PROPHYLACTIC ACTIVITY OF *DIODIA SCANDENS* CRUDE EXTRACTS ON SWISS ALBINO RATS

BY

OLOWOKERE, VICTORIA BUKOLA

MCB/11/0344

DEPARTMENT OF MICROBIOLOGY

FACULTY OF SCIENCE

FEDERAL UNIVERSITY OYE-EKITI

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CERTIFICATION

We certify that this research work was carried out by **OLOWOKERE, Victoria Bukola**, of the Department of Microbiology, Faculty of Science, Federal University Oye-Ekiti, under the supervision of DR S.K.S. OJO.

DR S.K.S OJO

SUPERVISOR

03-12-2015

DATE

PROF. BRYAN O. OGENEH

HEAD OF DEPARTMENT

04/12/2015

DATE

DEDICATION

This project work is dedicated to God Almighty, the source of all knowledge, the fountain of peace, success and the citadel of all wisdom.

ACKNOWLEDGEMENT

I have learnt in life that the greatest asset anyone can possess is the ability to give honest and sincere appreciation. I have grown up to know that whosoever is incapable of gratitude is incapable of any noble sentiment.

I am personally indebted to my parents for their care and unflinching support given me. I acknowledged them for giving me the best and greatest asset that money cannot buy. I am proud of them.

I am indeed very grateful to my wonderful supervisor, Dr. S.K.S OJO, who made sure that I did not only write project but also know every step involved in the methodology. He also painstakingly read through this report and makes necessary corrections despite his tight schedule. I say a big thank you to the Head, Department of Microbiology and all my Lecturers for the knowledge impacted on me for the past four years. May the good Lord crown your efforts.

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My special and worthy thanks to Dr. Ige for tremendous contributions towards the success of this work. May God continually bless him with the blessings of Heaven

This acknowledgement would be conspicuously incomplete without acknowledging Pastor Matthew Chukwu for his prayers, advice, encouragement and support. In fact, his encouragement has been a driving force in my life. Thanks for being a good and faithful shepherd. God is your reward.

To all my Microbiology class '15 students especially my group members, kudos to you.

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ABSTRACT

The aim of this study was to evaluate the prophylactic activity of *Diodia scandens* on Swiss albino rats challenged with *Staphylococcus epidermidis*. Twenty (20) Swiss albino rats of both sexes weighing (120-200) were housed in polycarbonated cages at ambient environmental condition of 23±2°C, 30-60% relative humidity. They were defasted for 4hour prior to treatment. Different concentrations of different extracts (50mg/kg and 100mg/kg) of *Diodia scandens* were given according to the weight of rats and were challenged with 0.5ml of 1×10^8 cfu/ml of *Staphylococcus epidermidis* in Mueller Hinton broth using 0.5 Mcfarland standard at the 4th day of the pre-treatment. The result showed aggressiveness, no fever or inflammation during the pre-treatment. After the organism challenge test it was observed that with some level of protection were offered by extract of *Diodia scandens* on the rats. The protection offered by *Diodia scandens* after organism challenged test has shown that the plant is potent, effective and had boosted the immunity of the rats against *Staphylococcus epidermidis*. It was concluded that 50mg/kg and 100mg/kg of *Diodia scandens* was effective during pre-treatment and was not toxic on the rats.

CHAPTER ONE

1.0. INTRODUCTION

There are high proportions of antibiotic resistance (ABR) in bacteria that cause common infections (e.g. urinary tract infections, pneumonia, bloodstream infections) in all regions of the world (Goossens *et al.,* 2005). A high percentage of hospital-acquired infections are caused by highly resistant bacteria such as multidrug-resistant Gram-positive bacteria. Patients with infections caused by drug-resistant bacteria are generally at increased risk of worse clinical outcomes and death, and consume more healthcare resources than patients infected with the same bacteria that are not resistant (WHO, 2014).

Staphylococci have again emerged as the predominant organisms causing infections in the hospital setting and are the leading cause of nosocomial infections and community-acquired infections including impetigo, folliculitis, furuncles, sycosis barbe, cellulitis, abscesses, osteomyelitis and bacteraemia (Gad *et al.*, 2010; Bashir *et al.,* 2007). Most developed countries have reported an increase in colonization and infection in hospitalized patients by Coagulase negative *Staphylococci* (CoNS) while there are scanty data in developing countries.

Since the introduction of antimicrobials, bacteria have developed mechanism for resisting the effects of antibiotics and the levels of antibiotic resistant infections in the developing world have increased steadily in the last few decades as a result of combination of microbial characteristics and the selective pressure of antimicrobial used (Bashir *et al.,* 2007; Blondeau and Tillotson, 2000). Several mechanisms by microorganisms in overcoming the activities of antimicrobial agents include the production of structure-altering or inactivating enzymes (e.g., beta-lactamase or aminoglycoside-modifying enzymes), alteration of penicillin-binding

proteins or other cell wall target sites, altered DNAase targets, permeability mutations, active efflux systems and ribosomal modification.

Multi-drug resistance bacteria in both the hospital and community environment are of importance to the clinician, as it is the major cause of failure in the treatment of infectious diseases, increased morbidity and mortality and the evolution of new pathogens (Akinjogunla and Enabulele, 2010).

Recently, the discovery and development of medicinal plants as drugs (especially from China, India and Nigeria and some African countries) has proven effective in the treatment of multi-drug resistance pattern among clinical and environmental isolates, which has plagued the healthcare system especially in developing and underdeveloped countries. The main essence of using plant-derived medicines are that they are with little or no side effects compare to than synthetic alternatives, offering profound therapeutic benefits and more affordable treatment (Ojo *et al.*, 2013a).

RESEARCH PROBLEM

The doubt that is often encountered in the use of traditional herbal medicine for the treatment of various pathological and physiological diseases due to the indeterminable toxic level of such traditional herbal medicine has flecked both primary and tertiary healthcare system in Nigeria.

