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ANTIBACTERIAL ACTIVITY, PHYTOCHEMICAL SCREENING AND TOXICITY STUDIES OF *Anogeissus leiocarpus* AND *Psidium guajava* ON SOME PATHOGENIC BACTERIA

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DEDICATION

This dissertation is dedicated to my family. May almighty Allah forgive and grant them Al-Jannatul Firdausi. Amen.

CERTIFICATION

This dissertation by AHMAD, Samira Yabo (1421307011) has met the requirement for the award of the Master of Science (Microbiology) in the faculty of science of Usmanu Danfodiyo University Sokoto, and is approved for its contribution to knowledge.

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ABSTRACT

This research determines the antibacterial activity of *Anogeissus leiocarpus* and *Psidium guajava* on *Escherichia coli* and *Staphylococcus aureus* isolated from clinical samples of patients attending Specialist Hospital Sokoto. The plants were obtained from Usmanu Danfodiyo University farm, Sokoto. The leaves of the plants were used. The plants leaves were extracted using Hexane, Methanol, Ethanol and Water. Various concentration were prepared (50, 25, 12.5 and 6.25mg/ml) sensitivity test was carried out using the crude extracts in various solvents. Agar well (Ditch) method of sensitivity was used. This was followed by the determination of minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC). The plants were also screened for phytochemical constituents and components were fractionated using column chromatography. The separated components were reassessed for antibacterial activity. Toxicity of the plants were determined using albino rats. The result of the sensitivity test showed the leaves extract of *Psidium guajava* is active against *Staphylococcus aureus* and *E. coli* with the highest zones of inhibition of 19mm and 9mm respectively. The minimum inhibitory concentration (MIC) was 12.5mg/ml for *S. aureus* while the minimum bactericidal concentration (MBC) was 25mg/ml for *S. aureus.* The phytochemicals screening revealed the presence of many phytochemicals including Alkaloids, flavonoids, tannins, saponins, etc. The column chromatography revealed five (5) different fractions with only fraction F4 having antibacterial activity against the *S. aureus*. The LD_{50} was above 5000mg/kgbw, hence consuming the leaves of *P. guajava* was found acutely harmless.

CHAPTER ONE

1.0 INTRODUCTCION

Antibiotic resistance is the ability of a microorganism to withstand the effect of antibiotic, it is a specific type of drug resistance and it has become a global challenge to health care providers. In recent years there is an increasing incidence of multiple antibiotic resistance among diseases causing microorganisms, largely due to the indiscriminate use of antimicrobial drugs commonly employed for the treatment of infectious diseases. The implication is that many antibiotics have failed in the treatment of some infectious diseases. This has forced scientist to search for new antimicrobial substances from various natural sources like medicinal plants (Parekh and Chanda, 2007).

Traditional medicine survived through cultural decade, providing indigenous basis of therapy for generations. In Africa, about 80% of the inhabitants rely on traditional medicines for therapeutics and well-being. In developed nations where chemotherapeutics are in the midstream of drug therapy, medicinal plants have often been used in complementary or alternative medicine (WHO, 2003). Traditional medicines practitioners use a variety of herbal preparations to treat different kinds of ailments, including microbial infections.

The World Health Organization (WHO) has catalogued more than 20,000 plant species with medicinal properties providing treatments for ailments such as pneumonia, ulcers, diarrhea, bronchitis, colds and diseases of the respiratory tract. One method, amongst the many ways in which plants are used, is to extract and consume essential plant oils. Essential oils are complex chemical mixtures, typically composed of more than a hundred compounds, by and large, are responsible for plant

aromas. The compounds are obtained from different parts of the plant; flowers, leaves, seeds, bark and tubers and many have medicinal properties. (Aridogan *et al.,* 2002). The plants used in this study are *Anogeissus leiocarpus* and *Psidium guajava.*

Anogeissus leiocarpus is a decidous tree growing up to 30m in height, typically 15-18m with light green foliage, Leaves are alternate to sub-opposite, elliptic to ovate-lanceolate in shape, and 2-8cm long and 1.5-3.5cm across (Klaus *et al.,* 2004). The stem are finely pubescent, the bark is grey to beige in colour, becoming blackish with age, and fibrous with thin scales. *Anogeissus leiocarpus* contains about 40 wind dispersed seeds of 10g each (Klaus *et al.,* 2004). (Appendix I)

Psidium guajava called guava is an evergreen small tree. The leaves are 2-6inches long and 1-2 inches wide, aromatic when crushed, and appeared dull-green with stiff but coriaceous with pronounced veins. *P. guajava* (guava) is commonly known for its food and nutritional values, the medicinal properties of the leaves are also well known in traditional system of medicine (Garode and Waghode, 2014). *P. guajava* is popularly used in Hausa community for the treatment of infant diarrhea caused by microorganisms.

Escherichia coli is the bacterial species most commonly recovered in the clinical laboratories and has been incriminated in infectious diseases involving virtually many human tissue and organ system. *E. coli* although an opportunistic pathogen, is one of the common organisms involved in gram-negative sepsis and endotoxin- induced shock. Urinary tract, wound infections, pneumonia in immunosuppressed hospitalized patients, and meningitis in neonates are other common infections caused by *E. coli* (Todar, 2008).

S. aureus is by far the most important human pathogen among the Staphylococci. Although this organism is frequently a part of the normal human microflora, it can

cause significant opportunistic infections under the appropriate conditions. (Jacquelyn, 2005).

The screening of plant extracts for antimicrobial activity has shown that higher plants represent a potential source of novel antibiotic. Considering these assumptions of medicinal values in plants, this study was conducted to determine the antibacterial potentials and phytochemical constituents of the crude extracts of *Anogeissus leiocarpus* and *Psidium guajava* leaves on selected pathogenic bacteria namely: *Escherichia coli* and *Staphylococcus aureus.*

1.1 Statement of the research problem.

Infectious diseases are one of the leading cause of premature death. And they account for approximately one-half of all death in the tropics (Iwu *et al.,* 1999). Resistance to antimicrobial agents is the major global health problem (Ibezim, 2005). Synthetic drugs are expensive, not always available, the good ones are not available and affordable in rural areas and are also associated with adverse side effect. This ugly development has strengthened the search for new drugs that are cheap, available, and affordable and with no or less side effects.

1.2 Justification for the research

Many infectious diseases are known to be treated with herbal medicines throughout the human civilization. Even today, plant materials continue to play major role in primary health care (Mann, 2012). Various part of the plants has 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.

Anogeissus leiocarpus and *Psidium guajava* ranked top in the list of medicinal plants in Northern Nigeria for their therapeutic benefits. However, not much research have been done to scientifically evaluate and reconcile the antibacterial potential of these plants with traditional claims, and of course to determine their level of safety. Previous studies, using these plants were limited at crude antibacterial activity and phytochemical screening. Toxicity studies and Screening of phytochemical components responsible for activity was not considered. Thus, this study will carry out toxicity studies and activity guided analysis of these plants components, with a view to address the gaps in the previous studies.

1.3 Aim and objectives

The study was aimed at determining the antibacterial potential and phytochemical constituents of the crude leaves extracts and fractions of *Anogeissus leiocarpus* and *Psidium guajava* (guava) against *Staphylococcus aureus* and *Escherichia coli* because of their close proximity with man.

The objectives of the study are:

1. To isolate and identify bacterial isolates from clinical samples.
2. To determine the antibacterial activity of the crude extracts of *Anogeissus leiocarpus* and *Psidium guajava* against the isolated bacteria.
3. To determine the minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) of the most active extract.
4. To identify the phytochemical constituents of the *Anogeissus leiocarpus* and *Psidium guajava* leaves and to fractionate the most active crude extract.
5. To carry out toxicity studies of the plant extract on albino rats.

CHAPTER TWO

2.0 LITERATURE REVIEW

2.1 Medicinal uses of the plants

2.1.1 *Anogeissus leiocarpus*

Anogeissus leiocarpus is used by the traditional medicine practitioners in the treatment of various conditions such as typhoid, diarrhea, fever, rheumatism, cough and skin diseases. Aqueous decoction of *A. leiocarpus* produces rapid relief from gastrointestinal disturbance (Burkill, 2004). In Nigeria some parts of the tree are boiled in water together with potash and the liquid is taken as a cure for stomach pains and for Schistosomiasis. The wood of *A. leiocarpus* is well appreciated as a carving wood and is used for construction and tool handles because it is fairly insects and termite resistant (Burkill, 2004). A cool infusion is considered palatable beverage and a decoction is given to a new-born baby to drink. The roots are used as chewing sticks for cleaning teeth, and the leaves as fodder for small ruminants. The bark itself is chewed to obtain the gum. Powdered bark is applied to wound and ulcers and a lotion made from the bark is similarly used. Roots-bark is considered by the soce natives of Senegal to be stimulant and aphrodisiac. The back have been recorded having 17% tannin, not a high enough concentration for profitable export, but usable locally for tanning (Burkill, 2004).

2.1.2 *Psidium guajava*

Psidium guajava is one of the plants in folk medicine that has been used for the management of various disease conditions and is believed to be active. The leaves and bark of the guava plant have been used to treat diarrhea, other gastrointestinal

disorders, toothaches, colds, and swelling, in areas such as Dutta *et al.,* (2000) in Africa, Rabe and van Staden., (1997), Lin *et al* (2002) in Hawaii, and Richard *et al*., (2013).

Guava is used for skin disorders such as an astringent for acne, rashes and ringworm. The southeast Nicaraguan indigenous communities of Cuna and Waunana, make tea from the leaves and bark of the guava for treatment of diarrhea and dysentery, while the Samu, Panamakha, and Bawihka tribes use it for upset stomachs, Vertigo, and to regulate menstrual cycles. Guava leaves and fruit juice has also been tested in treatment of infantile diarrhea and the results showed that, those who were treated with guava recovered at 3 days which was shorter than the controls and the study concluded that guava had good curative effect on infantile retroviral enteritis (Wei *et al*., 2000).

Bark and leaf extracts were shown to have *in vitro* toxic action against numerous bacteria. Gallocatechin isolated from the methanol extract of guava leaf showed anti mutagenic activity against *Escherichia coli (*Manosroi *et al.,* 2006). The antimicrobial activities of *P. guajava* and leaf extracts, determined by disk diffusion method (zone of inhibition), were compared to tea tree oil (TTO), doxycycline and clindamycin antibiotics. It was shown that *P. guajava* leaf extracts might be beneficial in treating acne especially those that have anti-inflammatory activities (Qadan *et al,* 2005). The active flavonoid compound - quercetin-3Oalpha- l-arabinopyranoside (guaijaverin) - extracted from leaves has high potential anti-plaque activity by inhibiting the growth of *Streptococcus mutans* (Limsong *et al.,* 2004). Guava leaf extract inhibited the growth of *Staphylococcus aureus* in a study carried out by disc diffusion method (Abdelrahim *et al*, 2002). Lin *et al*. (2002) tested *P. guajava* extracts for anti-microbial activities against different species of diarrheagenic *E. coli*, *Salmonella*, and

Shigella. *P. guajava* showed inhibitory activities against two species of *Salmonella, Shigella flexneri, Shigella Virchow,* and *Shigella dysenteriae,* and two varieties of enteropathogenic *E. coli.* Dutta *et al*., (2000) tested *P. guajava* against dermatophytes: *Trichophtyon tonsurans, Trichophyton rubum* and *Microsporum fulvum*. Almost all dermatophytes showed no growth with the exception of two: *Trichophyton beigelli* and *Candida albicans*. Extracts from both the bark and leaves were used, although the extracts from the bark were more efficient in inhibiting the dermatophytes than the leaves.

