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ANTIMICROBIAL ACTIVITY OF PSIDIUM GUAJAVA LEAVES EXTRACT AGAINST STAPHYLOCOCCUS AUREUS USING PURE EXTRACTS

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Chapter 1

INTRODUCTION

Rationale of the study

The key role in the treatment of the patients, is to use the pharmacologic therapy of the extract which can b used as an alternative, which is beneficial in terms of cost effectiveness, availability and potency. particularly to the emergence of resistant strains. It is therefore logical to divert some attention to possible alternatives that would create a revolution as far as cost-effective fight against bacterial pathogens is concerned. In relation to the understanding of the Microbial Activity of the Staphylococcus aureus infection, is that it has a role in delaying and poor wound healing and may develop into wide range of complications from mild skin infections to life threatening resistant associated infections.

Staphylococcus aureus, tends to mutate into several strains, giving rise to resistant strains, against not only by our immune system but also by common antimicrobial drugs, leading to severe complications, such complications are usually seen in immunocompromised, delayed wound healing, post-operative complications and Food poisoning.

Staphylococcus is one of the five most common causes of infections after injury or surgery. It affects around 500,000 patients in American hospitals annually. It is abbreviated to “S. aureus” or “Staph aureus” in medical literature. S. aureus was discovered in Aberdeen, Scotland in 1880 by the surgeon Sir Alexander Ogston in pus from surgical abscesses.

The rationale of this study is to bring cost effective fight against Staphylococcus aureus using pure leaf extracts from *Psidium guajava L.* as compared to to the commonly used

antimicrobial Amoxicillin, which has a lot of resistance tied to it, reducing morbidity and mortality associated to it, and hopefully improve the health sector as far as antimicrobial therapy is concerned. Hoping this lay a groundwork for establishing a more solid fight against this organism, to limit it as a normal flora, as it should be.

The researchers of this study are second year medicine students of Southwestern University Matias Aznar Memorial College of medicine. They chose to conduct this research in as much as they want to add knowledge to medicine. Their background on research in their premedical courses and their knowledge on pharmacology and microbiology will prove beneficial to them in conducting this study.

RELATED LITRATURE LITERARURE REVIEW

Psidium guajava L is a fruit-bearing tree commonly known as guava, which belongs to the family *Myrtaceae*. The French call it goyave or goyavier; the Dutch, guyaba, goeajaaba; the Surinamese, guave or goejaba; and the Portuguese, goiaba or goaibeira. Hawaiians call it guava or kuawa. In Guam, it is abas. In Malaya, it is generally known either as guava or jambu batu (Morton, 1987).

Fig. 1: Demonstration of Guava leaves.

Guava grows nearly throughout India up to 1500 m in height and is cultivated commercially in almost all states, the total estimated area being 50,000 hectares. The important guava-growing states in India are Uttar Pradesh, Bihar, Maharashtra, Assam, West Bengal and Andhra Pradesh. Cultivated varieties grow about 10 m in height and produce fruits within 4 years. Wild trees grow up to 20 m high and are well branched. The tree can be easily identified by its distinctive thin, smooth, copper-colored bark that flakes off, showing a

greenish layer beneath. Guava trees have spread widely throughout the tropics because they thrive in a variety of soils, propagate easily and bear fruits quickly. The fruits are enjoyed by birds and monkeys, which disperse guava seeds and cause spontaneous dumps of guava saplings to grow throughout the rainforest (Wealth of India, 2003). The leaves and bark of guava tree have a long history of medicinal uses.

Uses and Properties

It was reported that *Psidium guajava* leaf extract has a wide spectrum of biological activities such as anticough, antibacterial, haemostasis (Jaiarj et al., 1999; 2000), antidiarrhoeal and narcotic properties (Lozoya et al., 1990), and antioxidant properties (Qian and Nihorimbere, 2004).

According to Lutterodt and Maleque (1988) and Meckes et al.(1996), the leaf extract is used to treat diarrhoea, abdominal pain, convulsions, epilepsy, cholera, insomnia and has hypnotic effect.

Some studies reported that the leaf extract and its derivative identified as quercetin has effect on the intracellular calcium levels in gastrointestinal smooth muscle (Lutterodt, 1989; Lozoya et al., 1990), in cardiac muscle cell (Morales et al., 1994; Apisariyakul et al., 1999) and in neuromuscular junction (Re et al., 1999; Chaichana and Apisariyakul, 1996).

Assessment of two medicinal plants, Psidium guajava L. and Achillea millefolium L., in in vitro and in vivo assays: Study on the cytotoxicity and mutagenicity of the plants provide info on its safety for use as therapeutic agents.

Antihypertensives and Antidiarrheal: In the study, P guajava leaf extracts was more active than D mespiliformis in their antagonistic effects on caffeine-induced calcium release from the sarcoplasmic reticulum of rat skeletal muscle. Results might explain their use as antihypertensive and antidiarrheal agents in traditional medicine through an inhibition of intracellular calcium release.

Hypoglycemic / Hypotensive: The leaf of Psidium guajava is used extensively in African folk medicine. The study shows that the aqueous leaf extract of P. guajava possesses hypoglycemic and hypotensive properties and provides pharmacological credence to the folkloric use of the plant for type-2 diabetes and hypertension in some rural African communities.

Anti-Ulcer: Study showed rats pretreated with P guajava extract from fresh tender leaves showed antiulcer activity in aspirin-induced gastric ulcer model with a significant reduction of ulcer index, pepsin activity, free and total acidity, volume and mucus content of gastric juice. antioxidants and reduced oxidative stress and also increase the level of HDL cholesterol significantly.

Antibacterial: Study evaluated the antibacterial activities of aqueous and ethanol-water extracts from leaves, roots and stem bark of P. guajava. The AE of leaves roots and stems were active against gram-positive bacteria Staphylococcus aureus and B. subtilis and virtually ineffective against E. coli and P. aeruginosa. The EW showed higher activity than the AE.

Leaves Extracts / Differences in Hypoglycemic Potential: In a mice model, study showed the water soluble, edible alcohol, and edible alcohol-soluble extracts of wild Psidium guajava leaves may have different hypoglycemic potential.

Hepatoprotective / Leaves: Study in male and female rats showed the aqueous extract of P. guajava leaves may be hepatoprotective (not hepatotoxic), with hematopoietic potentials. Anticancer Activity / Review: Review of a limited number of studies revealed guava extracts may have anti-cancer activity. One study tested guava fruit extract against a proliferation of cancer cell lines. One study in mice used a combination of bark, leaf, and root extract to inhibit growth of B16 melanoma cells.

Corrosion Inhibition / Mild Steel: Study evaluated the corrosion inhibition behavior of an extract of guava leaves towards mild steel in HCl media. Results showed the extract has good inhibition efficiency (IE) and acts as a mixed-type inhibitor. As extract concentration increases, IE also increases.

Hepatoprotective / Leaves: Study evaluated the hepatoprotective activity of P. guajava in CCl4-, paracetamol-and thioacetamide-induced liver injury. Results showed significant reduction of liver enzymes and bilirubin. Higher doses prevented increases in liver weight. Antihyperglycemic/Unripe Fruit Peel: Study evaluated the glycemic potential of an aqueous extract of unripe fruit peel in STZ-induced diabetic rats. Results showed normal, mild, and severely diabetic rat models had hypoglycemic and antidiabetic effect.[28] Analgesic/ Antipyretic/Dried Leaves: Study of an ethanol extract produced significant reduction of pyrexia in yeast induced hyperpyrexia and hot plate latency assay. Analgesic activities were

observed in early and late phase of formalin induced paw licking tests in rats.[29]

Anti epileptic / Leaves: Study evaluated the anti epileptic activity of a leaves extract of P. guajava in seizure induced by maximal electroshock and pantaloon territorialize. Results showed the leaves extract at higher and medium doses produced highly significant and sustained increases in onset of convulsions and decrease in rate of convulsion. Activity may be due to presence of flavonoids and saponins.