AIM

To evaluate the prophylactic activity of *Diodia scandens* on swiss albino rats challenged with *Staphylococcus epidermidis.*

OBJECTIVES

- To confirm the viability of the reference strain using cultural and biochemical methods.
- To carryout crude extraction of bioactive ingredients using hot (soxhlet) and cold extraction method in different solvent of ethyl acetate and n-hexane.
- To perform pre-treatment of Swiss albino rat using different concentrations of *Diodia scanden* crude extracts.
- To determine the prophylactic activity of *Diodia scanden* on Swiss albino rats challenging with *Staphyloçoccus epidermidis.*

CHAPTER TWO

2.0 LITERATURE REVIEW

2.1 Medicinal Plants

Medicinal plants have been identified and used throughout human history. Plants have the ability to synthesize a wide variety of chemical compounds that are used to perform important biological functions, and to defend against attack from predators such as insects, fungi and herbivorous mammals. At least 12,000 such compounds have been isolated so far; a number estimated to be less than 10% of the total (Tapsell *et al.,* 2006, Lai, 2004). Chemical compounds in plants mediate their effect on the human body through processes identical to those already well understood for the chemical compounds in conventional drugs; thus herbal medicines do not differ greatly from conventional drugs in terms of how they work. This enables herbal medicines to be as effective as conventional medicines, but also gives them the same potential to cause harmful side effects (Tapsell *et al.,* 2006, Lai, 2004).

Ethnobotany (the study of traditional human uses of plants) is recognized as an effective way to discover future medicines. In 2001, researchers identified 122 compounds used in modern medicine which were derived from "ethnomedical" plant sources. Among these, 80% have had an ethnomedical use of identical or related to the current use of the active elements of the plant (Fabricant, 2004).

Many of the pharmaceuticals currently available to physicians have a long history of use as herbal remedies, including aspirin, digitalis, quinine, and opium (Swain, 2000). The use of herbs to treat disease is almost universal among non-industrialized societies, and is often more affordable than purchasing expensive modern pharmaceuticals.

The World Health Organization (WHO) estimates that 80% of the populations of some Asian and African countries presently use herbal medicine for some aspect of primary health care. Studies in the United States and Europe have shown that their use is less common in clinical settings, but has become increasingly more common in recent years as scientific evidence about the effectiveness of herbal medicine has become more widely available (WHO, 2014).

2.1.1 History of Medicinal Plants

The use of plants as medicine predates written human history. Many of the herbs and spices used by humans to season food also yield useful medicinal compounds. Studies show that in tropical climates where pathogens are the most abundant, recipes is the most highly spiced (Sumner, 2000). Further, the spices with the most potent antimicrobial activity tend to be selected. In all cultures, vegetables are spiced less than meat, presumably because they are more resistant to spoilage. Angiosperms (flowering plants) were the original source of most plant medicines. Many of the common weeds that populate human settlements, such as nettle, dandelion and chickweed, have medicinal properties (Stepp, 2004). A large amount of archaeological evidence exists which indicates that humans were using medicinal plants during the Paleolithic, approximately 60,000 years ago. Furthermore, animals such as non-human primates, monarch butterflies and sheep are also known to ingest medicinal plants to treat illness (Sumner, 2000).

2.1.2 Modern Study of Plant Medicines

Many of the pharmaceuticals currently available to physicians have a long history of use as herbal remedies, including opium, aspirin, digitalis, and quinine. The World Health Organization (WHO) estimates that 80% of the population of some Asian and African countries presently uses herbal medicine for some aspect of primary health care. Pharmaceuticals are prohibitively expensive for most of the world's population, half of which

lives on less than $2 U.S. per day (Edgar *et al.*, 2002). In comparison, herbal medicines can be grown from seed or gathered from nature for little or no cost. The use of, and search for, drugs and dietary supplements derived from plants have accelerated in recent years. Pharmacologists, microbiologists, botanists, and natural-products chemists are combing the Earth for phytochemicals and leads that could be developed for treatment of various diseases. In fact, according to the World Health Organization, approximately 25% of modern drugs used in the United States have been derived from plants (Edgar *et al.*, 2002).

Among the 120 active compounds currently isolated from the higher plants and widely used in modern medicine today, 80% show a positive correlation between their modern therapeutic use and the traditional use of the plants from which they are derived. More than two thirds of the world's plant species, at least 35,000 of which are estimated to have medicinal value come from the developing countries. At least 7,000 medical compounds in the modern pharmacopoeia are derived from plants. In many medicinal and aromatic plants (MAPs), significant variations of plants characteristics have been ascertained with varying soil traits, and the selective recovery and subsequent release in food of certain elements have been demonstrated. Great attention must be paid to choose soil and cropping strategies, to obtain satisfactory yields of high quality and best-priced products, respecting their safety and nutritional value (Edgar *et al.*, 2002).

2.1.3 Safety in the Use of Medicinal Plant

A number of herbs are thought to be likely to cause adverse effects. Furthermore, adulteration, inappropriate formulation, or lack of understanding of plant and drug interactions have led to adverse reactions that are sometimes life threatening or lethal (Ernst, 2007). Proper double-blind clinical trials are needed to determine the safety and efficacy of each plant before it can be recommended for medical use. Although many consumers believe

that herbal medicines are safe because they are "natural", herbal medicines and synthetic drugs may interact, causing toxicity to the patient. Herbal remedies can also be dangerously contaminated, and herbal medicines without established efficacy may unknowingly be used to replace medicines that do have corroborated efficacy (Ernst, 2007).