Existing literature has reported that *P. guajava* has demonstrated considerable anti-fungal and anti-bacterial effects. In this research, an experiment is designed to determine the effects of *P. guajava and A. leiocarpus on* the normal flora organisms of human *(S. aureus and E. coli*) that can turn to opportunistic pathogens.

2.2 Biological activity of *Anogeissus leiocarpus and Psidium guajava.*

Anogeissus leiocarpus.

Studied the in-vivo antiplasmodial activity of methanolic extracts of *Anogeissus leiocarpus* and its effects on oxidative stress and lipid profile in mice infected with *plasmodium bergheii* revealed that the rate of parasite clearance was higher in the group treated with 200mg/kg body weight of extract of *A. leiocarpus* when compared with the group treated with artesunate. Malondialdehyde (MDA) level was significantly higher ($p<0.05$) in the serum of negative control as compared with other groups which have received treatment (Akanbi *et al*., 2012). MDA level was moderately higher in the liver of homogenates of infected mice treated with artesunate than in the other group. Although the result obtained showed significant increases ($p<0.05$) in the level of serum and liver superoxide dismutase and high-density

lipoprotein (HDL) when compared with other groups. The study revealed that methanolic extract of *A. leiocarpus* has high antimalarial activities and high antioxidant property in malaria-infected organisms. Serum low density lipoprotein, total triglyceride, and cholesterol were moderately higher in the group treated with artesunate than other groups (Akanbi *et al.,* 2012).

Psidium guajava

The long history of guava use has led modern day researchers to study guava extracts. Many experiments have examined the antimicrobial properties *of Psidium guajava*. This medicinal plant has anti-proliferative effects on human mouth epidermal carcinoma and murine leukaemia cells using MIT assay, guava leaf showed anti-proliferative activity, which was 4.37 times more than Vincristine (Manosroi *et al*., 2006)

2.3. Antimicrobial properties of medicinal plants

Medicinal plant extracts are known to have antibacterial activities against certain strains of microorganisms as observed and documented from several antimicrobial screening studies carried out by some researchers (Hassan *et. al.,* 2006*).* In recent years, naturally-occurring bioactive compounds from medicinal plants have been used as chemotherapeutic agents to treat bacterial disease without risk assessments. Antimicrobial activities are measured *in-vitro* in order to determine the potency of a given antimicrobial agent in solution. The *in-vitro* activities of crude plant extract provide evidence to support the use of such plants (Sheila *et. al.,* 2008*).*

The volatile oils of black pepper *(Piper nigrum L.),* clove *(Syzygium aromaticum),* oregano *(Origanum vulgare spp.)* and thyme *(Thymus vulgaris)* were assessed for antibacterial activity against 25 different genera of bacteria. These included animal

and plant pathogens, food poisoning and spoilage bacteria. The volatile oils exhibited considerable inhibitory effects against all the test organisms while their major components demonstrated various degrees of growth inhibition (Dorman *et. al.*, 2000).

Searches for substances with antimicrobial activity are frequent, and medicinal plants have been considered interesting by some researchers since they are frequently used in popular medicine as remedies for many infectious diseases. In a study to verify synergism between 13 antimicrobial drugs and 8 plant extracts-"guaco" *(Mikania glomerata),* guava *(Psidium guajava),* clove *(Syzygium aromaticum),* garlic *(Allium sativum),* lemongrass *(Cymbopogon citratuss,* ginger *(Zingiber officinale),* carqueja *(Baccharis trimera)* and mint *(Mentha piperita*) against *Staphylococcus aureus.* In-*vitro anti-Staphylococcus aureus* activities of the extracts were confirmed, and synergism was verified for all the extract. Clove, guava and lemongrass presented the highest synergism with antimicrobial drugs while ginger and garlic showed limited synergistic capacity (Betoni *et. al.*, 2006*).*

2.4 Major plant components with medicinal value (phytochemicals)

The subject of phytochemistry or plant chemistry has developed in recent years as a distinct discipline, somewhere in between natural product, organic chemistry and plant biochemistry and is closely related to both. It is concerned with the enormous varieties of organic substances that are elaborated and accumulated by plant and deals with chemical structure of these substances, their biosynthesis, turnover and metabolism, their natural distribution and biological function (Harbone, 1991)

The main aim of phytochemical screening is to identify the nature of the compounds present in a given plant extract, which may be responsible for the observed biological effects. Medicinal action of some species of plant is as a result of the effect of the

plants constituents on some of the organs of an animal body. They clear up residual symptoms or destroy the cause of the disease in most cases of infections. They increase the body's resistance to disease, retard or ease the process of ageing. These components are responsible for a given therapeutic effect and they frequently serve as model for the synthetic preparation of new medicine (Harbone, 1991).

Phytochemical screening of the active compounds contained in plants were determined from the plant extracts by advances in understanding successful exploration techniques and continuing development of new techniques to solve outstanding problems as they appeared.

The analysis of chemical constituents of plants is very essential, because majority of the drugs being synthesized nowadays are of plant origin. Examples quinine, an alkaloid which occurs naturally in the bark of cinchona tree. These constituents of the plants include alkaloids flavonoids, tannins, saponins, glycosides, terpenoids, phlobatannin, anthraquinones etc Phytochemical study of plant will provide an information on the different substances accumulated in plants that serve the above and other purposes, and explanation of what constituents are responsible for the activity may be identified. Therefore, this may serve as a starting point for the production of many valuable products that are not easily obtained by synthesis (Harbone, 1991)

2.4.1 Alkaloids

These are heterocyclic compounds containing basic nitrogen derived from higher plants, often having marked physiological activity. Their physiological activities contribute to their wide use in medicine. There are about 5,500 known alkaloids comprising the largest single class of secondary plant substances. They are often toxic to man and a simple but by no means infallible test of alkaloids in fresh leaf or plant materials is the bitter taste they often impact to the tongue. Alkaloids generally

include those basic substances, which contain one or more nitrogen atoms, usually in combination as part of cyclic system. Alkaloids are often toxic and many have dramatic physiological activities, hence their wide use in medicine (Harbone, 1991).The occurrence of alkaloids in plants is not confined to certain specific organs. 'They are either found in all organs or specifically in certain organs, such as barks, fruit, seeds, leaves, rhizomes and roots. Alkaloids usually occur in plants, in form of salts of acetic, malic, oxalic, tartaric, succinic, tannic or other plant acids. A few alkaloids occur in glycosidal combination with sugars forming the so-called gluco-alkaloids (Harbone, 1991).

Alkaloids are usually odorless, colorless crystalline, non-volatile, bitter solids, but few are liquids at room temperature. Examples of alkaloids include moisture caffeine, cocaine, morphine and many others. Most free alkaloid bases are usually fairly soluble inorganic solvents, such as chloroform, either or other relatively non-polar solvents as well as alcohols. They are practically insoluble or very sparingly soluble in water. On the other hand, the alkaloid salts are generally soluble in water, less soluble in alcohol and mostly nearly insoluble or sparingly soluble in organic solvents. Alkaloid generally decomposes on heating, but few sublime without decomposition or degradation when allowed to stand at a temperature above 70°C for a long period of time. Most alkaloids exert some definite pharmacological action in many cases, small quantity of organs and tissues of animal are involve. Potency varies among different alkaloids some are narcotics and analgesics, while others are stimulants. Some will cause a rise in blood pressures, but others will produce a fall in excessive hypertension

2.4.2 Tannins

Tannins are complex chemical substances and consist of mixture of Polyphenols. Most of the time they occur in glycoside combination with sugar. They are non-crystallisable compounds and form colloidal solution with water Tannins readily combine with proteins rendering them resistant to proteolytic enzymes. They are of high molecular weight (500-5000); and also precipitate protein, gelatin and alkaloids to form solution and dark blue or greenish black compound with ferric chloride (Harbone, 1991). Two types of tannins exist:

a. The hydrolysable tannins, which hydrolyze upon heating with dilute acids giving either glucose or Gallic acid (Gallic-tannins) or both Gallic acid and allergic acids.
b. Condensed tannins, which upon heating with acids do not hydrolyze but oxidize and polymerize giving insoluble red amorphan precipitate

2.4.3 Saponins

Saponins are one of group of glycosides found in many plant species with known foaming properties when mixed with water. Saponins lower the surface tension of water allowing the formation of small stable bubbles. The amount of foam created by the crushed plant sample shaken with water in a jar is a good indication of the amount of saponins present (Harbone, 1991). Saponins are normally broken down in the digestive system and are toxic when absorbed into the blood stream. They cause haemolysis of red blood cells and destroy them. They are also toxic to fish and other cold- blooded animals and are precipitated by heavy metals. However not all substances having haemolytic activity are saponins.

2.4.4 Flavonoids

Flavonoids are mainly water soluble compounds. They occur both in Free State and as glycosides and are the largest group of naturally occurring phenols. They are formed

from acetate units and a phenyl propane units (Harbone, 1991).The flavonoids are generally found in the vacuole of plant cells although some are also found in chromoplast and chloroplast. Flavonoids normally occur in various parts of plant such as flower, fruits, and leaves and are commonly found in the form of their glycosides. The flavone is the generic name of a group of compounds produced from a skeleton of three rigs system called flavone nucleus with two sides aromatic ring A and Band a central oxygenated heterocyclic chalcone are not true flavonoids since they lack the characteristic central heterocycle of the flavone's nucleus. Flavones and flavonols are widely distributed in plants than other flavonoids. There are over a hundred flavonol aglycones known, only three are at all common, Kaempferol, quercetin and myricetin. (Harbone, 1991).

2.4.5 Glycoside

Natural glycosides are wide spread in nature and are diverse in structure. They are colorless, non-volatile, and crystalline. Mostly bitter testing solid compounds. Chemically on hydrolysis, the glycosides yield one or more sugar (glycones and aglycones). There are many classes of glycosides, some of which are cynogeruc, cauramin, saponin, anthraquinone, glycosides etc.

Glycoside often shows large variation in concentration during the different stages of the plant growth. Their concentrations often diminish or disappear completely, leaving only aglycones, due to enzymes action during drying of plant tissues. Glycosides are often associated with enzymes that are capable of synthesizing or hydrolyzing them.

According to Nuhu (2011), glycosides are non-reducing substances, which on hydrolysis brought about reagents or enzymes, yield one or more reducing sugar among the product of hydrolysis. The non-sugar components is known as the

aglycone while the sugar component is called glycone. The glycosidic linkages between the sugar and aglycone that is found in most glycosides is an oxygen linkage. It connects the reducing group of a sugar and an alcoholic or phenolic hydroxyl group of the aglycone. This is called D-glycosides. Others that occur includes S-glycosides where the sugar is linked to the sulfohyeldryl group of the aglycone example sinigrin, N- glycosides (strepticline moiety of streptomycin, glucosamine or adenosine).The sugar is linked to the amino group of the aglycone and C-glycosides example barbaloin, in which the sugar is linked to the aglycone by a carbon-to-carbon bond (Nuhu, 2011).