Antimicrobial Properties

More than twenty identified compounds from *Psidium guajava* leaf have been reported in (Seshadri and Vasishta, 1965; Osman et al., 1974; Lutterodt and Maleque, 1988). The major components are: β-selinene, β-caryophyllene, caryophyllene oxide, squalene, selin-11-en-4α-ol (Meckes et al., 1996), guaijavarin, isoquercetin, hyperin, quercitrin and quercetin-3-O-gentobioside (Lozoya et al., 1994), morin-3-O-α-L-lyxopyranoside and morin-3-O-α-L-arabopyranoside (Arima and Danno, 2002), β-sitosterol, uvaol, oleanolic acid and ursolic acid (Begum et al., 2004).

Our recent phytochemical screening of *Psidium guajava* leaf showed tannins in aqueous extract, anthocyans, alkaloids, flavonoids, tannins and steroids/terpenoids in ethanolic extract.

Guava is rich in tannins, phenols, triterpenes, flavonoids, essential oils, saponins, carotenoids, lectins, vitamins, fiber and fatty acids. Guava fruit is higher in vitamin C than citrus fruits (80 mg of vitamin C in 100g of fruit) and contains appreciable amounts of Vitamin A as well. Guava fruits are also a good source of pectin (Sunttornusk L, 2005).

The leaves of guava are rich in flavonoids, particularly quercetin. It has demonstrated antibacterial and anti-diarrheal effects and is able to relax the intestinal smooth muscle and inhibit bowel contractions. Guava has antioxidant properties attributed to polyphenols found in its leaves. Leucocyanidin, luectic acid, ellagic acid and amritoside have been isolated from the stem bark. Five constituents, including one new pentacyclic triterpenoid:guajanoic acid and four known compounds-beta-sitosterol, uvaol, oleanolic acid and ursolic acid, have been recently isolated from the leaves of *P. guajava* (Begum et al. , 2004).

Flavanoids

Flavonoids are polyphenolic compounds that are ubiquitous in nature and are categorized, according to chemical structure, into flavonols, flavones, flavanones, isoflavones, catechins,

anthocyanidins and chalcones. Over 4,000 flavonoids have been identified, many of which occur in fruits, vegetables and beverages (tea, coffee, beer, wine and fruit drinks). The flavonoids have aroused considerable interest recently because of their potential beneficial effects on human health-they have been reported to have antiviral, anti-allergic, antiplatelet, anti-inflammatory, antitumor and antioxidant activities.

In Vitro Flavonoids have been shown to have a wide range of biological and pharmacological activities in *in vitro* studies. Examples include anti-allergic,[14] anti-inflammatory,[14,15] antioxidant,[15] anti-microbial (antibacterial,[16,17] antifungal,[18,19] and antiviral[18,19]), anti-cancer,[20][15] and anti-diarrheal activities.[21] Flavonoids have also been shown to inhibit topoisomerase enzymes[22,23] and to induce DNA mutations in the mixed-lineage leukemia (*MLL*) gene in *in vitro* studies.[24] However, in most of the above cases no follow up *in vivo* or clinical research has been performed, leaving it impossible to say if these activities have any beneficial or detrimental effect on human health. Biological and pharmacological activities which have been investigated in greater depth are described below.

In Vivo Flavonoid-rich grape-seed extract has been shown to have antioxidant activity in *in vivo* studies with rats, protecting their gastrointestinal mucosa against the reactive oxygen species generated by acute and chronic stress.[25] In the absence of any additional *in vivo* data, it is impossible to say if these findings are generalizable to all flavonoids. Also, without any clinical studies, it is impossible to say if the antioxidant activity of grape-seed flavonoids offers any protection against oxidative stress in the human gastrointestinal tract.

Research at the Linus Pauling Institute and the European Food Safety Authority shows that flavonoids are poorly absorbed in the human body (less than 5%), with most of what is absorbed being quickly metabolized and excreted. These findings suggest that flavonoids have negligible systemic antioxidant activity, and that the increase in antioxidant capacity of blood seen after consumption of flavonoid-rich foods is not caused directly by flavonoids, but due to increased production of uric acid resulting from excretion of flavonoids from the body

Inflammation has been implicated as a possible origin of numerous local and systemic diseases, such as cancer, cardiovascular disorders, diabetes mellitus and celiac disease.

Preliminary studies indicate that flavonoids may affect anti-inflammatory mechanisms via their ability to inhibit reactive oxygen or nitrogen compounds. Flavonoids have also been

proposed to inhibit the pro-inflammatory activity of enzymes involved in free radical production, such as cyclooxygenase, lipoxygenase or inducible nitric oxide synthase, and to modify intracellular signaling pathways in immune cells.

Procyanidins, a class of flavonoids, have been shown in preliminary research to have anti-inflammatory mechanisms including modulation of the arachidonic acid pathway, inhibition of gene transcription, protein expression and activity of inflammatory enzymes, as well as secretion of anti-inflammatory mediators.

Antibacterial effects of Flavonoids have been shown to have (a) direct antibacterial activity, (b) synergistic activity with antibiotics, and (c) the ability to suppress bacterial virulence factors in numerous *in vitro* and a limited number of *in vivo* studies. Noteworthy among the *in vivo* studies[44,45,46] is the finding that oral quercetin protects guinea pigs against the Group 1 carcinogen *Helicobacter pylori*. Researchers from the European Prospective Investigation into Cancer and Nutrition have speculated this may be one reason why dietary flavonoid intake is associated with reduced gastric carcinoma risk in European women. Additional *in vivo* and clinical research is needed to determine if flavonoids could be used as pharmaceutical drugs for the treatment of bacterial infection, or whether dietary flavonoid intake offers any protection against infectio (McNaught, Alan D; Wilkinson, Andrew; IUPAC (1997).

Tannins

Tannins are polyphenolic compounds that are very complex. Because of the phenol group, the tannins can react with formaldehyde (condensation polymerization) to form thermosetting products that can be used as an adhesive. Antibacterial effectiveness of tannin contained in the leaves of plants such as guava is influenced by the concentration of tannins. The higher levels of tannin antibacterial activity will increase. Because of its importance in the treatment of guava leaf, the quality, safety and benefits should be improved through research and development

Literature of the Organism; *Staphylococcus aureus*

S. aureus causes numerous infections at various sites of the body. Some of these include: -

Skin infections – *S. aureus* causes boils, furuncles, stys, impetigo and other superficial skin infections in humans including Infections of surgical and traumatic wounds from those with chronic illnesses, diabetes, traumatic injuries, burns or immunosuppresed are susceptible to

more severe skin, deeper tissue infections and deep abscesses, such infections can actually be cured or healed by applying the *Psidium guajava* extract topically.

Food poisoning and gastrointestinal tract infections may be caused by consuming food contaminated with S. aureus which could be helped by drinking *Psidium guajava* extract juice.

Pathogenesis

S. aureus expresses quite a few extracellular proteins that are virulent to the host. For the majority of diseases caused by this organism, pathogenesis is multifactorial.

In order to initiate infection the bacteria needs to gain access to the host and attach to host cells or tissues. *S. aureus* has numerous surface proteins that promote attachment to host proteins such as laminin and fibronectin that form part of the extracellular matrix. Fibronectin is also present on epithelial and endothelial surfaces and is also a part of blood clots. The bacteria have a fibrinogen/fibrin binding protein that help it to attach to blood clots and traumatized tissue. This is the reason why *S. aureus* is capable of producing wound infections and post-surgery infections.