Standardization of purity and dosage is not mandated in the United States, but even products made to the same specification may differ as a result of biochemical variations within a species of plant. Plants have chemical defense mechanisms against predators that can have adverse or lethal effects on humans. Examples of highly toxic herbs include poison hemlock and nightshade. They are not marketed to the public as herbs, because the risks are well known, partly due to a long and colorful history in Europe, associated with "sorcery", "magic" and intrigue. Although not frequent, adverse reactions have been reported for herbs in widespread use. On occasion, serious untoward outcomes have been linked to herb consumption (Pinn, 2001).

2.2. Description of *Diodia scandens*

According to Essiett *et al.* (2010), *Diodia scandens* belong to the family Rubiacea; an evergreen perennial herb with slender angular stem of 1-3m long and the leaves are scabrid. *Diodia scandens* Sw has a dark green coloration, tasteless, odorless and has solitary inflorescence. It is a straggling herb, which has been in use in the Western African system of medicine. The stem and root roots had a higher concentration of active ingredient compared to the leaves which has an alternate leaf arrangement, and presence of petiole. It has enormous usefulness and importance with the whole parts of the plants useful in curing various ailments.

Plate 1. ***Diodia scandens*** **(Source: Federal University Oye-Ekiti and environs)**

2.2.1 Uses of *Diodia scandens*

Diodia scandens has enormous usefulness and importance. In Nigeria, the leaves are used for curing eczema (antifungal property); as fodder to poultry; its juice is used to stop bleeding; its extract is used to treat bruises and minor cuts and drunk as metric during treatment of ear problems (Etukudo, 2003). Generally, the plants medicinal value includes its use as antidotes (venomous stings, bites, etc.), painkiller, treatment of veneral diseases and cutaneous and subcutaneous fungal infections. However, the different parts of the plants- sap, leaf, stem and root, are used for various medicinal purposes. The leaf is used for treating arthritis, rheumatism, cutaneous and subcutaneous parasitic infection, diarrhea, dysentery and anti-abortifacients; the leaf plus roots are used for dropsy, swellings, edema, and gout and as lactation stimulants; while the sap is used for treating ear infections, paralysis, epilepsy, convulsions, spasm and pulmonary troubles, wounds, and ringworm (Etukudo, 2003).

2.2.2 Phytochemical Properties of *Diodia scandens*

In the recent years, secondary plant metabolites- known as phytochemicals have been extensively investigated for their potency as medicinal agents. Studies carried out on some plants showed that some plants contain many substances such as peptides, tannins, alkaloids, essential oils, phenols and flavonoids among others, which could serve as sources for antimicrobial production (Okoli *et al.,* 2009). *D. scandens* does not contain alkaloids, flavonoids, phobotannins and anthraquinones. However, it has saponins, which have anti-inflammatory, anti-yeasts, anti-fungal, anti-parasitic, anti-tumor, anti-viral and anti-abortifacient activities were present. Tannins, which have astringent and detergent properties were also present, and can be used against diarrhoea. According to Essiet *et al.* (2011), the photochemistry of the leaf extract revealed the presence of saponins, tannins, cardiac glycosides and absence of flavonoids, phlobatannins, alkaloids and anthraquinones.

2.2.3 Antimicrobial properties of *Diodia scandens*

D. scandens also has antimicrobial properties of which much is not known or documented yet. It is used in some localities in Nigeria to treat cutaneous and subcutaneous fungal infections. The procedure involves squeezing out the sap from the whole plant, freshly collected and appling the fluid on the infected skin by scrubbing. If done frequently for about 3-5days, the antifungal effect is seen. This agrees with the report of Essiett *et al.,* (2011), who stated that in Nigeria, the leaves are used for curing eczema, which is a fungal infection.

2.3. Description of Staphylococci

Taxonomically, the genus *Staphylococcus* is in the bacterial family Staphylococcacea. Staphylococci are non-motile Gram positive cocci being approximately 1µm in diameter, spherical with irregular clusters resembling bunch of grapes. Thus named as *Staphylococcus* Matthew *et al.* (2003). In 1884, Rosenbach described staphylococci according to the colony types they had and proposed the appropriate nomenclature: *Staphylococcus aureus* (yellow) and *Staphylococcus albus* (white). The latter species is now named *Staphylococcus epidermidis*. *Staphylococcus aureus* forms yellowish large colony and *Staphylococcus epidermidis* forms relatively small white colony. There are many other *Staphyloccus* species but these two are well defined in human diseases. Staphylococci are facultative anaerobes grow by aerobic respiration or by fermentation resulting in lactic acid production. They are oxidase negative and catalase positive. Their cell wall contains teichoic acid. Teichoic acid composition is also different among the Staphylococcal species. The cell wall contains ribitol teichoic acid (polysaccharide A) in *Staphylococcus aureus,* glycerol teichoic acid (polysaccharide B) in *S. epidermidis*. Staphylococci are common in skin, nasal cavity, oropharynx, gastrointestinal tract and in genitourinary tract flora (Matthew *et al.,* 2003).

2.3.1 Description of *Staphylococcus epidermidis*

Staphylococcus epidermidis is a coagulase negative, catalase positive bacteria usually resident in skin flora, in gut and in the respiratory tract. These coagulase negative staphylococci are known as the leading cause of the nosocomial sepsis. These bacteria are also known to cause medical device derived infections. Biofilm production is an important feature among staphylococci it may also be the reason why *Staphylococcus epidermidis* once thought to be non-pathogenic is considered to be pathogenic these days. Biofilms are the highly organized multicellular complexes occurring in two main steps: attachment of bacteria on solid surface and proliferation of cells in multilayers leading in the formation of the enclosed bacterial community in a polymeric matrix (Francois, 2008).

2.4 Pathogenesis of Staphylococci

Staphylococci cause a variety of suppurative (pus-forming) infections and toxinoses in humans. It causes superficial skin lesions such as boils, styes and furuncules; more serious infections such as pneumonia, mastitis, phlebitis, meningitis, and urinary tract infections; and deep-seated infections, such as osteomyelitis and endocarditis. *S. aureus* is a major cause of hospital acquired (nosocomial) infection of surgical wounds and infections associated with indwelling medical devices. *S. aureus* causes food poisoning by releasing enterotoxins into food, and toxic shock syndrome by release of superantigens into the blood stream.