Plant glycosides that are currently used in medicine, though not large in number, are important drugs. A great number of plant glycosides which are not currently used as medicinal agents are of interest to pharmacy and medicine because of their high toxicity or their possible use as potential sources of new drugs. Glycosides of medicinal plants may be used as cardiac stimulant, Laxatives, local irritants or against capillary fragility and have been documented to have an in-vitro antimicrobial benefit against pathogens -including *H pylori*, Epstein-Barr virus, and *Mycobacterium tuberculosis*.(Nuhu, 2011).

2.4.6 Anthraquinones

They are the largest group of natural quinine and historically the most important. Anthraquinones though not used extensively in medical practice, are the starting material for the preparation of several synthetic laxatives and represent the basic structure of a number of important laxatives and dyestuff. Anthraquinone is a tricyclic structure having weak reducing properties which may account for the use of anthronols and anthrones as antiseptics in certain skin diseases (Nuhu, 2011). Most plants contain the anthraquinone derivatives mainly as a glycosides, which on

hydrolysis yield aglycones (non-sugar part). Anthraquinone derivatives are often orange-red compounds and occur in different forms at different oxidation levels. They may occur as derivatives of anthraquinone, anthrore, axanthrone or of anthranol. When anthraquinone is reduced with tin and HCI, it yields anthrone, which when dissolved in warm alkali and precipitated by acids, forms anthranol. The sugars in these anthrone glycosides, may be attached at C-9 to the enol form of anthrone (anthranol) or linked through the phenolic hydroxyl group in the outside rings. The anthraquinone occurring as glycosides, can have their sugar attached in various position (Nuhu, 2011).

2.5 Clinical features of the pathogens

2.5.1 *Staphylococcus aureus*

S. aureus is by far the most important human pathogen among the staphylococci. Although this organism is frequently a part of the normal human microflora, it can cause significant opportunistic infections under the appropriate conditions.

2.5.1.1 Morphology:

S. aureus is Gram-positive cocci. The cocci are mainly arranged in grape-like clusters, but some, especially when examined in pathological specimens, may occur as single cells or pairs of cells. The organisms are non-sporing, non-motile and usually non-capsulated. When grown on many types of agar for 24 hours at 37°C, individual colonies are circular, 2-3mm in diameter with a smooth, shiny surface, (yellow, golden or creamy colonies appears opaque and are often pigmented, though few strains are unpigmented (Jawetz, *et al.*, 2000 and Cheesbrough 2000).

2.5.1.2 Pathogenesis

S. aureus may cause a variety of infectious processes, ranging from relatively benign skin infections to life-threatening systemic illness. Skin infections include simple

folliculitis (Superficial infection surrounding the hair follicles) and impetigo (a superficial skin infection frequently found in children), as well as furuncles and carbuncles involving subcutaneous tissues and causing systemic symptoms, such as fever. S. *aureus* is frequently isolated from post-surgical wound infections, which may serve as a nidus for the development of systemic infections (Jacquelyn, 2005).

Community - acquired staphylococcal broncho-pneumonia is usually seen in elderly persons and is associated with viral pneumonia as a predisposing factor. Nosocomial pneumonia caused by S. *aureus* occurs in the clinical settings of obstructive pulmonary disease, intubation, and aspiration. Underlying malignant diseases are recognized as important risk factors for the development of S. *aureus* bacteremia. Bacteremia may also seed distant sites throughout the body, leading to endocarditis, osteomyelitis, pyoarthritis, and metastatic abscess formation, particularly in the skin, the subcutaneous tissues, lungs, kidneys, and brain. Staphylococcal toxins are also responsible for toxic epidermal necrolysis (Staphylococcal scalded skin syndrome) and toxic shock syndrome. S. *aureus* may also cause food poisoning due to the elaboration of exotoxins during growth in contaminated foods.

2.5.1.3 Virulence factors of S. *aureus*

S. *aureus* possesses several properties that are believed to contribute to their ability to cause disease. These "virulence factors" are not found in all strains of S. *aureus,* however, and this organism continues to be constant source of surprise as new and different pathogenic properties are discovered (Jacquelyn, 2005).

2.5.1.4 Capsule formation: Some strains of *S aureus* produce an exopolysaccharide that may prevent ingestion of the organism by polymorphonuclear cells. These exopolysaccharides have been observed by electron microscopic examination of S. *aureus* infected pacemaker leads, peritoneal catheters, and intravenous lines and have

been demonstrated immunologically in vitro and *in-vivo*. This material may promote the adherence of the organism to cells and to prosthetic devices. Chemical isolates of S. *aureus* have been classified into eight types based on capsular polysaccharide immonotyping, and 70% to 80% of significant clinical isolates belong to capsular serotypes 5 or 8. These capsular types, particularly type 8, are also associated with other S. *aureus* virulence factors, such as the production of toxic shock syndrome toxin. In addition, a predominant number of S. *aureus* strains that are resistant to oxacillin, the most widely used antistaphylococcal penicillin, express the serotype 5 capsular polysaccharide (Jawetz *et al.*, 2000).

2.5.1.5 Protein A: *S. aureus* cell walls contain this unique protein, which has the ability to bind the Fc region of IgG molecules. Protein A is bound to the cell-wall peptidoglycan and is also shed into the medium during growth. Protein A functions as a virulence factor by interfering with opsonization and ingestion of the organisms by polymorphonuclear cells, by activating complement, and by eliciting immediate and delayed type hypersensitivity reactions. Protein A is immunogenic, and antibodies against it are found in persons with serious S. *aureus* infections. The presence of protein on S. *aureus* provides the basic for agglutination test procedures that are used in many clinical laboratories for organism identification and for detection of bacterial antigens in body fluids (Jawetz *et al.*, 2000).

2.5.1.6 Enzymes: S. *aureus* produces several enzymes that may contribute to its virulence. Catalase production by these organisms may function to inactive toxic hydrogen peroxide and free radicals formed by the myeloperoxidase system within phagocytic cells after ingestion of the microorganisms. Both free and bound coagulase may act to coat the bacterial cells with fibrin, rendering them resistant to opsonization and phagocytosis. Fibrinolysis may break down fibrin clots and allow spread of

infection to contiguous tissues. Similarly, hyaluronidase hydrolyzes the intercellular matrix of mucopolysaccharides in tissue and, thus, may act to spread the organism to adjacent areas. Strains of S. *aureus* causing chronic furunculosis have been found to be producers of potent lipases that may help to spread the organism in cutaneous and subcutaneous tissues. A phosphatidylinositol-specific phospholipase C has been described that is associated with strains recovered from patients with adult respiratory distress syndrome and disseminated intravascular coagulation. Tissues affected by this enzyme become more susceptible to damage and destruction by bioactive complement activation. Immunologic and substrate - specificity studies indicate that at least three different types of ~β lactamase enzymes are produced by S. *aureus,* production of these enzymes may be inducible (i.e. they are produced fully in the presence of –β lactam antimicrobial agent or constitutive and render these organisms resistant to penicillin and ampicillin. Genes coding for these enzymes usually reside on plasmids (extra chromosomal DNA) that also carry genes for resistance to several antibiotics, such as erythromycin and tetracycline. These resistance genes may be transferred to other bacterial by transformation and transduction (Jawetz *et al.*, 2000).

2.5.1.7 Cell wall constituents: *S. aureus* cell walls contains peptidoglycans (cross-linked polymers of N-acetylglucosamine and N-acetylmuramic acid), which are similar to those found in other gram-positive bacteria, and teichoic acids, which are unique ribitol (5 - carbon monosaccharide) - phosphate - polymers. Teichoic acids function in the specific adherence of gram-positive bacterial to mucosal surface. In addition to their role in providing rigidity and resilience to the staphylococcal cell wall, peptidoglycans and teichoic acids also have several biological activities that are believed to contribute to virulence. These properties include the ability to activate

complement, to inhibit chemotaxis of inflammatory cells, and to stimulate antibody production (Jawetz *et al.*, 2000).

2.5.1.8 Toxins (exotoxins): The α - toxins is a heterogeneous protein that acts on a broad spectrum of eukaryotic cell membranes the α - toxins is a potent hemolysin. The ~β toxin degrades sphingomyelin and therefore is toxic for many kinds of cells, including human red blood cells. The α - toxins lyses red blood cells from humans and animals. The δ - toxins are heterogeneous and dissociates into subunits in non-ionic detergents. It disrupts biologic membranes and may have a role in S. *aureus* diarrheal diseases (Jawetz *et al.*, 2000). Leucocidin is an exotoxin that exerts a direct toxic effect on human polymorphonuclear cell membranes, causing degranulation of the cytoplasm, cell swelling, and lysis. This toxin of S. *aureus* has two components. The two components act synergistically on the white blood cell membrane to form pores and increase cation permeability (Jawetz *et al.*, 2000). Exfoliate toxins - these epidermolytic toxins of S. *aureus* are two distinct proteins of the same molecular weight. Epidermolytic toxin A is a chromosomal gene product and is heat stable (resist boiling for 20 minutes). Epidermolytic toxin B is plasmid - mediated and heat - labile. The epidermolytic toxins yield the generalized desquamation of the staphylococcal scalded skin syndrome. In this condition, bullous formation occurs over large areas of the body, with subsequent sloughing of the superficial skin layers. This results in the exposure of large areas of denuded and raw skin. The disease is usually seen in neonates and infants. Both toxins are antigenic, and antibodies against them are protective (Jawetz *et al.*, 2000).

Toxic shock syndrome toxin- Most S. *aureus* strains isolated from patients with toxic shock syndrome produce a toxin called toxic shock syndrome toxin - 1 (TSST - 1), which is the same as enterotoxin F. TSST- 1 is the prototypical super-antigen, it binds

to MHC class II molecules, yielding T cell stimulation, which promotes the protein manifestations of the toxic shock syndrome. The toxin is associated with fever, shock, and multisystem involvement including a desquamated skin rash. The gene for TSST-l is found in about 20% of S. *aureus* isolates (Jawetz *et al.,* 2000). Enterotoxins - there are multiple (A-E, G-I, K-M) enterotoxins. Enterotoxins A through E are heat - stable molecules that are responsible for the clinical features of staphylococcal food poisoning. The exact mode of action of these enterotoxins is unknown, but it had been shown that the emetic effect of enterotoxin is probably the result of central nervous system stimulation (vomiting Centre) after the toxin acts on neural receptors in the gut (Jawetz *et al..*, 2000).

2.5.2 *Escherichia coli*

Theodor Escherich first described *E. coli,* as *Bacterium coli commune* which he isolated from the feces of newborns. It was later renamed *Escherichia coli* and for many years the bacterium was simply considered to be a commensal organism of the large intestine. It was not until 1935 that a strain of *E. coli* was shown to be the cause of an outbreak of diarrhea among infants (Todar, 2008). *E. coli* is the head of the large bacterial family, *Enterobacteriacea.* The enteric bacteria, which are facultative anaerobic, Gram negative rods that live in the intestinal tracts of animals. The enterobacteriacea are among the most important bacteria of medical importance. A number of genera within the family are human intestinal pathogens example *Salmonella, Shigella, and Yersinia.* Several others are normal colonists of the human gastrointestinal tract (e.g. *Escherichia, Enterobacter, Klebsiella),* but these bacteria, as well may occasionally be associated with diseases of humans (Todar, 2008).