S. aureus also has numerous factors that help it to evade the host defence mechanisms. For example, many of the strains carry a polysaccharide on their surface which may help them to resist phagocytosis by macrophages. Protein A is a surface protein of *S. aureus* which binds immunoglobulin G molecules by the Fc region. *S. aureus* can cause severe damage to the host. It makes several types of protein toxins which are probably responsible for symptoms during infections. These toxins damage the membrane of the red blood cells and lead to their breakdown. They also produce leukocidin that causes membrane damage to leukocytes. Systemic release of α-toxin causes septic shock, while enterotoxins and TSST-1 cause toxic shock.

Prognosis

Outcome varies depending on the individuals such as Immunocompromised, or just a simple cutaneous infection. Prognosis among the Immunocompromised varies depending if the individual harbors a resistant strain or not, and whether the individual had an earlier prompt treatment, or delayed. If the individual harbors such organism (resistant strain), without or delayed treatment, than the prognosis is likely poor, unless its just a simple Staphylococcal

infection which is purely cutaneous and hasn't disseminated (Morton, 1987).

Amoxicillin

Amoxicillin (INN), formerly amoxycillin (BAN), and abbreviated amox, Formulations of amoxicillin, a semisynthetic antibiotic, an analog of ampicillin, with a broad spectrum of bactericidal activity against many Gram-positive and Gram-negative microorganisms. Chemically, it is (2S,5R,6R)-6-[(R)-(-)-2-amino-2-(p-hydroxyphenyl)acetamido]-3,3-dimethyl-7-oxo-4- thia-1-azabicyclo[3.2.0]heptane-2-carboxylic acid trihydrate. Amoxicillin is one of the most common antibiotics prescribed for children. The drug first became available in 1972. Amoxicillin is susceptible to degradation by β-lactamase-producing bacteria, which are resistant to a broad spectrum of β-lactam antibiotics, such as penicillin. (http://en.wikipedia.org/wiki/Amoxicillin#Mechanism_of_action)

Indication

To reduce the development of drug-resistant bacteria and maintain the effectiveness of AMOXIL (amoxicillin) and other antibacterial drugs, AMOXIL should be used only to treat infections that are proven or strongly suspected to be caused by bacteria. When culture and susceptibility information are available, they should be considered in selecting or modifying antibacterial therapy. In the absence of such data, local epidemiology and susceptibility patterns may contribute to the empiric selection of therapy. Amoxicillin is indicated in the treatment of infections due to susceptible (ONLY β-lactamase–negative) isolates of the designated bacteria. (http://www.rxlist.com/amoxicillin-drug/indications-dosage.htm)

Drug Interaction

Probenecid decreases the renal tubular secretion of amoxicillin. Concurrent use of amoxicillin and probenecid may result in increased and prolonged blood levels of amoxicillin. Abnormal prolongation of prothrombin time (increased international normalized ratio [INR]) has been reported in patients receiving amoxicillin and oral anticoagulants. Appropriate monitoring should be undertaken when anticoagulants are prescribed concurrently. Adjustments in the dose of oral anticoagulants may be necessary to maintain the desired level of anticoagulation. The concurrent administration of allopurinol and amoxicillin increases the incidence of rashes in patients receiving both drugs as compared to patients receiving amoxicillin alone. It is not known whether this potentiation of amoxicillin rashes is due to allopurinol or the hyperuricemia present in these patients. Amoxicillin may affect the gut flora, leading to

lower estrogen reabsorption and reduced efficacy of combined oral estrogen/progesterone contraceptives. (http://www.rxlist.com/amoxicillin-drug/side-effects-interactions.htm).

Chloramphenicol, macrolides, sulfonamides, and tetracyclines may interfere with the bactericidal effects of penicillin. This has been demonstrated in vitro; however, the clinical significance of this interaction is not well documented.

Side Effects

Side-effects include nausea, vomiting, rashes, and antibiotic-associated colitis. Loose bowel movements (diarrhea) may also occur. Rarer side-effects include mental changes, lightheadedness, insomnia, confusion, anxiety, sensitivity to lights and sounds, and unclear thinking. Immediate medical care is required upon the first signs of these side-effects. The onset of an allergic reaction to amoxicillin can be very sudden and intense; emergency medical attention must be sought as quickly as possible. The initial onset of such a reaction often starts with a change in mental state, skin rash with intense itching (often beginning in fingertips and around groin area and rapidly spreading), and sensations of fever, nausea, and vomiting. Any other symptoms that seem even remotely suspicious must be taken very seriously. However, more mild allergy symptoms, such as a rash, can occur at any time during treatment, even up to a week after treatment has ceased. For some people who are allergic to amoxicillin the side-effects can be deadly.

(http://en.wikipedia.org/wiki/Amoxicillin#Mechanism_of_action)

Local Studies

In the study conducted by the National Institutes of Health, University of the Philippines Manila (2010). Fifty percent (50 percent) solution of the lyophilized leaf crude aqueous extract inhibited the growth of methicillin-resistant Staphylococcus aureus and methicillin-susceptible Staphylococcus aureus. The efficacy of guava as an antimicrobial agent on both MRSA and MSSA strains is greater than vancomycin but lower than fusidic acid. Based on the in vitro results, guava can be considered as a good alternative medication for Staphylococcal infections especially the resistant ones.

Foreign Studies

A study in Bangladesh by Hassan, Shahinuzzaman1, Ali Khan1,Uddin2 and Zaman (2011), that in Ethanol Extracted Guava (Psidium guajava) leave extract protected diarrhoea caused by common Diarrheal causing bacteria up to the level of 55.6%. Whereas the protection level of diarrhoea by loperamide was 75%. The Antimicrobial therapy shows that there is higher

sensitivity on *Staphylococcus aureus* and resistance of *Salmonella* and *Shigella* to Guava leaves. Cytotoxicity study shows wide LC 50, indicating low toxicity level once ingested (Hassan el al, 2011).

Gonclaves, Neto, Bezerra, Macrae, Sousa, Filho & Viera (2008) showed that the antimicrobial effect of essential oils and methanol, hexane, ethyl acetate extracts from guava leaves. The extracts were tested against diarrhea-causing bacteria: *Staphylococcus aureus, Salmonella spp. and Escherichia coli*. Strains that were screened included isolates from seabob shrimp, *Xiphopenaeus kroyeri* (Heller) and laboratory-type strains. Of the bacteria tested, Staphylococcus aureus strains were most inhibited by the extracts. The methanol extract showed greatest bacterial inhibition. No statistically significant differences were observed between the tested extract concentrations and their effect. The essential oil extract showed inhibitory activity against *S. aureus* and *Salmonella spp* (Gonclaves et al, 2008)

Mohamed Ismail, Minhas,Fathima, Khanu, Sahana and Sowmya (2008), The antibacterial testing of Guava (*Psidium Guajava*) leaves extract was carried out by Agar well diffusion method. Amongst the tested extracts the result suggested that methanolic extracts of leaves showed significant antibacterial activity compared with standard drug (liprofloxacin).

The guava leaves exhibited a distinct resistance in some strains of bacteria involved in present study at the concentration of 200ug/ml. In comparison to liprofloxacin the methanolic extract offered significant protection against *Staphylococcus aureus* & *Escherichia coli*. The antibacterial potential exhibited by guava leaves extract may be contributed to the presence of the Flavanoids as found in the preliminary phytochemical investigation (Ismail et al, 2012).