However, there are correlations between strains isolated from particular diseases and expression of particular virulence determinants, which suggests their role in particular diseases. The application of molecular biology has led to advances in unraveling the pathogenesis of staphylococcal diseases. Genes encoding potential virulence factors have been cloned and sequenced, and many protein toxins have been purified. With some

staphylococcal toxins, symptoms of human disease can be reproduced in animals with the purified protein toxins, lending an understanding of their mechanism of action. Human staphylococcal infections are frequent, but usually remain localized at the portal of entry by the normal host defenses. The portal may be a hair follicle, but usually it is a break in the skin which may be a minute needle-stick or a surgical wound. Foreign bodies, including sutures, are readily colonized by staphylococci, which may make infections difficult to control. Another portal of entry is the respiratory tract. Staphylococcal pneumonia is a frequent complication of influenza. The localized host response to staphylococcal infection is inflammation, characterized by an elevated temperature at the site, swelling, the accumulation of pus, and necrosis of tissue (Reusch *et al.,* 2008).

Around the inflamed area, a fibrin clot may form, walling off the bacteria and leukocytes as a characteristic pus-filled boil or abscess. More serious infections of the skin may occur, such as furuncles or impetigo. Localized infection of the bone is called osteomyelitis. Serious consequences of staphylococcal infections occur when the bacteria invade the blood stream. A resulting septicemia may be rapidly fatal; a bacteremia may result in seeding other internal abscesses, other skin lesions, or infections in the lung, kidney, heart, skeletal muscle or meninges (Reusch *et al.,* 2008).

Various virulence factors are associated with the pathogenicity of staphylococci infection, which is as follows:

I. **Adherence**

Staphylococci express certain surface proteins that are necessary for binding throughout the body. These surface proteins typically promote attachment to laminin and fibronectin. Most strains also express a clumping factor, coagulase that promotes attachment to blood clots and traumatized tissue. Fibronectin and fibrinogen-binding proteins are also produced by *S.*

aureus as virulence factors. Mutations of these proteins that have been studied drastically decrease the bacteria's virulence. Adhesins that bind to collagen are significant in infections that cause osteomyelitis. Once the bacteria have adhered, they can secrete a biofilm that make them difficult to eradicate (Papadakis, 2014).

II. **Invasion**

Invasins help to promote bacterial spread within the tissues of the body. Alpha toxin is the most characterized and potent membrane-damaging toxin secreted by staphylococci. It is originally expressed as a monomer that binds to the surface of susceptible cells before becoming oligamerized into a heptomeric ring that causes a pore in the membrane of the attacked cell, which causes the contents of the cell to leak out. Platelets and monocytes are especially susceptible to this toxin. ß-toxin is a sphingomyelinase toxin that damages lipid membranes that are rich in sphingomyelin. It is not often expressed in human isolated strains of the bacteria.

Staphylococc isolates expresses leukocidin, a multi-component protein that acts to severely damage cell membranes, but 90% of *S. aureus* strains isolated from severe dermonecrotic lesions are found to express this toxin. This correlation suggests that the toxin is a large component of necrotizing skin infections. Leukocidin forms a hetero-oligamerictransmembrane pore made from four LukF and four LukS subunits. This toxin is hemolytic, but not as hemolytic as alpha toxin. S. aureus also produces a host of proteases, lipases, and DNase, and FAME (fatty acid modifying enzyme). FAME may be important for virulence in abscesses, where it could prolong bacterial survival by modifying anti-bacterial lipids in the cell (Papadakis, 2014).

III. Avoidance

To avoid the immune system, *S. aureus* produces a microcapsule composed of surface

polysaccharide that can only be detected by electron microscopy. Strains of the bacteria isolated from infections have been found to produce this capsule in high amounts. This capsule helps the bacteria evade phagocytosis in the absence of complement. Protein A is an Fc receptor on the surface of the pathogen that specifically binds IgG antibody in the wrong orientation. This incorrect orientation of the antibody disrupts opsonization and phagocytosis of the bacteria, allowing it to evade the immune system. Leukocidin also helps in avoidance of the immune system, as acts specifically on phagocytic cells called polymorphonuclear leukocytes (Papadakis, 2014).

IV. Toxins

This bacterium produces several toxins. A few are listed above, such as invasion toxins. *S. aureus* also releases other exotoxins and enterotoxins. Exfoliatoxin comes in two distinct forms (ETA and ETB) and causes scalded skin syndrome in infants. These bacteria secrete two different types of toxins that are considered to have "Superantigen activity," enterotoxins and Toxic Shock Syndrome Toxin (TSST-1). There are six types of enterotoxins (SE-A, B, C, D, E, and G) which cause food poisoning in the host. Enterotoxins B and C are also responsible for 50% of non-menstrual related cases of TSS.

S. aureus enterotoxins and TSS toxins work by stimulating T-cells nonspecifically without normal antigen recognition, causing them to release cytokines in large amounts, which cause the symptoms of TSS. These super antigens bind directly to MHC II receptors rather than in the antigen binding groove of the MHC (Papadakis, 2014).