Physiologically, *E. coli* is versatile and well adapted to its characteristic habitats. It grows in media with glucose as the sole organic constituent. Wild type *E. coli* has no

growth factor requirements and metabolically it can transform glucose into all of the macromolecular components that make up the cell. The bacterium can grow in the presence or absence of O_2, under. Anaerobic conditions it will grow by means of fermentation, producing characteristic "mixed acids and gas" as end products. However, it can also grow by means of anaerobic respiration, since it is able to utilize $N0_3$, $N0_2$ or fumarate as final election acceptors for respiratory electron transport processes. In part, this adapts *E. coli* to its intestinal (anaerobic) and its extra intestinal (aerobic or anaerobic) habitats (Todar, 2008).

E. coli can respond to environmental signals such as chemicals, pH, temperature, osmolality, etc. in a number of very remarkable ways considering it as a unicellular organism. For example, it can sense the presence or absence of chemicals and gases in its environment and swim towards or away from them. Or ' it can stop swimming and grow fimbriae that will specifically attach it to a cell or surface receptor. In response to change in temperature and osmolality, it can vary the pore diameter of its outer membrane porins to accommodate large molecules (nutrients) or to exclude inhibitory substances. With its complex mechanism for regulation of metabolism the bacterium can survey the chemical contents in its environment in advance of synthesizing any enzymes that metabolize these compounds. It does not wastefully, produce enzymes for degradation of carbon sources unless they are available, and it does not produce enzymes for synthesis of metabolites if they are available as nutrients in the environment (Todar, 2008).

2.5.2.1 Pathogenesis of *E. coli*

Over 700 antigenic types (serotypes) of *E. coli* are recognized based on O, H, and K antigens. At one time serotyping was important in distinguishing the small number of strains that actually cause disease. Thus, the serotype 0157: H7 (0 refers to somatic

antigen; H refers to flagella antigen) is uniquely responsible for causing HUS (hemolytic uremic syndrome). Pathogenic strains of *E. coli* are responsible for three types of infections in humans. Urinary tract infections (UTI), neonatal meningitis, and intestinal disease (gastroenteritis). The disease caused or not caused, by particular strain of *E. coli* depend on distribution and expression of an array of virulence determinants, including adhesions, invasions, toxins, and abilities to withstand host defenses (Todar, 2008).

2.5.2.2 Urinary tract Infection:

Uropathogenic *E. coli* (UPEC) cause 90% of the Urinary tract infections (UTI) in automatically - normal, unobstructed urinary tracts. The bacteria colonize from the feces or perineal region and ascend the urinary tract to the bladder. Bladder infections are 14 times more common in females than males by virtue of the shortened urethra. The typical patient with uncomplicated cystitis is a sexually active female who was first colonized in the intestine with an uropathogenic *E. coli* strains. The organisms are propelled into the bladder from the periurethral region during sexual intercourse. With the aid of specific adhesions they are able to colonize the bladder (Jawetz *et al.*, 2000).

The adhesin that has been most closely associated with uropathogenic *E. coli* is the P fimbria or otherwise known as pyelonephritis- associated pili (PAP). The latter designation is derived from the ability of P fimbriae to bind specifically to the P blood group antigen which contains a D-galactose - D - Galactose residue. The fimbriae bind not only to red cells but to a specific galactose disaccharide that is found on the surfaces uroepithelial cells in approximately 99% of the population. The frequency of the distribution of this host cell receptor plays a role in susceptibility and explains why certain individuals have repeated UTI caused by *E. coli.* Uncomplicated *E. coli* UTI

virtually never occurs in individuals lacking the receptors. Uropathogenic strains of *E. coli* possess other determinants of virulence in addition to P. fimbriae. *E. coli* with P fimbriae also possess the gene for Type 1 fimbriae, and there is evidence that P fimbriae are derived from Type 1· fimbriae by insertion of a new fimbrial tip protein to replace the mannose - binding domain of Type 1 fimbriae. In any case, Type 1 fimbriae could provide a supplementary mechanism of adherence or play a role in aggregating the bacteria to a specific manosyl-glycoprotein that occurs in urine (Todar, 2008). Uropathogenic strains of *E. coli* usually produce siderophores that probably play an essential role in iron acquisition for the bacteria during or after colonization. They also produce hemolysins which are cytotoxic due to formation of transmembranous pores in host cell membranes. One strategy for obtaining iron and other nutrients for bacterial growth may involve the lysis of host cells to release these substances. The activity of hemolysins is not limited to red cells since the alpha-hemolysins of *E. coli* also lyse lymphocytes, and the beta-hemolysins inhibit phagocytosis and chemotaxis of neutrophils. Another factor thought to be involved in the pathogenicity of the uropathogenic strains of *E. coli* is their resistance to the complement dependent bactericidal effect of serum. The presence of K antigen is associated with upper urinary tract to infections, and antibodies to the K antigens have been shown to afford some degree of protection in experimental infections. The K antigens of *E. coli* are "capsular" antigens that may be composed of proteinaceous organelles associated with colonization, or made of polysaccharides. Regardless of their chemistry, these capsules may be able to promote bacterial virulence by decreasing the ability of antibodies and / or complement to bind to the bacterial surface, and the ability of phagocytes to recognize and engulf the bacterial cells. The best studied K antigen, K-I, is composed of a polymer of N - acetyl neuramic acid

(sialic acid), which besides being antiphagocytic, has the additional property of being an antigenic disguise (Todar, 2008).

2.5.2.3 Neonatal meningitis:

E. coli strains invade the blood stream of infants from the nasopharynx or GT tract and are carried to the meninges. The K-I antigen is considered major determinant of virulence among strains of *E. coli* that causes neonatal meningitis. K-I is a homopolymer of sialic acid. It inhibits phagocytosis, complement, and responses from the host's immunological mechanisms. K-I may not be the only determinant of virulence, however, as siderophore production and endotoxin are also likely to be involved (Todar, 2008). Epidemiologic studies have shown that pregnancy is associated with increased rates of colonization by K-I strains and that these strains become involved in the subsequent causes of meningitis in the newborn. Probably, the infant GT tract is the portal of entry into the blood stream. Fortunately, although colonization is fairly common, invasion and the catastrophic sequelae are rare (Todar, 2008).

2.5.2.4 Intestinal disease caused by *E. coli:*

As a pathogen *E. coli* is known for its ability to cause intestinal diseases. Five classes of *E. coli* that cause diarrhea disease are now recognized. Enterotoxigenic *E. coli* (ETEC), entroinvasive *E coli* (EIEC), enterohemorrhagic *E coli* (EHEC), entropathogenic *E. coli* (EPEC), and enteroaggregative *E coli* (EAEC) . Each class falls within a serological subgroup and manifests distinct features in pathogenesis (Jawetz *et al.*, 2000).

1. Enterotoxigenic *E. coli* (ETEC): ETEC is an important cause of diarrhea in infants and travelers in developing countries or regions of poor sanitation. In the USA, it has been implicated in sporadic waterborne outbreaks, as well as due to the

consumption of soft cheeses, Mexican-style foods and raw vegetables. The diseases vary from minor discomfort to a severe cholera-like syndrome. ETEC are acquired by ingestion of contaminated food and water, and adults in endemic areas evidently develop immunity. The disease requires colonization and elaboration of one or more enterotoxins. Both traits are plasmid-encoded (Todar, 2008).

ETEC may produce a heat-labile enterotoxin (L T) that is similar in molecular size, sequence, antigenicity and function to the cholera toxin (Ctx). It is an 86kDa protein composed of an enzymatically active (A) subunit surrounded by 5 identical binding (B) subunits. It binds to the same identical ganglioside receptors that are recognized by the cholera toxin (i.e. GMI), and its enzymatic activity is identical to that of the cholera toxin. ETEC may also produce a heat-stable toxin (ST) that is of low molecular size and resistant to boiling for 30 minutes. There are several variants of ST, of which STla or STp is found in *E. coli* isolated from both humans and animals, while STlb or STn is predominant in human isolates only. The ST enterotoxins are peptides of molecular weight about 4,000 Daltons. Their small size explains why they are not inactivated by heat. ST causes an increase in cyclic GMP in host cell cytoplasm leading to the same effects as an increase in cAMP. STla is known to act by binding to a guanylate cyclase that is located 'on the apical membranes of host cells, thereby activating the enzyme. This leads to secretion of fluid and electrolytes resulting in diarrhea (Todar, 2008).

The infective dose of ETEC for adults has been estimated to be at least 10 cells, but the young, the elderly and the infants may be susceptible to lower numbers. Symptoms of ETEC infections include diarrhea without fever .The colonize the GI tract by means of a fimbrial adhesin e.g. CFAI and CFA II, and are non-invasive, but produce either the LT or ST toxin.

11. Enteroinvasive *E. Coli* (EIEC): There are known animal reservoirs of EIEC. Hence the primary source for EIEC appears to be infected humans. EIEC closely resemble *Shigella* in their pathogenic mechanisms and kind of clinical illness they produce. EIEC penetrate and multiply within epithelial cells of the colon causing widespread cell destruction. The clinical syndrome is identical to *Shigella* dysentery and includes dysentery like diarrhea with fever. Although the infective dose of *Shigella* is low (in the range of 20 to few hundred cells) volunteer feeding studies showed that at least 10^6 cells EIEC organisms are required to cause illness in healthy adults. EIEC apparently lack fimbrial adhesions but do possess a specific adhesins that, as in *Shigella,* is thought to be an outer membrane protein. Unlike typical *E. coli,* EIEC are non-motile, do not decarboxylate lysine and do not ferment lactose. Pathogenicity of EIEC is primarily due to its ability to invade and destroy colonic tissue (Jawetz *et al.,* 2000)

111. Enteropathogenci *E. coli* (EPEC): EPEC induce a profuse watery, sometimes bloody, diarrhea. They are a leading cause of infantile diarrhea in developing countries. Outbreaks have been linked to the consumption of contaminated drinking water as well as some meat products. Pathogenesis of EPEC involves a plasmid - encoded protein referred to as EPEC adherence factor (EAF) that enables localized adherence of bacterial to intestinal cells and a non fimbrial adhesin designated intimin, which is an outer protein that mediates the final stages of adherence. They do not produce ST or LT toxins. Adherence of EPEC strains to the intestinal mucosa is a very complicated process and produces dramatic effects in the ultra-structure of the cells resulting in rearrangement of actin in the vicinity of adherent bacteria. The phenomenon is sometimes called "attachment and effacing" of cells. EPEC strains are said to be "moderately invasive" meaning they are not as invasive as *Shigella,* and

unlike ETEC or EAEC, they cause an inflammatory response. They diarrhea and other symptoms of EPEC infections probably caused by bacterial invasion of host cells and interference with normal cellular" signal transduction, rather than by production of toxins (Todar, 2008).

1V. Enteroaggregative *E. coli* (EAEC): The distinguishing feature of EAEC strains is their ability to attach to tissue culture cells in aggregative manner. These strains are associated with persistent diarrhea in young children. They resemble ETEC strains in that the bacteria adhere to the intestinal mucosa and cause non-bloody diarrhea without invading or causing inflammation. This suggests that the organisms produce an enterotoxin of some sort.

Recently, a distinctive heat-labile plasmid-encoded toxin has been isolated from these strains, called the EAST (Entero-Aggregative ST) toxin. They also produce a hemolysin related to the hemolysin produced by *E. coli* strains involved in Urinary tract infections. The role of toxin and the hemolysin in virulence has not been proven. The significance of EAEC strains in human disease is controversial (Todar, 2008).