Richard, Joshua and Philips (2013), investigated the effects of *Psidium guajava* on organisms responsible for skin disorders, specifically the fungi *Microsporum gypseum* and *Trichophyton mentagrophytes* and bacteria *Staphylococcus aureus*, and *Staphylococcus epidermidis*. The leaves and bark of the *P. guajava* plant was harvested from Obasa farm Ijero, Ekiti-State, Nigeria during the beginning of rainy season in March, 2009. Aqueous solutions were obtained by grinding the leaves and the bark. Mueller Hinton agar was used to grow the bacteria *S. aureus* and *S. epidermidis* Sabouraud Dextrose broth was used to grow the fungi *Trichophyton mentagrophytes* and *Microsporum gypseum*. Analysis of the antibacterial action of the extracts of guava leaves and bark was carried out at different concentrations, by comparing the mean diameter of the inhibition haloes as a variable. Values were represented

as mean ± S.E. An ANOVA Tukey's test was performed to determine the mean difference between the control and the two treatments (S1 and S2). In comparing the tetracycline positive control to both solutions, tetracycline had a significantly ($p < 0.05$) stronger inhibition effect than both solutions. This could be due to the fact that tetracycline is a pure chemical while the *P. guajava* solutions were crude extracts. Both *P. guajava* solutions were effective against inhibiting the growth of bacteria *S. aureus* and *S. epidermidis*, and fungi *M. gypseum* and *T. mentagrophytes*. This supports the reported use of *P. guajava* in many countries as a traditional herbal medicine (Richard et al, 2013).

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Related Studies

Heller, (2008). Antibacterial Acitivity of Guava, *Psidium guajava* Linnaeus, Leaf extract on diarrhea causing enteric bacteria isolated from seabob shrimp. *Xiphopenaeus kroyeri.*

Statement of the problem

This study aims to investigate the antimicrobial activity of *Psidium guajava L* extract against commonly isolated gram positive Staphylococcus aureus.

General Objective

This study aimed to determine the antimicrobial effect of the pure extract of ***Psidium guajava*** against *S. aureus*

Specific Objectives

1. To determine the minimum dose of concentration that will produce a zone of inhibition against *Staphylococcus aureus*

a. Guava Leaves extract (experimental Group)
b. Amoxicillin (Positive control Group)
c. 80% Methanol (Negative Control Group)

2. To compare the results of antimicrobial effects of *Psidium guajava* extract from the Standard antimicrobial therapy of amoxicillin against *Staphyloccous aureus*.

a. Guava Leaves extract and Amoxicillin
b. Guava leaf extract and 80% ethanol

Statement of null hypothesis

Null hypothesis (H0)

1. There is no significant appearance of the zone of inhibition produced by the specific phytochemicals of Psidium Guajava methanolic extract.
2. There is no significant difference in the proportion of sensitive samples between the Guava leaf extract and 95% methanol on Staphylococcus aureus.

Althernative hypothesis (Hi)

1. There is a significant difference in the zone of inhibition produced by the specific phytochemicals of Psidium guajava methanolic extract.
2. There is a significant difference in the proportion of sensitive samples between the guava leaf extract and 80% methanol on staphylococcus aureus.

Significance of the study

This study seeks to determine the effectiveness of the ethanolic leaf extract of *Psidium guajava L.* in preventing Staphylococcus aureus specific diseases. Several therapeutic effects have been established in the general community. However, this research hopes to add upon the studies of treatment while also providing a more affordable and accessible alternative.

This study will be significant in promoting the use of the plant, Psidium guajava leaves in combating microbial activity and alleviating its signs and symptoms; and encouraging the use of beneficial natural substances. Individuals suffering from infections which were caused by any microbes will have an alternative which will benefit them medicinally and commercially providing a cheaper and accessible Antimicrobial alternative to high-priced, synthesized Antimicrobial Drugs.

Furthermore, the findings of the study may benefit the following:

Patient with S. areus infection

This will give patients, especially who live in rural areas, an alternative
source of herbal medicine to treat their infection. Psidium guajava L. leaf is abundant and this would be a cheap alternative for antibiotics sold in the market.

Pharmaceutical Companies

This study can be used as a basic structure for the development of new drugs that can be effective and inexpensive.

Future Researchers

This study can be a basis for researchers who would like to expound in this topic for further possible health benefits of Guava leaves to other bacteria related diseases.

Chapter 2

RESEARCH METHODOLOGY

Research Design

The type of research design which we will deploy is an experimental study. All the experiments in this study will be done in vitro. The Staphylococcus aureus will be obtained by either from the Hospital or from the Microbiology department. This Research was chosen to because we wanted to observe the growth as well as the zone of inhibition from the specific phytochemicals extract of Guava leaves on S. aureus, with ethanol as the solvent and

subject it to a soxhlet extractor then separation of the solvent from the extract using a rotary vapor.

Research Environment

The environment which we will conduct our experiment will be in *Matias H. Aznar Memorial* College of Medicine laboratory (MHAMCM), Microbiology department for incubation of the organism, Medical technology laboratory for autoclaving and preparation of the agar and college of pharmacy for the extraction process. Practicing of proper waste disposal will be done to keep out of contamination of the environment and proper use of all facilities and equipments,

Research Subjects and Respondents

Laboratory safety precautions will be applied to ensure the well-being of the researchers involved in said experiment.

Bacteriological laboratory experimentation will be performed aseptically and efficiently in order to prevent contamination of the cultures made of Mueller Hinton Agar which was used for susceptibility testing of antibiotics.

Staphylococcal aureus sample will either be obtained from the Microbiology department in the college of medicine or from the hospital laboratories. The sample which includes inoculation techniques will all be handled aseptically using heat sterilization.

Research Instruments

The instruments and tools we used in this study include analytical scales, petridish, stir bar, beaker, beakers, pipettes, Erlenmeyer flask, aluminum foil, sterile cotton swab, filter paper, the rotary vapor extractor and the Soxhlet extraction apparatus.

Materials used in this study is the guava leaves, distilled water, technical ethanol 96%, Tryptic soy broth, MacFarlands standard, and the Mueller hinton agar. The Sample will be quantified based on growing the population in a broth then it will be inoculated in a culture for proper growth. An autoclave will be used for sterilization depending on the type available.

Research Procedure

Phase I (Pre- Experimental phase)

Preparation of the agar

Mueller Hinton Agar

Agar will be prepared by the researchers in the laboratory using the steps below:

1. Prepared by mixing 7.6g of MHA and 200 ml Distilled water, after mixture on a beaker or a large flask, the agar must be heated in a microwave until boiling in order for the components to dissolve completely while stirring.
2. Autoclave, for sterilization purposes
3. cooling period to 45 – 50C
4. dispense into 100 ml sterile plates in 20 ml amounts (4mm depth)
5. allow agar to dry before storage
6. refrigerate

Staphylococcus aureus specimen will be used, and to get a standard concentration of the specimen, the S. aureus will be mixed in a Tryptic Soy Broth.

Tryptic Soy Broth (TSB)

TSI is an all purposes medium that supports the rapid growth of most organisms, including Streptococci without supplements. TSB contains trypticase and phytone as protein sources, sodium chloride for osmotic stability, and glucose as a fermentable carbohydrate and dipotassium phosphate as buffer. TSB contains glucose, which when fermented can lower the pH which can kill other organisms such as Streptococci pneumoniae. Principles of the Procedure

Enzymatic Digest of Casein and Enzymatic Digest of Soybean Meal are nitrogen sources in TSB. Dextrose is the carbon energy source that facilitates organism growth. Sodium Chloride maintains osmotic balance; dipotassium Phosphate is a buffering agent.