2.5 Epidemiology of staphylocci infections

S. epidermidis is distributed worldwide and is most often found in hospitals and nursing homes, but community settings are becoming increasingly contaminated. Scientists typically

distinguish between CA-MRS and HA-MRS when studying epidemiology. *Staphylococcus aureus* was first isolated by a surgeon in the 1890's. The first strain of MRS was observed in 1961, and now approximately 35% of hospital strains of *S. epidermidis* are methicillin resistant. Staphylococci have the ability to synthesize and secrete many factors that either allow the bacteria to survive in the host or cause damage to host tissues (Batabyal *et al.*, 2012). The list below summarizes these substances and their effects on the host:

- Surface proteins (for example, capsular polysaccharide, protein A) to enhance attachment to host cells; others reduce phagocytosis (host immune cell's ability to ingest and kill bacteria)
- Membrane-altering toxins (for example, alpha, beta toxins plus leukocidin) all damage host cells by making holes in their membranes
- Exfoliatin toxins (exotoxins ETA and ETB) cause scalded skin syndrome (exfoliation of skin after erythematous cellulitis) in infants and children
- Enterotoxins (exotoxins secreted into the gastrointestinal tract termed SE-A, B, C, D, E, and G); cause nausea and vomiting associated with food poisoning
- Toxic shock syndrome toxin (TSST-1 toxin) syndrome of rapidly onset of fever (102 F or higher), low blood pressure (hypotension), watery diarrhea, muscle aches, weakness, and a rash after about 24 hours associated with staph infections. This occurs usually in females with tampons in place but occasionally occur in males and females with other staph infections such as wound infections.
- Coagulase; possibly protects staph bacteria from host immune cells by causing bacterial aggregation
- Slime (a biofilm secreted by *S. epidermidis*); Coats and protects bacteria from host's immune cells
- Antibiotic resistance; Staphylococci strains have developed remarkable resistance to a

number of drugs which make antibiotic treatment frequently complex.

- Other factors produced by these bacteria that may play a role in causing disease are hyaluronidase, kinases, clotting factor, and others, but their disease-causing potentials are still being evaluated (Kluytmans *et al.,* 2003).

2.6. Clinical symptoms

S. epidermidis can cause a whole host of different diseases and symptoms depending on where the infection is occurring in the body. The most well known Staphylococci infection is that of the skin, with many other diseases. Diagnosis of the infection depends on where the bacteria have caused infection.

I. Bacteremia

Bacteremia, also known as "blood poisoning" can occur when bacteria enter into the blood stream and begin to colonize. In some cases, bacteremia is self-limiting and asymptomatic but in other cases is known to lead to septic shock, which is fatal (Goldstein *et al., 2005*). Bacteremia may be characterized by one of more of the following symptoms: fever, chills, malaise, abdominal pain, nausea, vomiting, diarrhea, anxiety, shortness of breath, and confusion. Bacteremia is diagnosed by culturing blood for bacteria. The blood might also reveal elevated white blood cell levels. The bacteria can travel to locations deep within your body, to produce infections affecting: Internal organs, such as your brain, heart or lungs, Bones and muscles, and Surgically implanted devices, such as artificial joints or cardiac pacemakers (Francois, 2008).

II. Endocarditis

Endocarditis occurs when the endocardium of the heart muscle is damaged, allowing bacteria in the blood stream to become lodged onto the heart valves or lining and cause infection. Endocarditis is not characterized by a single symptom, but can be detected by many

symptoms such as a mild fever, chills, weakness, cough, trouble breathing, headaches, aching joints, and loss of appetite. It also causes heart murmurs and regurgitation in the heart valves. This disease usually affects people between the ages of 15-60. A risk that accompanies endocarditis is the formation of emboli. This occurs when bacteria break off from the site of colonization in clumps and become lodged in blood vessels. This can lead to organ failure as nutrients are blocked from accessing certain areas of the blood stream. Diagnosis of this is usually done by taking a sample of blood from the patient to test it for bacteria. Another way to diagnose Endocarditis is echocardiography, which uses ultrasound waves to make an image of the heart. This allows doctors to check for abnormalities like bacterial vegetation (Francois, 2008).

III. Skin infections

According to Stevens *et al.* (2010), skin infections caused by staphylococci include;

- Boils: The most common type of staphylococci infection is the boil, a pocket of pus that develops in a hair follicle or oil gland. The skin over the infected area usually becomes red and swollen. Boils occur most often under the arms or around the groin or buttocks.
- Impetigo: This contagious, often painful rash can be caused by staphylococci. Impetigo usually features large blisters that may ooze fluid and develop a honey-colored crust.
- Cellulitis: This an infection of the deeper layers of skin, causing skin redness and swelling on the surface of your skin. Sores (ulcers) or areas of oozing discharge may develop. Cellulitis occurs most often in the lower legs and feet.
- Staphylococcal scalded skin syndrome: Toxins produced as a result of staphylococci infection may lead to staphylococcal scalded skin syndrome. Mostly affecting newborns and children, this condition features fever, a rash and sometimes blisters. When the blisters break, the top layer of skin comes off leaving a red, raw surface that looks like a

burn.

IV. Food poisoning

Staphylococci are one of the most common causes of food poisoning. Symptoms come on quickly, usually within hours of eating a contaminated food (Le Loir *et al., 2003*). Symptoms usually disappear quickly, too, often lasting just half a day. A staphylococci infection in food usually doesn't cause a fever. Signs and symptoms you can expect with this type of staphylococci infection include: Nausea and vomiting, Diarrhea, Dehydration and Low blood pressure

2.7 Treatment

Treatment of staphylococci infection may include surgical and antibiotic treatment. In most patients who require surgical treatment, antibiotic treatment is also required. Pus drainage is the main surgical treatment; however, surgical removal of sources of infection (for example, intravenous lines, artificial grafts, heart valves, or pacemakers) may be required. Other sites of infection, such as joint infections (especially in children), osteomyelitis, or postoperative abscesses, may require surgery. Any tissue site that continues to harbor the bacteria may require surgical intervention (Markowitz *et al.,* 2002).

Antibiotics commonly prescribed to treat staphylococci infections include certain cephalosporins, nafcillin or related antibiotics, sulfa drugs or vancomycin. Vancomycin increasingly is required to treat serious staphylococci infections because so many strains of staphylococci have become resistant to other traditional medicines (Papadakis, 2014).