V. Enterohemarrhagic *E. coli* (EHEC): EHEC are recognized as the primary cause of hemorrhagic colitis (HC) or bloody diarrhea, which can progress to the potentially fatal hemolytic uremic syndrome (HUS). EHEC are characterized by the production of verotoxin or shiga toxins (Stx). Although Stxl and Stx2 are most often implicated in human illness, several variants of stx2 exist (Cheesbrough, 2000)

CHAPTER THREE

3.0 MATERIALS AND METHODS

3.1 Collection and identification of plant materials

Fresh leaves samples of the two plants *Anogeissus leiocarpus* and *Psidium guajava* were collected from the Usmanu Danfodiyo University, Sokoto farm. The plants were botanically authenticated and samples of the plants were prepared according to the botanical standards and carefully brought to the department of microbiology Usmanu Danfodiyo University Sokoto. Voucher specimen was kept in the herbarium of Botany unit in the department of biological sciences. Voucher No. (UDUS/ANS/OI67).

3.2 Preparation of media.

The media that were used for Isolation, biochemical identification and antibacterial Sensitivity tests include Nutrient agar, Mueller Hinton (MH) agar, MacConkey agar, Eosin Methylene Blue (EMB) agar, Manitol salt (MSA) agar, Triple sugar iron (TSI) agar, Simmons Citrate agar and Tellurtte-Cefixime Sabitol MacConkey agar (CT-SMA). And all were prepared according to manufacturer's label accompanying the containers. (Appendix II)

3.2.1 Preparation of plants materials for extraction

The leaves of the plants *Anogeissus leiocarpus* and *Psidium guajava* were rinsed with clean water and air dried at room temperature (25^{o}c) for two weeks. The air dried leaves were milled in to powder by pounding manually with a clean pestle and mortar, the powdered leaves were sieved, weighed and stored in a clean sterile containers (Oyeleke and Manga, 2008)

3.2.2 Extraction of the plants materials

Using a soxhlet apparatus, the soxhlet method of extraction was employed in extracting the crude leaves extract using hexane, methanol, ethanol, and water. A weight of 500g of each of the powdered plants leaves samples were placed in the upper chamber in a thimble, and 500ml of the solvent in the bottom flask. The Pulverized leaves of both *A. leiocarpus* and *Psidium guajava* were defatted exhaustively with the above solvents by successive soxhlet extraction method of NIPRD Protocol (2004). The extracted solutions were concentrated in a rotary evaporator. According to Oyeleke and Manga (2008) the extracts were weighed and kept in well labelled sterile sample bottles for further analysis.

3.2.3. Preparation of concentrations of the crude leaves extracts

A test stock concentration of 500mg/ml for methanol, ethanol, and water extract of *A. leiocarpus and P. guajava* (leaves) were prepared by dissolving 1g of each extract in 2mls of sterile distilled water in separate test tubes. Non-polar hexane extract was first homogenised in 0.1ml of dimethyl sulfoxide (DMSO) and then added to 1.9ml of distilled water. 0.1ml of the stock concentration equivalent to 50mg was used to prepare different concentrations of the plants extract by doubling dilution (50, 25, 12.5 and 6.25mg/ml). And the concentrations were assayed for crude antibacterial activity on the test bacteria. The positive control drugs used is Tetracycline (0.5mg/ml).

3.2.4 Samples collection and inoculation

The urine: Fifty (50) Samples were collected from the patients attending Specialist Hospital Sokoto. The samples were collected in clean sterile, leak free containers from different patients' children, adults (married, non-married) and were transported to Microbiology Research laboratory Usmanu Danfodiyo University Sokoto. The samples were centrifuged at 2000RPM for ten (10mins) the supernatant was decanted leaving only the sediments in the tube. A sterile wire loop was used to take a drop of each sample and inoculated on nutrient agar, MacConkey and Eosin methylene agar plates by streak plate method. (Koneman *et al.,* 2005)

The wound: fifty (50) wound samples were collected using sterile cotton swabs from patients with different types of wounds such as burn, surgical and accidental wounds. All the samples were labelled and brought immediately to the laboratory for analysis. The pus specimen was streaked on nutrient agar and MacConkey agar and manitol salt agar plates. Streaked plates were incubated at 37ºC for 24hrs. Bacterial colonies on the agar plates where later gram stained. (Koneman *et al.,* 2005)

3.2.5 Identification and characterization of bacterial isolates: The bacterial isolates were identified and characterized based on macroscopy, microscopy(shape, grams reaction) and standard biochemical methods which include catalase, coagulase, indole, methyl red, voges-proskauer, hydrogen sulphide production, citrate, sugar utilization test. The isolates were identified by comparing their characteristics with those of known taxa, as described by (Cappuccino and Sherman, 2011). The organisms were maintained on slants until required for assay.

3.2.6 Gram staining:

A smear was prepared by placing a drop of sterile distilled water on a clean, grease free slide. A sterilized wire loop was used to pick colony and smear was prepared on

the slide. The smear was heat fixed by passing the slide over flame. The smear was flooded with crystal violet and allowed to stand for one minute. It was washed with distilled water without blotting. This was followed by pouring lugol's iodine solution and allowed to stand for one minute. It was washed with distilled water and then flooded with ethanol (decolorized) for 30 seconds. This was washed off with distilled water and flooded with safranin and left for 1 minute after which it was washed and allowed to dry (Cheesbrough, 2000). The dried slides were then viewed with oil immersion objectives lens x100 and the result recorded.

3.3 Biochemical identification

The following biochemical tests were carried out for the identification of the isolated bacteria. The tests include; catalase, coagulase, methyl-red, voges- proskaure test, citrate utilisation, indole, hydrogen sulphide production and sugar utilization tests.

3.3.1 Catalase test

A drop of 3% hydrogen peroxide was placed on a clean glass slide. A sterile wire loop was used to pick the colony and emulsified with a drop hydrogen peroxide. Observation for gas bubbles was done and the result recorded (Oyeleke and Manga, 2008).

3.3.2 Indole production test

The test organism was inoculated into 5ml of peptone water in a test tube by emulsifying few colonies picked with a sterile wire loop and incubated at 37^{o}C for 24hrs to give optimum accumulation of indole. A 0.5ml of kovacs reagent was added and shaken. Observation for red colouration at uppermost layer was done and the result recorded (Cappuccino and Sherman, 2011).

3.3.3 Methyl red test

A colony of the bacteria was inoculated in MR-VP medium using sterile wire loop and then incubated at 37^{o}C for 48hrs. After the incubation period three (3) drops of methyl red were added. Observation for colour change was done and the result recorded (Cappuccino and Sherman, 2011).

3.3.4 Voges-proskaure test

Five (5) drops of 40%KOH and 15 drops of ethanol were mixed, shaken, loosen the cap of the test tubes and placed in a sloping position. Observation for red colour was done and the result recorded (Cappuccino and Sherman, 2011).

3.3.5 Citrate utilization test

The test organism was inoculated in to a Simmons citrate agar slant with sterile wire loop and incubated at 37^{o}C for 48hrs. Observation for colour change from green to blue was done and the result recorded (Cappuccino and Sherman, 2011).

3.3.6 Triple sugar-iron (TSI) agar test

Using a sterile straight wire a colony of test organism was streaked on the surface of the slant and stabbed the butt 2 times. The test tubes were cap loosely and incubated at 37^{o}C for 24hrs. Observation done and the results were read. Isolates were classified based on their ability to ferment the sugars with or without the production of gas, color change from red to yellow and the production of hydrogen sulphide (H_2S) which is indicated by blackening of the medium (Oyeleke and Manga, 2008).

3.3.7 Coagulase test

Two (2) colonies were emulsified in 0.5ml of normal saline contained in a clean serological tube, and 1ml of human plasma added and incubated at 35^{o}C. Observation for increase in viscosity or clotting was done after 1hour, 2 hours, 3 and 4 hours of incubation and the result recorded. (Oyeleke and Manga, 2008).

3.4 Identification of the strains of *E. coli*

Pure cultures of all positive *E. coli* identified from biochemical tests and growing on EMB agar were streaked onto Tellurtte-Cefixime Sorbitol MacConkey agar (CT-SMA) plates and incubated at 37^{o}C for 24hrs. A suspected *E. coli* 0157:H7 appearing as non-sorbitol fermenter colonies (NSFC) characterized as having a slightly transparent, almost colourless with a weak pale brownish appearance were observed. The presumptive colonies were gram stained for the presence of gram negative rods. Individual discrete colonies were picked and sub-cultured on to nutrient agar slants, incubated at 37^{o}C for 24hrs and refrigerated for further serological analysis (Reuben and Makut, 2014).

3.4.1 Serological test (Confirmatory test)

E. coli 0157:H7 positive colonies were serologically confirmed using *E. coli* 0157:H7 latex agglutination assay, containing latex particles coated with antibodies specific for *E. coli* 0157 and *E. coli* H7 antigen. Identification of *E. coli* 0157:H7 was carried out following the manufacturer's instructions. Hence colonies that agglutinated to the separate antisera were considered to be *E. coli* 0157:H7 (Reuben and Makut, 2014).

3.5 Preparation of the Inoculum

The standardization of the inoculum was carried out in accordance with the methods described by Oyeleke and Manga, (2008). The isolates were sub-cultured from slant

bottles using sterile wire loop in to fresh Nutrient agar plates and incubated at 37ºC for 24hrs. After incubation period, 5ml of distilled water was introduced in to different universal bottles and was used to prepare the size of inoculums. The bacterial suspension and optical density was compared with 0.5 tube of the McFarland standard which is equivalent to 10^8 cfu/ml (0.5ml of barium chloride + 9.5ml of sulphuric acid solution). (Appendix III)

3.6. Antibacterial Activity of the crude extracts

The suspension of the bacterial inoculums (*S. aureus* and *E. coli*) that matched McFarland scale 0.5, were spread on the surface of the Muller Hinton agar plate with sterile glass rod. A standard cork borer of 6mm in diameter was used to bore well at the centre of each inoculated plate. A molten nutrient medium was used to seal the bottom. Zero point one millilitre (0.1ml) of each extracts (at concentrations of 50, 25, 12.5 and 6.25mg/ml) were introduced into the wells. The plates were incubated at 37^oC for 24hrs, and observed for the zone of inhibition of the growth. The zones were measured with a transparent ruler and the result recorded in millimetres. The screening was done in triplicates. Sterilized distilled water was used as negative control. (Hugo and Russel, 1992).

3.6.1 Determination of Minimum Inhibitory Concentration (MIC) For Crude Extract

Minimum inhibitory concentration (MIC) was determined for the crude extracts of the leaves of *A. leiocarpus and P. guajava* that showed appreciable activity against the test organisms. The broth dilution method described by Ibekwe *et al.* (2001) with slight modifcation was employed. Twelve test tubes numbered 1-12 were used. 1ml of nutrient broth was dispensed in to tubes 2-12 each. Then stock

concentration of the crude extract was prepared (50mg/ml). 1ml of 50mg/ml of stock concentration was introduced into tube 1, and another 1ml into tube two. Then doubling dilution of the extract in the broth was made from tube 2 to tube 10, to obtain 25, 12.5, 6.25, 3.125, 1.562, 0.781, 0.390, 0.195, and 0.097mg/ml. zero point one millilitre (0.1ml) of the standardized inoculums of the bacteria was inoculated into the different concentrations from tube 1 to 11 of the extract in the broth. Tube 12 contain another 1ml of sterile broth. The test tubes of the broth were incubated at 37^{o}C for 24hrs and were observed for turbidity due to growth of the organisms. The lowest concentration which showed no turbidity in the test tube was recorded as the Minimum Inhibitory Concentration.