Preparation of The culture media

1. 3g of TSB mixed with 100 ml of distilled water
2. Dissolve dehydrated medium in distilled water. Warm slightly to dissolve completely
3. dispense in 4 ml amount in test tubes and cover with cotton plugs
4. Autoclave for 15 minutes
5. Store at room temperature

After the Staphylococcus aureus is mixed on the TSB and left to incubate for 18 to 24 hours, TSB tends to be turbid which indicates that the Staphylococcus aureus bacteria multiplied. The turbidity is compared with the turbidity of the Macfarlands solution.

McFarland standards are used as a reference to adjust the turbidity of bacterial suspensions so that the number of bacteria will be within a given range.
Original McFarland standards were mixing specified amounts of barium chloride and sulfuric acid together. Mixing the two compounds forms a barium sulfate precipitate**,** which causes turbidity in the solution. A 0.5 McFarland standard is prepared by mixing 0.05 mL of 1.175% barium chloride dihydrate ($BaCl_2 2H_2O$), with 9.95 mL of 1% sulfuric acid (H_2SO_4).

After comparison the Staphylococcus aureus was streaked or inoculated on the agar and after it was inoculated in an aseptic manner, a sterilized filter paper will be soaked on the extract and gently pressed on the middle of the petri dishes and then incubated for 18 to 24 hours in an incubator. In the next day a zone of inhibition is seen around the filter paper computing the length of one of inhibition in millimeters differences in terms of different concentrations used.

In terms of preparation of the control, Kirby-Bauer antibiotic testing (KB testing or disk diffusion antibiotic sensitivity testing) is a test which uses antibiotic-impregnated wafers to test whether particular bacteria are susceptible to specific antibiotics. Known quantities of bacteria are grown on agar plates in the presence of thin wafers containing relevant antibiotics. If the bacteria are susceptible to a particular antibiotic, an area of clearing surrounds the wafer where bacteria are not capable of growing (called a zone of inhibition). Amoxicillin will be used as our standard control drug; with 14 – 17mm inhibition indicates intermediate susceptibility while more than 18mm indicates severe susceptibility.

Preparation of the Specimen

The Specimen Staphylococcous aureus will be taken from the Microbiology Laboratory sample specimen from Matias. H Aznar Memorial.

Preparation of the Extract

Extraction of the Guava was done by using a Soxhlet Apparatus. Leaves were inserted within 3 condensers, they were subjected to 80% Ethanol and extracted for nine hours with three cycles in each condenser and the extract was filtered. Then, the extract was separated from

the 80% Ethanol concentrate NS extract by using a Rotatory evaporator.

Fig. 2: Demonstration of a Soxhlet apparatus.

Preparation of the antimicrobial discs

In the experimental group a Sterilized filter paper discs (Whatman No. 1; 6 mm in diameter) soaked in the test tube containing the dissolved extracts of different concentrations were taken out with sterilized forceps and air-dried and placed on plates with the staphylococcus aureus.

Phase II (Experimental phase)

Minimum Inhibitory Concentration determination

1. **Preparation of the Extract:** The Extract is stored at freezing temperature until the day where the serial dilution is conducted.
2. **Preparation of the extract dilution range:** Using a ratio of 1:1 of both the extract and the diluent.
3. **Preparation of agar dilution plates:** The prepared agar was poured in fourteen previously sterilized petri dishes.
4. **Preparation of inoculum:** The prepared dilution range separated to a 14 test tubes using serial dilution method and labeled according to its specific concentration.
5. **Inoculation:** A prepared blank disk with a diameter of 6mm used, dipped in the guava extract and inoculated at the corresponding plate that has the same concentration.
6. **Incubation:** Plates were incubated for 18 to 24hours.
7. **Reading and interpreting results:** Reading and measuring of the plates using vernier caliper and scores were recorded.

Preparation of the experimental group, positive control and negative control

Cultured S. aureus in a Mueller Hinton Agar were inoculated into 60 plates per Group (Test Group, Positive control and negative control). In each petri dish of the positive control an antimicrobial disk (amoxicillin) put at the center of the plate, and for each petri dish of the test group a plain disk immersed in the guava extract and put at the center of the plate, while the negative control left with no antimicrobial activity as plain distilled water.

After an Incubation period of 18 – 24hours, plates were measured using vernier caliper for the zone of inhibition and compared with the standard Kirby bauer chart.

Statistical Analysis

In terms of Treatment of the Data, The statistical tool we will be using in our research paper is the Z test for two samples since the negative control has a value of zero therefore it is not significant. It is a statistical test for which the distribution of the test statistic under the null hypothesis can be approximated by a normal distribution.

Antimicrobial Assay

Antimicrobial Susceptibility Test Procedure (Kirby- Bauer Method)

Standard for Amoxicillin against staphylococcus aureus

Table 1: Control Group. Source:

Sensitive(Amoxicillin) 0.25ug	Moderate	Resistant
>18 mm	14 - 17mm	<13mm

Standard for Guava leaf extract against staphylococcus aureus

Table 2: Experimental Group. Source:

sensitive (Guava extract)	Moderate	Resistant
>13mm	12mm – 9mm	0 – 8mm

Definition of Terms

1. **Moderate inhibition:** Inhibition of growth of Staphylococcus aureus ranging from 14 to 17mm using Amoxicillin and 21 – 28mm using Flavonoids.
2. **Psidium guajava:** Apple guava or the common guava.
3. **Psidium guajava leaf extract:** Extract of the leaves of the guava.
4. **Resistant:** Mild to no inhibition of Staphylococcus aureus, less than 13mm using Amoxicillin and less than 20mm using Flavonoids.
5. **Susceptibility:** Inhibition of the growth of Staphylococcus aureus, with above 18mm

using Amoxicillin and 29mm if with Flavonoids.

6. **Zone of inhibition:** The clear region around the paper disc saturate with an antimicrobial agent on the agar surface.

80% Ethanol

Antimicrobial activity: Is the property of psidium guava leaf extract to inhibit or kill staphylococcus aureus in vitro.

Culture: Is the microbiology technique of growing bacteria specifically staphylococcus aureus in an artificial environment like the culture media.

Staphylococcus aureus - is the organism used to demonstrate the antimicrobial property of the 80% ethanolic guava leaf extract.

Amoxicillin - is the positive control which is active against staphylococcus aureus strains.

Confidence level - This reflects the confidence with which you would like to detect a significant difference between the two proportions.

Chapter 2

RESULTS, DISCUSSION AND DATA ANALYSIS

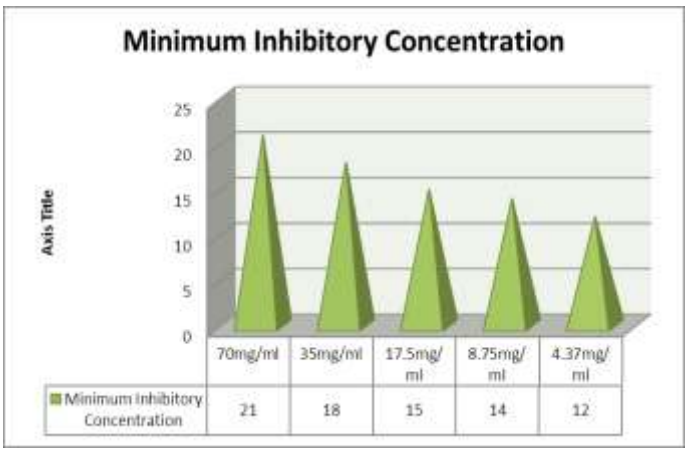

Fig 3: A chart showing the minimum inhibitory concentration used in this study.