2.7.1. Antibiotic Resistance in Staphylococci

Antibiotic resistance is a growing world-wide issue because of its effect on the rapid spread of threatening diseases and infections and the inability to control them. Resistance occurs both naturally and due to human action. While natural resistance is a large issue, recently the

involvement of modern medicine and over use of antibiotics have caused about 23,000 people to die each year due to antibiotic resistant bacterial infections, while about 2 million people experience these same infections (CDC, 2014).

Antibiotic resistant infections can occur anywhere in the community but recently there has been an increase of infections that come from hospitals and other medical facilities. Resistance in bacteria is a large issue because as bacteria evolves and forms ways of counteracting the antibiotics, the antibiotics become ineffective and harmful, making bacteria to thrive in multiple environments (Williams, 2003).

Several mechanisms have evolved in bacteria which confer them with antibiotic resistance. These mechanisms can chemically modify the antibiotic, render it inactive through physical removal from the cell, or modify target site so that it is not recognized by the antibiotic (Benveniste and Davies, 2003). The most common mode is enzymatic inactivation of the antibiotic. An existing cellular enzyme is modified to react with the antibiotic in such a way that it no longer affects the microorganism. An alternative strategy utilized by many bacteria is the alteration of the antibiotic target site. Antibiotic inactivation mechanisms are more commonly associated with bacterium that gains resistance overtime from human action. An example of this can be traced through *Staphylococcus aureus*, which fights antibiotics by deactivating β-lactam binding proteins.

2.8 Laboratory Diagnosis

Depending upon the type of infection present, an appropriate specimen is obtained accordingly and sent to the laboratory for definitive identification by using biochemical or enzyme-based tests. A Gram stain is first performed to guide the way, which should show typical Gram-positive bacteria, cocci, in clusters (Mackay, (2007).

The following specimens should be collected to confirm the diagnosis;

I. Pus from abscesses wounds and burns.

II. Sputum from cases of pneumonia

III. Faeces or vomit from patience with suspected food poisoning or remains of implicated food.

IV. Blood from patience with suspected bacteriamia i.e septic shock, osteomyelitis and endocarditis.

V. Mid stream urine from patient with suspected cystitis.

VI. Anterior nausea or swab (moisten in sterile water or saline) from suspected carriers

Specimen can also be demonstrated by microscopic and culture on blood agar, inoculated on mannitol salt agar, which is a selective medium with 7–9% NaCl that allows *S. aureus* to grow, producing yellow-colored colonies as a result of mannitol fermentation and subsequent drop in the medium's pH, which is used to differentiate between *Staphylococcus aureus* from Coagulase negative staphylococci (Francois, 2008).

2.8.1 Biochemical Identification

Assignment of a strain to the genus *Staphylococcus* requires it to be a Gram-positive coccus that forms clusters, produces catalase, has an appropriate cell wall structure (including peptidoglycan type and teichoic acid presence) and G + C content of DNA in a range of 30–40 mol% (Harris *et al.*, 2002). *Staphylococcus* species are facultative anaerobes (capable of growth both aerobically and anaerobically) and can be differentiated from other aerobic and facultative anaerobic, Gram-positive cocci by several simple tests. All species grow in the presence of bile salts. It was believed that all species were coagulase-positive, however it is now known that not all *Staphylococcus* are coagulase positive (Ryan and Ray, 2004).

Growth can also occur in a 6.5% NaCl solution. On Baird Parker medium, *Staphylococcus* spp. grow fermentatively, except for *S. epidermidis*, which grows oxidatively. *Staphylococcus* spp. is resistant to bacitracin and susceptible to furazolidone. Further biochemical testing is needed to identify to the species level. (Matthews *et al.,* 2003)

Coagulase production

One of the most important phenotypical features used in the classification of staphylococci is their ability to produce coagulase, an enzyme that causes blood clot formation (Jin *et al.,* 2004). *S. aureus,* a coagulase positive specie produces enzyme coagulase while coagulase negative specie include: *S. epidermidis*, *S. saprophyticus*, *S. lugdunensis*, *S. schleiferi*, and *S. caprae*.

2.9 Prevention

Staphylococci infections will never be completely controlled because of the asymptomatic carrier state in humans in the home and hospital. The spread of infection can be limited only by proper hygienic care and disposal of contaminated materials. The following precautionary measures as described by (Parsonnet *et al.,* 2006) can help lower the risk of staphylococcal infections:

I. Washing of hands: Careful hand-washing is the best defense against germs. Wash your hands briskly for at least 15 to 30 seconds, then dry them with a disposable towel and use another towel to turn off the faucet. If your hands aren't visibly dirty, you can use a hand sanitizer containing at least 62 % alcohol.

II. Keep wounds covered: Keep cuts and abrasions clean and covered with sterile, dry bandages until they heal. The pus from infected sores often contains staphylococci, and keeping wounds covered will help keep the bacteria from spreading.

III. Reduce tampon risks: Toxic shock syndrome is caused by staphylococci. Since

tampons left in for long periods can be a breeding ground for staphylococci, thus frequent change (at least every 4-8 hours) of tampon can reduce the chance of getting toxic show shock syndrome. The use of the lowest absorbency tampon and alternating it with sanitary napkins also serve as precautionary measures. (Parsonnet, 2006)

IV. Keep personal items personal: Avoid sharing personal items such as towels, sheets, razors, clothing and athletic equipment. Staphylococcal infections can spread on objects, as well as from person to person.

V. Wash clothing and bedding in hot water: Staphylococci can survive on clothing and bedding that isn't properly washed. To get bacteria off clothing and sheets, wash them in hot water whenever possible. Also, use bleach on any bleach-safe materials. Drying in the dryer is better than air-drying, but staphylococci may survive the clothes dryer (Moesby *et al.,* 2005).