3.6.2. Determination of the Minimum Bactericidal Concentration (MBC) For the Crude Extract.

The Minimum bactericidal concentration (MBC) was determined by the method described by Ibekwe *et al*. (2001). The contents of the negative tubes from MIC above were inoculated on sterile nutrient agar plates and incubated for 37^{o}C for 24hours. The tubes with least concentration of the crude extract that show no growth on sub-culturing was recorded as minimum bactericidal concentration.

3.7. Phytochemical screening of the crude extract of the plants.

The crude leaves extract of the two plant samples were screen for the determination of phytochemical constituents using the procedures described by Oyeleke and Manga (2008).

3.7.1 Test for alkaloids

Hager's reagent, Meyer's reagent and Wanger's reagent were used for this test. Zero point five grams (0.5g) of the leaf extracts was mixed by stirring with 5ml of 1% aqueous hydrochloric acid on a steam bath. It was then allowed to cool and filter. A 1ml of the filtrate was treated separately with all the reagents (Hager's, Meyers and Wanger's reagents). A deep brown creamy precipitate indicate positive test. (Oyeleke and Manga, 2008)

3.7.2 Test for balsam

Two (2) drops of alcoholic ferric chloride solution was added to 5ml of 99% ethanolic extract. Observation for colour change was done and the result recorded (Oyeleke and Manga, 2008).

3.7.3 Test for carbohydrate (molisch test)

3 drops of molisch reagent was added to 2ml of aqueous crude extracts of the leaves, this was followed by adding small quantity of concentrated sulphuric acid and the content was allowed to form a lower layer. This was then followed by diluting the mixture with 5ml of water. Observation for purple precipitate was done and the result recorded (Oyeleke and Manga, 2008).

3.7.4 Test for flavonoids

One millilitre (1ml) of methanolic crude leaves extract was mixed with sodium hydroxide (NaOH) solution. The appearance of a yellow solution, which disappears on addition of hydrochloric acid, this indicates the presence of flavonoids. (Oyeleke and Manga, 2008).

3.7.5 Test for free anthraquinones (Borntrager's test)

Ten millilitre of chloroform was added to 0.5g of the methanolic crude leaves extract in a clean test tube and shake for 5 minutes. It was then filtered with wart-man No. 1 filter paper. Observation for bright pink colour in the upper aqueous layer was done and the result recorded (Oyeleke and Manga, 2008).

3.7.6 Test for cardiac glycosides (Keller-killini test)

For each of the plants extract a quantity of 0.5g was added to 3ml of FeCl in a glacial acetic acid and left for 1 minute. A quantity 1.50ml of H_2SO_4 was ran down the side of the tube. Observation for blue layer at the interphase was done and the result recorded (Oyeleke and Manga, 2008).

3.7.7 Test for tannins

One in four (1:4) dilutions in water of the aqueous leaves extract was prepared and mixed with few drops of 10% ferric chloride solution. Observation for Development of blue or green color was done and the result recorded (Oyeleke and Manga, 2008).

3.7.8 Test for steroid

Fifteen millilitre (15ml) of concentrated H_2SO_4 was added to 1ml of each crude extract. Observation reddish brown color was done and the result recorded (Oyeleke and Manga, 2008).

3.7.9 Test for saponins

Ten (10) ml of distilled water was added to 0.5ml of each crude extract and was shaken vigorously for 2 mins. Observation for bubbles was done and the result recorded (Oyeleke and Manga, 2008).

3.8. Separation of plant components by column chromatography

The chromatographic media (silica gel) was used for the chromatographic separation. The lower end of a glass column 10cm long and 1.5cm internal diameter was plug with glass wool. The column was packed with wet silica gel by pouring the silica gel on to the column in a stepwise manner and a glass wool was placed. The plant materials was poured on to the glass wool and air bubble released were trapped with flat end of a packed rod. Three grams (3g) of the most active extracts was drawn on to the absorbent and eluted with solvents. The components of the sample were separated in to narrow band as they move down the column at different rates, depending on how strongly they were absorb from the column materials (Banu and Catharine, 2015).

3.8.1 Antibacterial activity of the bioactive compounds from the column chromatographic fractions

The plant components (fractions) that were obtained from column chromatography, were reconstituted (10, 5, 2.5 and 1. 25mg/ml), and tested against S *aureus* and *E. coli* by agar well diffusion method described by Hugo and Russel (1992). The wells of the inoculated media plate was filled with the diluted extract and the plates were incubated for 24hours at 37^{o}C. The zones of inhibition were measured and express in millimetre.

3.9 Toxicity studies

3.9.1 Test animals.

Thirty (30) Adult albino rats weighing 150-200g of the same age group were used after being certified healthy by a veterinary doctor. The animals were kept at animal house in a cage. Where they were maintained under veterinary supervision and were fed well with standard growers “vital feed” and with access to water for 3days to

acclimatize before commencement of the experiment. The animals were housed and cared for in accordance with good laboratory practice (GLP) regulation of WHO (2008).

3.9.2 Acute toxicity studies.

The test was carried out according to the method described by Aboudoulatif *et al,* (2010) with slight modification, thirty (30) albino rats were purchased from the animal house Usmanu Danfodiyo University Sokoto. And were allowed to acclimatize under appropriate condition. The limit test dose of 2000mg/kg and 5000mg/kg body weight as stipulated in organization for economic corporation Development (OECD) guidelines were used. The rats were grouped in to three (3) each group made up of ten (10) rats each, then 2000mg/kg and 5000mg/kg body weight of methanolic leave extract of *P. guajava* were administered orally with cannula attached to a graduated syringe to the first two groups, while the last group received only distilled water and served as control. The animals were observed individually for the first eight (8) hours for acute toxicity signs, behavioural changes, and at least once daily for 14 days, the first day was taken as D_0 whereas the day of sacrifice was designated as D14. The rats were sacrificed after 14days and the sacrificed rats were prepared for further analysis.

3.9.3 Preparation of blood samples and Estimation of biochemical parameters

Blood samples were immediately collected from each sacrificed rat into well labelled clean plastic test tubes the blood samples were allowed to stand to ensure complete clotting. The clotted blood samples were centrifuged at 3000rpm for 10mins, serum were collected and stored under frozen condition. The effect of the crude extracts on the biochemical parameters (Alanine aminotransferase (ALT), Aspartate

aminotransferase (AST), Alkaline phosphate (ALP), Direct bilirubin (DB), Total bilirubin (TB), Urea and Creatinine level etc) were determined at Usman Danfodiyo University Teaching Hospital Sokoto. Using commercial kit obtained from Aggappe Diagnostics Switzerland.

3.10 Statistical analysis

The antibacterial sensitivity test results were expressed as mean of the zones of inhibition. The result of toxicity studies were expressed as mean value ± standard deviation (S.E.M) within the group, and comparisons were performed between the control and treated groups using analysis of variance ANOVA test.

CHAPTER FOUR

4.0 RESULTS

Ten (10) bacterial isolates identified include; *Staphylococcus aureus* (24.73%), *E. coli* (16.13%), *Proteus spp* (16.13%), *Pseudomonas auroginosa* (11.84%), *Acinobacter spp* (9.68%), *Klebsiella spp* (8.60%), Coagulase *negative staphylococcus* (4.30%), *B-haemolytic streptococcus* (3.22%), *Enterobacter spp* (3.22%) and *S. pyogenes* (2.15%).(appendix 1V). The bacteria isolated from the urine and wound samples were identified based on their cultural, morphological and biochemical characteristics. The bacterial isolates identified from urine samples indicated that *E. coli* was present in most of the urine samples (13 out of 20) collected from married patients, and none found in any of the urine samples (20) collected from children.

The result of the biochemical tests are presented in Table 4.1 the test carried out include Catalase, Coagulase, Indole, Citrate utilization, Methyl-Red-Voges-Proskauer and sugar utilization tests.

Out of the 15 *E. coli* isolated from the urine samples four (4) were found to be *E. coli* 0157:H7 Table 4.2

The result of physical appearance and yield of *Anogeissus leiocarpus* and *Psidium guajava* are presented in Table 4.3. The result obtained revealed that methanol extract of *P. guajava* recorded the higher yield of 6.71g compared to Hexane extract of *Anogeissus leiocarpus* and *Psidium guajava*.

Table 4.1 Biochemical Identification of the Isolates.

Identified organism	Gram Rxn	Glu	Lact.	Suc	H_2S	Gas	Mot	Cit	Ind	MR	VP	Cat	Coa
S. aureus	+	-	+	-	+	+	-	-	-	-	+	+	+
E. coli	-	+	+	+	-	+	+	-	+	+	-	+	-

Key: Gram= Gram Reactions, Glu= Glucose, Lact= Lactose, Suc= Sucrose, H_2S= Hydrogen Sulphide, Gas, Mot= Motility, Cit= Citrate, Ind= Indole, MR= Methyl Red, VP= Voges Proskauer, Cat= Catalase and Coa= Coagulase

Table 4.2: Serological identification of *E. coli* 0157:H7

Isolate (*E. coli*)	Agglutination with antisera specific to 0157:H7
E1	**-**
E2	**+**
E3	**-**
E4	**+**
E5	**-**
E6	**-**
E7	**+**
E8	**-**
E9	**-**
E10	**-**
E11	**-**
E12	**-**
E13	**-**
E14	**-**
E15	**+**

Key: E = *E. coli*, + =positive, - = negative

Table 4.3: Physical appearance and yield of *Anogeissus leiocarpus* and *Psidium guajava* leaves

Solvent	Physical appearance and yield of the plants in various solvents			
	Anogeissus leiocarpus	**Gram**	***Psidium guajava***	**Gram**
Hexane	Greasy/light green	1.8	Greasy/light green	2.1
Methanol	Molten/brown	4.0	Brown/green	6.7
Ethanol	Powdered green	3.2	Dark green	4.6
Aqueous	Brittle/brown	2.7	Brown	2.6

The results of the crude antibacterial activity of the *Anogeissus leiocarpus* leaves against *Staphylococcus aureus* and *E. coli*. Are presented in Table 4.4. At 50mg/ml, methanol leaves crude extract and aqueous leaves crude extract inhibited the growth of *Staphylococcus aureus* by 15mm and 9mm respectively. At 25mg/ml also the methanol and aqueous extract inhibited the growth of *Staphylococcus aureus*, by 13mm and 7mm respectively. At 12.5mg/ml only the methanol leaves crude extract inhibited the growth of *Staphylococcus aureus* by 11mm.

The results of the crude antibacterial activity of *Psidium guajava* leaves crude extract against *Staphylococcus aureus* and *E. coli* are presented in Table 4.5. At 50mg/ml, methanol leaves cruse extract inhibited the growth of both *Staphylococcus aureus* and *E. coli* by 19mm and 9mm respectively. Similarly, ethanol leaves crude extract inhibited the growth of *Staphylococcus aureus at* 50mg/ml by 12mm.