Cultured S. aureus were inoculated into 60 plates per Group (Test Group, Positive control and negative control). In each petri dish of the positive control an antimicrobial disk (amoxicillin) put at the center of the plate, and for each petri dish of the test group a plain disk immersed in the guava extract and put at the center of the plate, while the negative control left with no antimicrobial activity as plain distilled water.

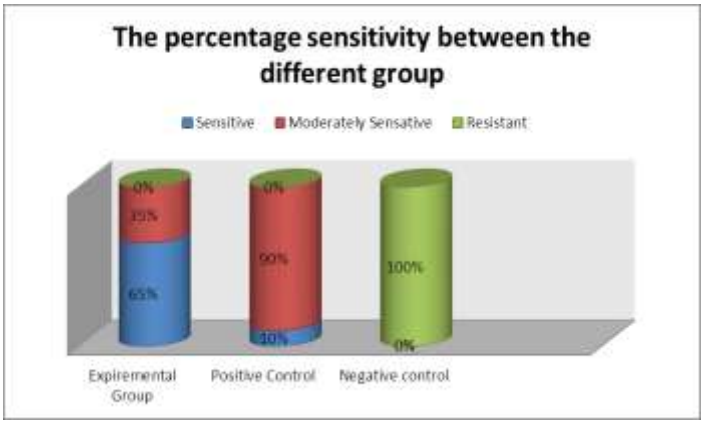

Figure 4: A chart showing the percentage sensitivity between different groups used in this study.

After 24 hours incubation, zone of inhibition were read and recorded. Amoxicillin exhibited an intermediate zone of inhibition with a percentage of 10%, while the Guava leaf ethanolic extract had the greatest zone of inhibition with an average of 65%. The distilled water negative control did not show any zone of inhibition.

The different activities of each test group shown in the table. Amoxicillin showed 10% sensitivity to S. aureus, on the other hand, Guava leaf ethanolic extract showed 65% sensitivity. The negative control showed 100% resistance.

Investigating the antimicrobial effect of guava leaves involved a comparison of its 80% Ethanolic extract with commercially available antibiotics by comparing the inhibition zones. We have noted that the Guava extract showed a larger inhibitory effect than commercially available antibiotic. This is not surprising and reinforces the position that Guava leaf extract should be used in treatments whenever available.

The demonstration of activity of the extract against both gram positive and gram negative bacteria is an indication of the broad spectrum of activity and thus can be used to source antibiotic substances for drug development that can be used in the control of these bacterial infections.

Table 3: A tabulated form of the computation of the Z value.

Groups	Proportion of sensitive cases	Computed z-score	Critical z value at 0.05	p-value	Decision	Interpretation
Sample	0.65	6.223	±3.291	0.000	Reject Ho	Significant
Positive	0.1					
p < .05 = Significant						

This study used Z test two sample formula to determine the proportion of sensitivity between the different groups and to calculate the Z value. The proportion of sensitivity for the guava extract is 0.65 and the proportion for the Positive Control is 0.1, knowing that the negative Control had no value and hence there is no propensity for it.

The computed Z value is 6.223 while the critical Z value for this study at 0.05 is ±3.291, this means that the decision is rejected, and there is a significant different between the test sample and the commercially drug used, therefore it oppose our null hypothesis.

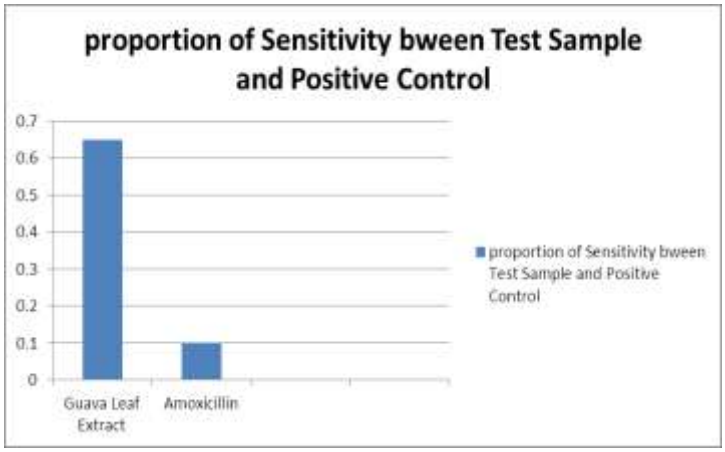

Figure 4: This chart shows the proportion sensitivity between test sample and positive control.

On the other hand, the antibacterial activity of guava leaf ethanolic extract is due to its phytochemical content. The antibacterial activity exhibited by the extract may be associated with the presence of some natural plant substances namely, alkaloids, tannins, saponins, anthraquinons in addition to flavonoids which was known to be responsible for the antimicrobial properties of some ethanolic medical plants. The mechanism of activity of flavonoids include their ability to complex with extracellular and soluble proteins and to complex with bacterial cell walls as well as the ability of lipophilic flavonoids to disrupt microbial membranes, while that of alkaloids is attribiuted to their ability to intercalate with bacterial DNA.

The action of tannins maybe related to their ability to inactivate microbial adhesions, enzymes, cell envelope transport protein and the ability to complrx with polysaccharides.

In order to illustrate the significant difference in the proportion of sensitive organisms between the different groups of this study, sensitivity of Staphylococcus aureus on the three

groups – Guava leaf ethanolic extract, Amoxicillin as the positive control and the negative control were compared.

S. aureus plates demonstrated 10% highly sensitivity to Amoxicillin; 90% highly sensitivity for guava leaf ethanolic extract and 100% resistance for the negative control.

Chapter III

SUMMARY OF FINDINGS, CONCLUSION AND RECOMMENDATION

Summary of findings

The study was conducted to determine the antibacterial activity of *Psidium guajava L* 80% ethanolic leaf extract against *Staphylococcus aureus.*

Using the proportion sensitivity, the percent population of the extract that showed a zone of inhibition that is sensitive, moderlately sensitive and resistant was obtained.

With *Staphylococcus aureus,* the Kirby bauer test result showed that Amoxicillin (0.25mg) had 10% sensitive susceptibility against *S. aureus*. While Guava leaf extract showed highest sensitivity with 65% sensitivity and the negative control distilled water did not show any antibacterial activity. Moreover, among the three treatments only Guava leaf extract have showed highest antibacterial activity with 65% of the total proportion, the positive control showed an intermediate activity with a 10% sensitivity of the total proportion and it was also evident that the absolute ethanol had showed 100% resistance against S. aureus.

In the terms of the proportion of sensitive plates, the results showed that there is a significant antimicrobial activity of the Guava leaf extract against S. Aureus compared to the amoxicillin.

CONCLUSION

As the rapid emergence of drug-resistant organisms necessitates the continuous search of new antimicrobial substances, natural products may act as alternative for antibiotics in certain circumstances. The results showed that the 80% ethanolic extract of P. guajava leaf was able to inhibit all of the bacteria used in this study with different degree of inhibition. The information obtained may provide validation for its reported medicinal uses.

In conclusion, the guava leaf 80% ethanolic extract is more effective against the staphylococcus aureus than the commercially antibiotic used amoxicillin and the negative

control.

Based on the results and statistical analyses, it is therefore concluded that guava leaf extract has a potential antimicrobial activity against staphylococcus aureus preventing its growth and spread of its colonies.

This antimicrobial activity could be attributed to the presence of Plant Natural substances like tannins, alkaloids, saponins, anthraquinons and flavonoids which has been proven to be present in guava leaves using the phytochemical screening tests.

After the statistical analysis it showed that there were significant differences between the sensitive proportion between the Guava leaf extract and the positive controls (amoxicillin), therefore concludes that the extract have an effective antimicrobial activity which is higher than the amoxicillin concluding that it can be applied as an alternative for amoxicillin agains S. aureus for the treatment of diseases.