CHAPTER THREE

3.0 MATERIALS AND METHODS

3.1 Test organism

The test organism, a referenced pure culture of antibiotic resistance Coagulase Negative *Staphylococcus epidermidis* with reference strain number: CoNS10b-OJO was obtained from Drug Discovery and Developmental Research unit of the Department of Microbiology, Federal University Oye-Ekiti, Ekiti State.

3.2 Confirmation of Test Organism

Colonies growing on slants were streaked on top of freshly prepared plates of mannitol salt agar and incubated again at 35°C. Primary characterization of isolates was based on Gram stain, morphological and cultural characteristics, growth on nutrient agar, fermentation on mannitol salt agar, catalase and coagulase tests, citrate utilization tests and other sugars (maltose, lactose, sucrose and fructose) were performed as described by Ojo *et al.* (2013b).

3.3 Plant Collections

The whole plants of *Diodia scandens* were obtained from Federal University Oye-Ekiti and its environs at latitude $7^{0}8$'N and longitude $5^{0}33$'N. The harvested plants were washed thoroughly with distilled water and air dried. The plants stored at room temperature prior to use.

3.4 Preparation of Extracts

The dried plants were pulverized into powdered form using household blender (Nakai Magic blender, Model no S1-889BD). The powdered material was subjected to hot and cold extraction of ethyl acetate and n-hexane to obtain respective extracts. The hot extraction was

done with soxhlet apparatus using 75g of the plant in 500ml of n-hexane and ethyl acetate respectively for 5-6hours, while the cold extraction was carried out by weighing 75g of pulverized plant into the beaker and 500ml of respective solvents were added, covered with foil paper and kept for 48 hours. Whatman No.1 filter paper was use to filter the extract out and were concentrated for 3 hours until the solvent used is extracted out using a rotary evaporator (Senco Technology, Model no- R205, SN- 13605) at a speed of 39-40 rpm.

3.5 Animal study

Twenty (20) Swiss albino rats of both sexes weighing (120-200g) were purchased from the Animal house of Afe Babalola University, Ado Ekiti, Ekiti State. The animals were housed in polycarbonated cages at ambient environmental condition of 23±2°C, at 30-60% relative humidity. The animals were fed with rodent commercial diet and water adlibitum as described by Jena *et* al. (2012). The animals were allowed to acclimatize to the environment, and subjected to light and dark cycles of 12 hours respectively for 7 days before the experiment.

3.6 Prophylactic Treatment

For prophylactic studies 20 Swiss albino rats were used. The rats were sorted into four groups of five according to their body weights and a control group.

Group1 with average weight of 126.5kg

Group2 with average weight of 150kg

Group3 with average weight of 165kg

Group4 with average weight of 180kg

Group 5 (control) with average weight of 190kg.

They were defasted for 4hour prior to treatment. The extract dosages were given according to the weight of rats and the solvents of extractions. The dosages were administered orally using

an oral canular with 1ml syringe of 0.5ml sterile extracts. Group 1 received 50mg/kg of 0.5ml of *Diodia scandens* ethyl acetate extract. Rats in group 2 received 100mg/kg of 0.5 ml of *Diodia scandens* ethyl acetate extract. Group 3 received 50mg/kg of 0.5ml of *Diodia scandens* n-hexane extract. Group 4 received 100mg/kg of 0.5ml of *Diodia scandens* n-hexane extract, while group 5 which serve as the control received 0.5ml of DMSO and distilled water mixture (2.5ml of DMSO in 15ml of distilled water) for 7days respectively. They were observed for 1 hour, 2 hours and 4hours after treatment and intermittently for 24hours for 7 days for clinical signs such as weakness, aggressiveness, loss of weights, diarrhea, discharge from eyes and ears, inflammation on skin and number of deaths.

3.7 Organism challenge tests

The rats were challenged with 0.5ml of 1×10^{8} cfu/ml of *S. epidermidis* in Mueller Hinton broth using 0.5 McFarland standard at the 4th day pre-treatment. The control group was also challenge with the test organism. (Mahapatra *et al.,* 2013). The protections offered by the extracts were determined by recording the observed signs and mortality rate of rats in each group.

Plate 2. Swiss albino rats in polycarbonated metabolic cage

CHAPTER FOUR

4.0 RESULTS

4.1 Microscopic examination and biochemical characterization of staphylococci

The referenced pure culture of antibiotic resistance strain of CoNS obtained on confirmation showed Gram positive cocci, coagulase negative, do not ferment mannitol and produced acid and gas on lactose, maltose, fructose and sucrose, while citrate was also utilize (Table 1).

4.2 Dose administration and response on *Diodia scandens* pre-treatment of swiss albino rats

Each groups received 0.5ml of plant extracts (*Diodia scandens*) while the control group received DMSO and distilled water. After an hour of intubation, the response on *Diodia scandens* pre-treatment of swiss albino rats on each groups showed clinical sign of aggressiveness but no report on inflammation, weakness, redness of eyes/skin, diarrhea, discharge in the eyes and death as presented in table 2. One of the rats in group 1 showed some clinical signs such as fever, weakness, redness of the eyes, and diarrhea on day 3, while two in group 3 only showed signs of weakness. On the 4th day the same rat in group 1 showed two clinical signs of weakness and redness of the eyes and one in group 4 showed a sign of weakness (Table 2).

4.3 Prophylactic activity of *Diodia scandens* extracts on Organism challenged Swiss albino rats

After an hour of the organism challenge test, response to dosage by the three groups (non-treated group, treated group1 and treated group 2) was at the same level, as they were observed to be feverish. From the 4th hour to 72nd hour of been challenged, the non treated group was still feverish while the treated group 1and 2 appeared to be normal.

