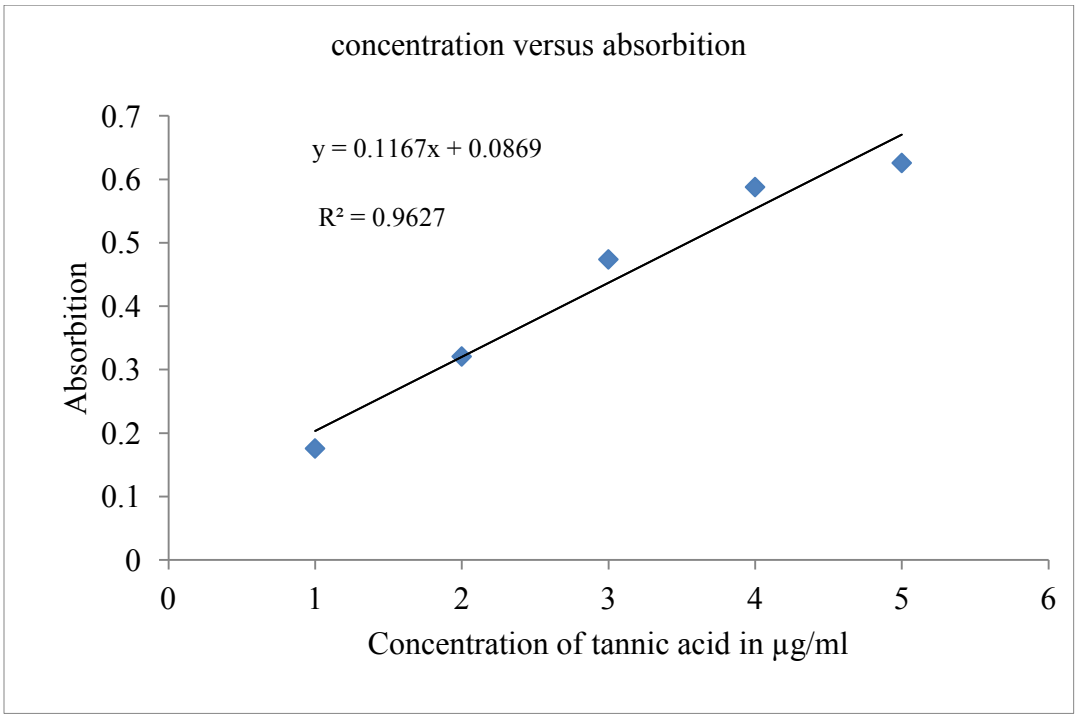

Figure 1: Standard curve of tannic acid

Figure 2: plant sample preparation