RECOMMENDATION

In view of the above findings and conclusions, the following recommendations are suggested:

1. In vivo application of psidium guajava leaf extract as an antibacterial agent in experimental animals.
2. Determine the minimum inhibitory concentration of guava plant against MRSA.
3. In vitro application of a variety of guava plant extracts to have a further study on its antimicrobial activity and conduct a comparison.
4. Encourage researches to use other parts of the guava plant such as the roots, fruits, flower, stem and young leaves in order to have a further study on its antibacterial activity against MRSA.
5. Conduct other research studies including different organisms which are claimed to be sensitive against guava plant extract
6. Evaluate the toxicity level of the guava leaf extract.
7. Isolation of specific substance which is claimed to have the antimicrobial property in guava leaves.

APPENDIX II

Kingdom	Plantae
Subkingdom	Tracheobionta
Superdivision	spermatophyte
Division	Mangoliophyla
Class	Mangoliopsida
Subclass	Rosidae
Order	Myrtales
Family	Myrtaceae
Genus	Psidium L.

APPENDIX III A

Sample size determination of two proportions

Confidence level is 95%

Power = 80%

Proportion of group 1 = 70

Proportion of group 2 = 90

Recommended sample size = **59**

Actual Sample size used = **60**

$$n = (Z_{\alpha/2}+Z_{\beta})^{2} * (p_1 (1-p_1) + p_2 (1-p_2)) / (p_1-p_2)^{2}$$

$$= \frac{(1.96 + 0.84)^2 * (0.70(1-0.70) + 0.90 (1 - 0.90))}{(0.70 - 0.90)^2}$$

$$= \frac{(2.8)^2 * (0.70 * 0.30) + (0.90 * 0.10)}{(-0.2)^2}$$

$$= \frac{(2.8)^2 * (0.21 + 0.09)}{0.04}$$

$$= \frac{7.84 * 0.3}{0.04}$$

$$= \frac{2.352}{0.04}$$

= **58.8 » 59 » 60**

APPENDIX IIIB

Statistical computation for proportion

A. Simple Percentage

% = X/N * 100

Where:

% = Percentage

X = Frequency of Reaction (Sensitive, Moderately Sensitive and Resistant)

N = Total Number of Plates

P = Total number of plates with sensitivity susceptibility x 100

Total number of Plates.

1) Control (Amoxicillin) = 60 plates

Sensitive = 6/60 * 100 = 10% are sensitive

Moderately Sensitive = 54/60 * 100 = 90% are Moderately Sensitive

Non are Resistant.

2) Test Sample (Guava Extract) = 60 plates

Sensitive = 39/60 *100 = 65% are Sensitive

Moderately Sensitive = 21/60 * 100 = 35% are Moderately Sensitive

Non are Resistant.

3) Negative Control (Distilled Water) = 60 plates

Sensitive = 0/60 * 100 = 0

Moderately Sensitive = 0/60 *100 = 0

Resistant = 60/60 * 100 = 100 % are Resistant

APPENDIX III C

Statistical computation for Z – test

(Two Sample Proportion)

$$z = \frac{\hat{p}_1 - \hat{p}_2}{\sqrt{\frac{\hat{p}_1(1-\hat{p}_1}{n_1} + \frac{\hat{p}_2(1-\hat{p}_2)}{n_2}}}$$

Where

$P^1 - P^2$ = Proportion from groups 1 and group 2, respectively.

P = Proportion of subjects with attributes in two groups.

Q = P – 1

n^1 and n^2 = number of subjects in groups 1 and 2, respectively.

Computation between Guava Leaf Extract and Amoxicillin

$P^1 = 39/100$

$= 0.65$

$p^2 = 6/60$

$= 0.1$

$$P = \frac{60 + 39}{60 + 6}$$

$= 1.5$

$$Z = \frac{(0.65-0.1)}{1.5(1-1.5)\ 1/60 + 1/60}$$

=

APPENDIX III D

Statistical computation for the guava extract

Extract

300 grms (leaves): 2100ml (Ethanol) = X : 343mL (extract)

- 300 grms (343mL) = 2100mL (X)
- X = 49 grams

Serial Dilution

- 49grams per 343mL » 0.14 grams per 1 mL
- 0.14 gram x 1000 = 140 mg (extract per mL)
- Dilute to first test tube 140 mg / 2 = 70mg
  - 70 mg / 2 = 35mg
  - 35 mg / 2 = 17.5 mg
  - 17.5 mg / 2 = 8.75 mg.

Our Minimum Inhibitory concentration is 8.75 mg.

APPENDIX IV

Kirby Bauer Sensitivity Interpretation

80% Guava leaf extract (psidium guava) compared to amoxicillin and the negative control.

Table 4: Zone of inhibition scores between the different groups.

Sample	Result	Interpretation	Positive Control (10mcg Amoxicillin)	Result	Interpretation	Negative Control (Distilled water)	Result	Interpretation
1	13mm	Sensitive	**1**	16mm	ModSensitive	**1**	0mm	Resistant
2	12.5m	ModSensitive	**2**	16mm	ModSensitive	**2**	0mm	Resistant
3	18mm	Sensitive	**3**	17mm	ModSensitive	**3**	0mm	Resistant
4	15mm	Sensitive	**4**	18mm	Sensitive	**4**	0mm	Resistant
5	11mm	ModSensitive	**5**	18mm	Sensitive	**5**	0mm	Resistant
6	12.5m	ModSensitive	**6**	16mm	ModSensitive	**6**	0mm	Resistant
7	19mm	Sensitive	**7**	16mm	ModSensitive	**7**	0mm	Resistant
8	13mm	Sensitive	**8**	17mm	ModSensitive	**8**	0mm	Resistant
9	13mm	Sensitive	**9**	18mm	Sensitive	**9**	0mm	Resistant
10	13mm	Sensitive	**10**	15mm	ModSensitive	**10**	0mm	Resistant
11	14mm	Sensitive	**11**	16mm	ModSensitive	**11**	0mm	Resistant
12	13mm	Sensitive	**12**	18mm	Sensitive	**12**	0mm	Resistant
13	16mm	Sensitive	**13**	18mm	Sensitive	**13**	0mm	Resistant
14	14mm	Sensitive	**14**	16mm	ModSensitive	**14**	0mm	Resistant
15	19mm	Sensitive	**15**	17mm	ModSensitive	**15**	0mm	Resistant
16	12.5m	ModSensitive	**16**	17mm	ModSensitive	**16**	0mm	Resistant
17	16mm	Sensitive	**17**	19mm	Sensitive	**17**	0mm	Resistant
18	18mm	Sensitive	**18**	16mm	ModSensitive	**18**	0mm	Resistant
19	18mm	Sensitive	**19**	16mm	ModSensitive	**19**	0mm	Resistant
20	14mm	Sensitive	**20**	16mm	ModSensitive	**20**	0mm	Resistant
21	15mm	Sensitive	**21**	16mm	ModSensitive	**21**	0mm	Resistant
22	12.5m	ModSensitive	**22**	16mm	ModSensitive	**22**	0mm	Resistant
23	12.5m	ModSensitive	**23**	16mm	ModSensitive	**23**	0mm	Resistant