The result of the minimum inhibitory concentrations (MIC) of methanol and aqueous extract of *Anogeissus leiocarpus* leaves are presented in Table 4.6. The result revealed that the minimum inhibitory concentration of crude methanol lea extract for *Staphylococcus aureus* to be 12.5mg/ml and that of aqueous crude extract for *Staphylococcus aureus* to be 25mg/ml. similarly, the minimum bactericidal concentrations (MBC) of methanol and aqueous crude extract of *Anogeissus leiocarpus* leaves are presented in Table 4.7. The result revealed that the minimum bactericidal concentration of crude methanol leaves crude extract for *Staphylococcus aureus* to be 25mg/ml and that of aqueous crude extract for *Staphylococcus aureus* to be 50mg/ml.

Table 4.4: Antibacterial activity of the crude extract of *Anogeissus leiocarpus* leaves.

Conc. (mg/ml)	Mean of the zone of inhibition (mm)/Conc. of extracts(mg/ml)							
	hexane		methanol		Ethanol		aqueous	
	S. aureus	*E. coli*	*S. aureus*	*E. coli*	*S. aureus*	*E. coli*	*S. aureus*	*E. coli*
6.25	-	-	-	-	-	-	-	-
12.5	-	-	11	-	-	-	-	-
25.0	-	-	13	-	-	-	7.0	-
50.0	-	-	15	-	-	-	9.0	-
Control drug (tetracycline)	28	22	26	22	28	18	30	28

Key: - = No inhibition

Table 4.5: Antibacterial activity of the crude extract of *Psidium guajava* leaves.

Conc. Mg/ml	Mean of the zone of inhibition (mm)/Conc. of extracts (mg/ml)							
	hexane		methanol		ethanol		aqueous	
	S. aureus	*E. coli*	*S. aureus*	*E. coli*	*S. aureus*	*E. coli*	*S. aureus*	*E. coli*
6.25	-	-	-	-	-	-	-	-
12.5	-	-	10	-	6	-	-	-
25.0	-	-	15	6	10	-	-	-
50.0	-	-	19	9	12	-	-	-
Control drug (Tetracycline)	30	28	28	28	28	28	28	28

Key: - = No inhibition

Table 4.6: Minimum Inhibitory concentration (MIC) of methanolic and aqueous extract

Anogeissus leiocarpus* against *E. coli* and *S. aureus

Test Bacteria	**Methanol Extract**	**Aqueous Extract**
Escherichia coli	_	_
Staphylococcus aureus	12.5	25

Key**: - = No inhibition**

Table 4.7: Minimum Bactericidal Concentration (MBC) of methanolic and aqueous extract of *Anogeissus leiocarpus* against *E. coli* and *S. aureus*

Test Bacteria	Methanol Extract	Aqueous Extract
Escherichia coli	_	_
Staphylococcus aureus	25	50

Key**: - = No inhibition**

The result of the minimum inhibitory concentrations (MIC) of methanol and ethanol extract of *Psidium guajava* leave are presented in Table 4.8. The results indicated that the minimum inhibitory concentration of the crude methanol leaves extract for both *E. coli* and *Staphylococcus aureus* was 12.5mg/ml while that of ethanol crude extract was 12.5mg/ml for *Staphylococcus aureus* only.

The minimum bactericidal concentration (MBC) of methanol and ethanol extract of *Psidium guajava* leaves extract are presented in Table 4.9. The result suggested that the minimum bactericidal concentration of crude methanol leaves extract for *E. coli* was 50mg/ml and 25mg/ml for *Staphylococcus aureus.* The minimum bactericidal concentration of ethanol crude leaves extract for *Staphylococcus aureus* was 50mg/ml.

The results of the phytochemical screening of the crude methanolic leaves extracts of *Anogeissus leiocarpus and Psidium guajava* are presented in Table 4.10. The result indicated the presence of alkaloids, flavonoids cardiac glycosides, saponins, saponins glycosides, glycosides, tannins and steroids in the methanolic extracts of both *A. leiocarpus and P guajava*.

The results of the phytochemicals constituents of the fractions from methanol extract of *Psidium guajava* are presented in Table 4.11. The result revealed that fraction F4 which represent ethyl acetate/methanol 50:50 contains alkaloids, flavonoid, tannins and steroids.

Table 4.8: Minimum Inhibitory concentration (MIC) of methanolic and ethanolic extract of *Psidium guajava* against *E. coli* and *S. aureus*

Test Bacteria	Methanol Extract	Aqueous Extract
Escherichia coli	12.5	_
Staphylococcus aureus	12.5	12.5

Key**: - = No inhibition**

Table 4.9: Minimum Bactericidal concentration (MBC) of methanolic and ethanolic extract of *Psidium guajava* against *E. coli* and *S. aureus*

Test Bacteria	Methanol Extract	Aqueous Extract
Escherichia coli	50	_
Staphylococcus aureus	25	50

Key**: - = No inhibition**

Table 4.10: Phytochemical constituents of methanolic leaves extract of *Anogeissus leiocarpus* and *Psidium guajava.*

Bioactive compounds	***Anogeissus leiocarpus***	***Psidium guajava***
Alkaloids	+	+
Balsam	_	++
Anthraquinones	_	_
Flavonoids	++	++
Cardiac glycosides	+	+
Saponins	++	+++
Saponins glycosides	+	+++
Glycosides	++	+++
Steroids	+	++
Tannins	+++	+++
Volatile oil	_	++

Key- = absent, + = minimally present, ++ = moderately present and +++ = highly present.

Table 4.11: Phytochemical constituents from fractions of methanol extract of *Psidium guajava*

Phytochemicals	F1	F2	F3	F4	F5
Alkaloids	-	+	+	++	-
Flavonoids	-	-	+	+++	+
Tannins	+	+++	-	++	+
Volatile oil	++	-	-	-	+
Saponins	-	++	++	-	+
Antraquinones	-	-	-	-	-
Steroid	++	+	+	+	_
Glycosides	+	+	+	-	+

Key - = absent, + = minimally present, ++ = moderately present and +++ = highly present.

The antibacterial activity of the fractions from methanol leaves extract of *Psidium guajava* are presented in Table 4.12. The result suggested that only F4 fraction inhibited the growth of *Staphylococcus aureus* with zone of inhibition of 16mm.

The results of the general appearance and behavioural observation for the treated animal are presented in Table 4.13. The results indicated that there were no any physical or behavioural changes in all the albino rats following the administration of the methanolic leaves extract of *P. guajava* at 2000 and 5000mg/kgbw doses.

The result of the biochemical parameters in the serum samples of the albino rats treated with methanol leaves crude extract of *P. guajava* are presented in Table 4.14. It evaluates the function of liver using key parameters such as alkaline phosphate (ALP), aspartate transaminase (AST), alanine transaminase (ALT), albumin, total bilirubin and divided bilirubin, the result indicated significant increase in the level of ALP.

The result of kidney function test is presented in Table 4.15. The parameters evaluated are urea, creatinine, sodium, potassium and chlorine ions. The tables shows the mean and the standard deviation of the treated and control group.

Table 4.12: Antibacterial activity of fractions from methanol extract of *Psidium guajava* (mm)

Fractions	***Escherichia coli***	***Staphylococcus aureus***
F1	-	-
F2	-	-
F3	-	-
F4	-	16
F5	-	-
Controldrug (tetracycline)	28	28

Key: - = No activity

Table 4.13, Appearance and Behavioural Observations of the control and treated rats

	Control group		Treated group	
Observation	**8hrs**	**14days**	**8hrs**	**14days**
Activity	Active	Active	Active	Active
Breathing	Normal	Normal	Normal	Normal
Convulsions	Absent	Absent	Absent	Absent
Ears and eyes	Normal	Normal	Normal	Normal
Injury	Absent	Absent	Absent	Absent
Skin changes	Absent	Absent	Absent	Absent
Salivation	Absent	Absent	Absent	Absent
mortality	Absent	Absent	Absent	Absent

Table 4.14, Effect of methanol leaves crude extract of *P. guajava* on liver

Parameters	Control group	Group treated with 2000mg/kgbw	Group treated with 5000mg/kgbw
AST	70.6±2.79	68.1±5.23	64.6±15.2
ALP	273±1.30	836±5.03	923±9.91
ALT	110±0.26	139±11.2	139±9.50
Albumin	2.80±2.10	2.67±0.24	2.88±0.12
Total bilirubin	0.73±1.55	0.86±0.12	0.80±0.10
Divided bilirubin	0.13±1.24	0.08±1.27	0.06±1.45

Key: Values are mean± S.E.M for n=10

Mg/kgbw = Milligram per Kilogram body weight

AST = Aspartate transaminase, ALP = Alkaline phosphate, ALT = Alanine transaminase

Table 4.15, Effect of the methanol leaves crude extract of *P. guajava* on the kidney

Parameters	Control group	Group treated with 2000mg/kgbw	Group treated with 5000mg/kgbw
Urea	6.79±0.44	6.96±1.16	7.0±0.73
Creatinine	0.73±0.17	0.72±0..22	0.78±0.17
Na+	135.5±0.84	134.2±0.78	135.8±1.47
K+	4.51±0.12	4.41±0.26	4.44±0.06
CL-	96.3±0.94	95.9±1.91	97.3±1.25

Key: Values are mean ± S.E.M for n=10

Mg/kgbw = Milligram per kilogram body weight

Creatinine, Urea, Na+ = Sodium ion, K+ = Potassium ion and CL-=Chlorine

CHAPTER FIVE

5.0 DISCUSSION

The result obtained from the isolation and identification of bacteria revealed that different types of bacteria were found in the samples (urine and wound) with varying frequency of occurrence. It also indicated that the same organisms were found in both the samples collected (*Pseudomonas auroginosa, Klebsiella and Proteus*). However, out of the fifty (50) wound samples examined *Staphylococcus aureus* was the most common type of bacteria isolated and this is in agreement with the findings of Mahdi *et al.,* (2000) which indicated that *Staphylococcus aureus* was the most frequently isolated microorganisms from pus caused by incision to reach pus or fluid collection under the skin surface.

Crude antibacterial activity for both *Anogeissus leiocarpus* and *Psidium guajava* leaves extract in this study showed that at concentration of 6.25mg/ml all the test organisms were not susceptible to all the extracts. This suggest that the susceptibility of all the bacteria to the extract starts at concentration of 12.5mg/ml through 50mg/ml, which showed that the higher the concentration of the extract, the higher the antibacterial activity, thus, this exhibit the concentration dependent activity similar to the concentration dependent nature of antibiotics in which the rate of bacterial eradication increases with increase in concentration of the drug.

The result obtained from antibacterial activity of *Anogeissus leiocarpus* leaves extract shows that methanol and aqueous extract have inhibition ability. But, methanol extract show higher activity than the other solvents. This result indicated that methanol stands as the most effective solvent for the extraction of *Anogeissus*

leiocarpus. Similar findings (Alzoreky and Nakahara, 2003) have reported methanol to be more efficient in extracting substances from medicinal plants. This may have been due to the better solubility of the bioactive agents as polarity increased (El-mahmood *et al.*, 2010).

In most cases bioactivity has been associated with specific compounds or classes of secondary metabolites; hence the inability of the hexane extract to inhibit the growth of any of the test bacteria may be due to the presence in trace amount of phytochemicals because of non-polar nature of hexane. Noteworthy is the fact that solubility of bioactive agents in plant increases with increasing polarity (El-mahmood *et al.,* 2010). This may have contributed to the inability of hexane to elicit significant inhibitory activity on the tests bacteria.

In this research also, methanol and ethanol extract of *Psidium guajava* exhibited antibacterial activity against both the test organisms (*E. coli* and *Staphylococcus aureus*). This is line with the work of Abdelrahim *et al*, (2002) who reported that Guava leaves extract inhibited the growth of *Staphylococcus aureus* in a study carried out by disc diffusion method. However, the finding of this research is also in contrast to the results reported by Jaiarj *et al.,* (1999) testing for antibacterial activity of aqueous, methanol and chloroform extracts of *P. guajava*, on strains of *S. aureus* isolated from clinical patients, which obtained better results from aqueous extracts than from methanol extract. However, the difference in the extract performance suggest that there are multiple and different antimicrobial agents present in each type of extract acting in different ways on different bacterial strains.

The result of phytochemical analysis indicates the presence of flavonoids, alkaloid, cardiac glycosides, saponins, saponins glycosides, glycosides, steroids and tannins in both *Anogeissus leiocarpus* and *Psidium guajava.* In addition, balsam and volatile oil

are present only in *Psidium guajava.* These phytochemicals have been associated with antimicrobial activity (Aliyu *el al.,* 2008).

Flavonoids which was found abundantly in the methanolic extract of *P. guajava* in this research work, may have work synergistically with the other compounds in inhibiting the test organisms. This also agrees with the findings of Aliyu *et al.,* (2008) who reported that flavonoids have been known for its exceptional antioxidant activity and has attracted considerable attention due to its phenolic hydrogen which is very effective against bacteria, fungi, and virus, allergic and inflammatory reactions (Aliyu *et al.,* 2008).

Phytochemical screening indicated that methanol leave extract contain higher amount of tannins. Tannins are water soluble polyphenols and they have been found to form irreversible complexes with proline rich proteins resulting in the inhibition of the cell protein synthesis. Tannins also have binding capacity of bacterial protein through hydrogen binding hydrophobic effects and covalent bond formation (Shimada, 2006).

This investigation the antibacterial effect of *Anogeissus leiocarpus* and *Psidium guajava* leaves involved comparison of their effect with commercially prepared antibiotics. The findings of this study show that the commercial antibiotics have a larger inhibitory effect than the *Anogeissus leiocarpus* and *Psidium guajava* leaves. This could be due to the fact that the Tetracycline is a pure chemical while the *Anogeissus leiocarpus* and *P. guajava* solutions were crude extracts. This results support the findings of Vieira *et al.,* (2001) which reinforces the positions that commercially perfected and tested antibiotics should be used in the treatment of disease.

However, it is in contrast with the findings of Wei *et al*., (2000) which shows that Guava leaves and fruit juice has been tested in treatment of infantile diarrheal and the results showed that, those who were treated with guava recovered at 3 days which was shorter than the controls.

The test microorganisms exhibited appreciable antibacterial susceptibility to the extract of *A. leiocarpus*, especially methanolic leaf extract however, this study has a limitation because the yield from these extract were low to allow for further studies. Therefore *P. guajava*, which not only had abundant yield but also comparable or even higher antibacterial activity than *A. leiocarpus* was selected for further studies.

Column chromatography of the methanolic leaf extract of *P. guajava* in this study showed five (5) different fractions (F1-F5) in which only F4 was active against one of the bacterial isolate *(S. aureus*) with the zone of inhibition of 16mm. the remaining fractions were not active against both the test bacterial isolates. This is in line with the work of Abdulhamid *et al.,* (2017) which showed the column chromatographic fractions of *carica papaya* methanol leaves extract to have seven fractions with fraction 5 having the zone of inhibition against *s. aureus.* The crude has high antibacterial activity compared with fractions. This is may be explained from the stand point that the secondary metabolites in the crude elicited bioactivity synergistically, whereas, the fractions may have contained phytochemicals which acted singly, thus, supporting the suggestion of Okili and Ireogbu (2005) that crude extracts sometimes exhibit higher bioactivity than fractions.

Acute Toxicity study was conducted at doses of 2000mg/kg and 5000mg/kg body weight and it is usually carried out to determine whether or not a substance is safe for human use, in this investigation different biochemical parameters were analysed from

the serum of the albino rats such as alkaline phosphate (ALP), alanine transaminase (ALT), aspartate transaminase (AST), total bilirubin, direct bilirubin, urea, creatinine, sodium, potassium and chlorine to evaluate the liver and kidney functions.

In this research there was significant elevation ($p<0.05$) in the alkaline phosphate (ALP) and alanine transaminase (ALT) levels among the rats treated with the extract as compared with the control group, the increase in ALP level may be due to increased functional activity of the liver and this finding is consistent with the previous report of John *et al.,(*2014) which shows that lowered level of ALP are less common than elevated levels. ALP has been the marker enzyme for plasma membrane and is required in certain amount for proper functioning of organs. Alkaline phosphate (ALP) is a hydrolase responsible for removing phosphate groups from many types of molecules, including nucleotides, proteins and alkaloids. High ALP levels can occur if bile ducts are obstructed. ALP level increases if there is active bone formation occurring.

Alanine transaminase (ALT) is a transaminase, formerly called serum glutamate-pyruvate transaminase (SGPT). ALT is the most common in liver but may also be found in the blood. Significantly elevated level of ALT often suggest the existence of other medical problems such as liver damage, viral hepatitis, diabetes, heart failure, bile duct problems, infectious mononucleosis or myopathy. The increase in the ALT level in this work may be due to the liver damage. And this may be in of line with the findings Hydar *et al.*, (2013) which shows that liver damage and its recovery are usually assessed by measuring the level of serum enzymes-transaminases, particularly (ALT). All other biochemical parameters analysed there was no significant difference between the treated groups and the control.

In toxicology, the median lethal dose LD_{50} is a standard measurement of acute toxicity that is in milligrams (mg) of toxin per kilogram (kg) body weight. Thus, the value of LD_{50} for substance is the dose required to kill half the members of a tested population after a specific test duration. The result obtained in the present study showed that there was no mortality at the dose limit of 2000 and 5000mg/kgbw following oral administration with methanolic leaf extract of *P. guajava* and there was no any sign of toxicity in all the treated rats. This suggest that the methanolic leaf extract of *P. guajava* is therefore relatively harmless acutely. This is in agreement with the findings of Kabiru *et al.,* (2013) that revealed that the methanolic extract of *E. camaldulensis* leaf exhibited LD_{50} above 5000mg/kgbw and was therefore non-toxic acutely.

5.1 Conclusion

This research revealed that different bacterial species are isolated from urine and wound samples with varying degree of occurrences. *E. coli* was the most common in the urine while *S. aureus* was the most frequently isolated bacteria from the wound samples. The research also indicated that the leaves crude extract of *Psidium guajava* is active against *Staphylococcus aureus* and *E. coli* with the highest zones of inhibition of 19mm and 9mm respectively. The minimum inhibitory concentration (MIC) was 12.5mg/ml for *S. aureus* and 25mg/ml for *E. coli* while the minimum bactericidal concentration (MBC) was 25mg/ml for *S. aureus* and 50mg/ml for *E. coli*. The antibacterial activity differs with the extractive solvent and the efficacy is attributed to the phytochemicals it contains. The phytochemicals screening revealed the presence of many phytochemicals including Alkaloids, flavonoids, tannins, saponins, saponins glycosides, cardiac glycosides, glycosides, balsam and volatile oils in both the crude extracts and column chromatography fractions. The column

chromatography revealed five (5) different fractions with only fraction F4 having antibacterial activity against the *S. aureus* with the zone of inhibition of 16mm, the remaining fractions were inactive against both the test bacteria. The LD_{50} was above 5000mg/kgbw, hence consuming the leaves of *P. guajava* was found acutely harmless as it causes not any variation in the physical appearance and behaviours of the treated albino rats, however oral consumption of the leaves of this medicinal plant can cause elevation in the ALP level.

5.2 Recommendations

I. The ability of *Psidium guajava* to exert appreciable inhibitory effect on *Staphylococcus aureus* and *Escherichia coli* is a huge milestone in the search for new treatment strategies to manage infections caused by these microorganisms. These antibacterial activities support further studies to investigate/discover new chemical structures that can contribute to alleviate or cure diseases associated with these microorganisms.

II. Also the effectiveness of the medicinal plant has been established in the present study and the LD_{50} is above 5000mg/kgbw, hence, it indicates that the crude extract of *P. guajava* is acutely non-toxic. Therefore the medicinal plant is recommended for usage on short term basis by the local populace. This can be beneficial in providing low cost therapy for ailments in local communities.

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APPENDIX 1

Plate 1*: Psidium guajava*

Plate 11: *Anogeissus leiocapus*

APPENDIX 11

Preparation of media

Nutrient Agar

-28g weighed and poured in to sterile conical flask

-1000ml of distilled water added and dissolved

- The medium was heated on hot plate to dissolve well

-The medium was sterilise in an autoclave at 121ºC for 15min

- The medium was allowed to stand for some minutes before being poured asceptically in to sterile petri dishies.

MacConkey agar

-50g weighed and poured in to sterile conical flask

-1000ml of distilled water added and dissolved

- The medium was heated on hot plate to dissolve well

-The medium was sterilise in an autoclave at 121ºC for 15min

-Medium was allowed to cool down on the media bench and poured asceptically in to sterile plates.

Eosin methylene blue (EMB) agar

-35.5g weighed and poured in to sterile conical flask

-1000ml of distilled water added and dissolved

- The medium was heated on hot plate to dissolve

-The medium was sterilise in an autoclave at 121ºC for 15min

-medium was allowed to cool down on the media bench and poured asceptically in to sterile plates.

Manitol salt agar (MSA)

111g weighed and poured in to sterile conical flask

-1000ml of distilled water added and dissolved

- The medium was heated on hot plate to dissolve well

-The medium was sterilise in an autoclave at 121ºC for 15min

- The medium was allowed to stand for some minutes before being poured asceptically in to sterile petri dishies.

APPENDIX 111

McFarland Turbidity standard

1. 1% solution (w/v) of anhydrous barium chloride ($Bacl_2$).

2. 1% solution (v/v) of sulfuric acid (H_2SO4) prepered.

3. The two solutions were mixed using these ratio to obtain the desired McFarland scale:

Scale	**Amt of 1% $Bacl_2$ (ml)**	**Amt. of 1% H_2SO4 (ml)**	**Estimated Bacterial Conc. X 10^6**
0.5	0.5	9.95	150

1	0.1	9.9	300
2	0.2	9.8	600
3	0.3	9.7	900
4	0.4	9.6	1200
5	0.5	9.5	1500
6	0.6	9.4	1800
7	0.7	9.3	2100
8	0.8	9.2	2400
9	0.9	9.1	2700
10	1.0	9.0	3000

APPENDIX 1V

S/N	Isolate/ Parentage	No. of occurrence in urine sample	No. of occurrence in wound sample

1	*Staphylococcus*	*aureus*	4	19
2	24.73%		15	-
3	*Escherichia*	*coli*	10	5
4	16.12%		6	5
5	*Proteus*	*spp*	5	4
6	12.02%		5	3
7	*P.*	*auroginosa*	3	-
8	11.82%		2	2
9	*Acinobacter*	*spp*	-	3
10	9.67%		-	2
	Klebsiella spp	7.52%		
	Enterococcus	*spp*		
	3.22%			
	Coagulase	*-ve staph*		
	4.30%			
	B-haemolytic	*streph*		
	3.22%			
	S.	*pyogenes*		
	2.15%			