24	15mm	Sensitive	**24**	16mm	ModSensitive	**24**	0mm	Resistant
25	18mm	Sensitive	**25**	16mm	ModSensitive	**25**	0mm	Resistant
26	12.5m	ModSensitive	**26**	16mm	ModSensitive	**26**	0mm	Resistant
27	21mm	Sensitive	**27**	16mm	ModSensitive	**27**	0mm	Resistant
28	14mm	Sensitive	**28**	16mm	ModSensitive	**28**	0mm	Resistant
29	14mm	Sensitive	**29**	16mm	ModSensitive	**29**	0mm	Resistant
30	12.5m	ModSensitive	**30**	16mm	ModSensitive	**30**	0mm	Resistant
31	13mm	Sensitive	**31**	16mm	ModSensitive	**31**	0mm	Resistant
32	11mm	ModSensitive	**32**	16mm	ModSensitive	**32**	0mm	Resistant
33	16mm	Sensitive	**33**	16mm	ModSensitive	**33**	0mm	Resistant
34	14mm	Sensitive	**34**	16mm	ModSensitive	**34**	0mm	Resistant
35	12.5m	ModSensitive	**35**	16mm	ModSensitive	**35**	0mm	Resistant
36	12mm	ModSensitive	**36**	16mm	ModSensitive	**36**	0mm	Resistant
37	14mm	Sensitive	**37**	16mm	ModSensitive	**37**	0mm	Resistant
38	18mm	Sensitive	**38**	16mm	ModSensitive	**38**	0mm	Resistant
39	17mm	Sensitive	**39**	16mm	ModSensitive	**39**	0mm	Resistant
40	10mm	ModSensitive	**40**	16mm	ModSensitive	**40**	0mm	Resistant
41	13mm	Sensitive	**41**	16mm	ModSensitive	**41**	0mm	Resistant
42	18mm	Sensitive	**42**	16mm	ModSensitive	**42**	0mm	Resistant
43	12mm	ModSensitive	**43**	16mm	ModSensitive	**43**	0mm	Resistant
44	15mm	Sensitive	**44**	16mm	ModSensitive	**44**	0mm	Resistant
45	18mm	Sensitive	**45**	16mm	ModSensitive	**45**	0mm	Resistant
46	15mm	Sensitive	**46**	16mm	ModSensitive	**46**	0mm	Resistant
47	12mm	Modsensitive	**47**	16mm	ModSensitive	**47**	0mm	Resistant
48	11mm	Modsensitive	**48**	16mm	ModSensitive	**48**	0mm	Resistant
49	16mm	Sensitive	**49**	16mm	ModSensitive	**49**	0mm	Resistant
50	14mm	Sensitive	**50**	16mm	ModSensitive	**50**	0mm	Resistant
51	14.8m	Sensitive	**51**	16mm	ModSensitive	**51**	0mm	Resistant
52	12.7m	Modsensitive	**52**	16mm	ModSensitive	**52**	0mm	Resistant
53	12.5m	Modsensitive	**53**	16mm	ModSensitive	**53**	0mm	Resistant
54	15mm	Sensitive	**54**	16mm	ModSensitive	**54**	0mm	Resistant

55	20mm	Sensitive	**55**	16mm	ModSensitive	**55**	0mm	Resistant
56	11mm	Modsensitive	**56**	16mm	ModSensitive	**56**	0mm	Resistant
57	10mm	Modsensitive	**57**	16mm	ModSensitive	**57**	0mm	Resistant
58	13mm	Sensitive	**58**	16mm	ModSensitive	**58**	0mm	Resistant
59	10mm	Modsensitive	**59**	16mm	ModSensitive	**59**	0mm	Resistant
60	12.5m	Modsensitive	**60**	16mm	ModSensitive	**60**	0mm	Resistant

Guava Extract: ***Resistant*** = 0 – 8mm; ***Moderately Sensitive*** = 9 – 12mm; ***Sensitive*** >13mm

Amoxicillin: ***Resistant*** = <13; ***Moderately Sensitive*** = 14-17mm; ***Sensitive*** >18mm

APPENDIX V

Phytochemical Screening

A. Alkaloid

To 100 mg of extract small quantity of Wagner's reagent [Solution of iodine in potassium iodide] was added, Presence of reddish brown precipitate if alkaloids are present.

To 100 mg of extract small quantity of Hager's reagent [saturated solution of Picric acid] was added. Formation of yellow precipitate indicated the presence of alkaloids.

B. Tannins

To 5 Ml of extract few drops of 5% FeCl3was added. Presence of deep blue black color indicated the presence of tannins.

C. Flavonoids

To 5 Ml of extract 5 Ml of 95% ethanol was added along with dilute HCl from sides of test tube. Few fragments (0.5 g) of magnesium turnings were also added. Presence of slight pink color indicated the presence of flavonoids.

To 5 Ml of extract few drops of NaOH solution was added. Formation of an intense yellow color, which turns to colorless on addition of few drops of dil. H2SO4 indicated the presence of flavonoids.

D. Proteins

A little extract was taken with 2 Ml of water and 0.5 Ml of concentrated HNO3 was added to it. Yellow color is obtained if proteins are present.

To 5 Ml of extract 4% NaOH was added along with few drops of 5% CuSO4 solution. Violet or pink color appeared indicated the presence of proteins.

E. Fixed Oils

Small quantity of extract was pressed the between two filter papers, the stain on 1st filter paper indicated the presence of fixed oils.

F. Triterpenoids

Extract (5 mL) was treated with 5 mL CHCl3 with few drops of conc. H2SO4, shaked well and allowed to stand for some time. Formation of yellow colored lower layer indicated the

presence of triterpenoids.

G. Carbohydrate

Table 5: The Phytochemical screening test performed in this study.

Table 1. Phytochemical Screening Test Results			
Plant constituent	**Expected Results**	**Actual Results**	**Interpretation of results**
Flavonoids	Presence of slight pink color	No Color change	Negative
Alkaloids	Orange brown precipitate	Orange – Brown precipitate	Positive
Tannins	Appearance of a White precipitates	Appearance of a White precipitates	Positive
Fixed oils	Stain on 1st filter paper	Presence of stain	Positive
Proteins	Yellow color is obtained	No Color change	Negative
Carbohydrate	Violet ring formed at the junction	Violet ring formed at the junction	Positive
Triterpenoids	Formation of Yellow color	Dark Deep Yellow	Positive

In a test tube containing 5 mL of extract, few drops of freshly prepared 10% alcoholic solution of α- naphthol was added and shaken/stirred for few min. Then 5 mL of conc. H2SO4 was added from sides of the test tube. Violet ring was formed at the junction of two liquids, indicated the presence of carbohydrates.

APPENDIX VI

Time Table

Table 6: Time table of the activities done since this research started.

Date of Research Activities	Research Activities
June 2013	Research Grouping with corresponding leader and group members were identified
July 2013	Each group member submitted a possible research topic
september 25, 2013	Submission of the Proposal
Nov 20, 2013 to Nov 31, 2013	Buying of the materials (Guava leaves), Antibiotic (Positive Control)
December 1, 2013 to December 2, 2013	Inquiring of the agar powders and the hopefully the specimen from the Microbiology department or the Medical technology Department
December 6, 2013	Orientation to the Pharmacology Department and laboratories
Feb 12, 2014	Start of the Extraction
Feb 21, 2014 to Feb 22, 2014	Serial dilution and Preparation of Agars and Petridishes
Feb 22, 2014	Testing of the ratios on the agars for the Minimum inhibitory concentration
March 1, 2014 to December 14, 2013	Checking of the results and choosing the lowest ratio that causes the Minimum inhibitory concentration, and Start of

	Antimicrobial thearpy (+, - control), first 10 samples
March 2, 2014	Reading of the results for the first fourteen samples, including the distance of inhibition (either minimal, moderate or maximal inhibition or sensitive, moderate or resistant)
March 2, 2014	Continuing of the Antimicrobial therapy on the rest of the samples
March 2, 2014	Reading of the rest of the samples (possibly 60 more)
March 10, 2014	Use of the Z test to determine whether there is a significance inhibitory activity

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