Table 1. Biochemical tests on referenced Coagulase Negative *Staphylococcus*

Test Organism	Coagulase	Catalase	MSA	Citrate	Lactose		Maltose		Fructose		Sucrose	
					A	G	A	G	A	G	A	G
CoNS 10b-OJO	-	+	-	+	+	+	+	+	+	+	+	+

Key: A Acid production, G Gas production, + Positive, Negative

Table 2. Dosage administration and response of pre-treated Swiss albino rats with *Diodia scandens* extracts.

				Day 1								Day 2							
				Response to dosage								Response to dosage							
Group Mw(kg)	Treatment	Conc (mg/kg)	Dosage (ml)	Fever (n)	Agg (n)	Inf (n)	W (n)	R (n)	D (n)	Diar (n)	De (n)	Fever (n)	Agg (n)	Inf (n)	W (n)	R (n)	D (n)	Diar (n)	De (n)
1 (126.5)	EA_1	50	0.5	0	5	0	0	0	0	0	0	0	5	0	0	0	0	0	0
2 (150)	EA_2	100	0.5	0	5	0	0	0	0	0	0	0	5	0	0	0	0	0	0
3 (165)	n-H_1	50	0.5	0	4	0	0	0	0	0	0	0	4	0	0	0	0	0	0
4 (180	n-H_2	100	0.5	0	4	0	0	0	0	0	0	0	4	0	0	0	0	0	0
Control (190)	DMSO + DW	_	0.5	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0

KEY: EA_1-Ethyl acetate , EA_2--Ethyl acetate 2, n-H_1--n-Hexane 1, n-H_2-- n-Hexane,DMSO +DW--Dimethyl sulphoxide + distilled water, Conc—Concentration, Agg—Aggressive, N—Number, Inf – Inflammation, W—Weakness, R--Redness of eyes/skin, D--Discharge in eyes, Diar—Diarrhea and De—Death

Table 2. continued

				Day 3 Response to dosage								Day 4 Response to dosage							
Group Mw(kg)	Treat ment	Conc (mg/kg)	Dosage (ml)	Fever (n)	Agg (n)	Inf (n)	W (n)	R (n)	D (n)	Diar (n)	De (n)	Fever (n)	Agg (n)	Inf (n)	W (n)	R (n)	D (n)	Diar (n)	De (n)
1 (126.5)	EA_1	50	0.5	1	5	0	1	0	0	0	0	1	5	0	1	1	0	0	0
2 (150)	EA_2	100	0.5	0	5	0	0	0	0	0	0	0	5	0	0	0	0	0	0
3 (165)	n-H_1	50	0.5	0	4	0	2	0	0	0	0	0	4	0	0	0	0	0	0
4 (180	n-H_2	100	0.5	0	4	0	0	0	0	0	0	0	4	0	0	1	0	0	0
Control (190)	DMSO + DW	_	0.5	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0

KEY: EA_1-Ethyl acetate , EA_2...Ethyl acetate 2, n-H_1--n-Hexane 1, n-H_2... n-Hexane,DMSO +DW--Dimethyl sulphoxide + distilled water, Conc—Concentration, Agg—Aggressive, N—Number, Inf – Inflammation, W—Weakness, R--Redness of eyes/skin, D--Discharge in eyes, Diar—Diarrhea and De—Death.

Table 3. Protection offered by *Diodia scandens* on challenged swiss albino rats with *Staphylococcus epidermidis*

Type	Group	Extract injected per rats	Response to dosage(hrs)					Number of deaths(hrs)		
			1	4	24	48	72	24	48	72
Non treated	Control (n=4)	0.5ml DMSO+H20	F	F	F	F	F	0	0	0
Treated	Group1(n=9)	50mg	F	N	N	N	N	0	0	0
Treated	Group2(n=9)	100mg	F	N	N	N	N	0	0	0

KEY:n----Number of rats, F ---Feverish, N—Normal, DMSO +H_20--Dimethyl sulphoxide + distilled water,

CHAPTER FIVE

5.0 DISCUSSION

5.1 Microscopic and Biochemical characterization of *Staphylococcus epidermidis*

Staphylococcus epidermidis are bacteria that usually reside in skin flora, in gut and in the respiratory tract. They are known as the leading cause of the nosocomial sepsis and are also known to cause medical device derived infections. *Staphylococcus epidermidis* are spherical gram-positive cocci arranged in irregular grapelike clusters. Parallel results have also been reported by Matthew *et al.* (2003) that other means of identifications that differentiate it from other members of staphylococcal species include, citrate test, catalase test, sugar fermentation Coagulase test which is the major means of identification and MSA. *Staphylococcus epidermidis* are coagulase negative staphylococci, catalase positive, are able utilize citrate, produces acid and gas in lactose, maltose, sucrose, fructose and does not ferment mannitol.

5.2 Dose Administration and Response on *Diodia scandens* Pre-treatment of Swiss Albino Rats

According to Hidayathulla *et al.* (2011), *Diodia scandens* is a potential source of new antibacterial agent. This study observed clinical symptoms such as aggressiveness and weakness after an hour of pre-treatment, however reduction in activity and reaction in the control group given only DMSO and distilled water was observed.

The findings by Ankit *et al.* (2009), observed analgesic activity and anti-inflammatory potential of *Diodia scandens* at 150mg/kg and 100mg/kg which corroborate with the report of this study on the non-inflammatory response recorded at the dosage of administration (50mg/kg and 100mg/kg). However, inflammation and redness of eye was observed in one member of the group 4.

5.3 Prophylactic Activity of *Diodia scandens* Extracts on Organism Challenged Swiss Albino Rats

In this study, it was observed that the dosage of administration could successfully protect the animals from the lethal infection of *Staphylococcus epidermidis*. The protection offered by *Diodia scandens* on the rats against *Staphylococcus epidermidis* after 72 hour of investigation revealed no clinical signs and symptoms. This work, in relation to earlier findings by Mazumder *et al*. (2004) and Mishra *et al.* (2008) noted that strains of *S. aureus* and *Bacillus* sp. were remarkably sensitive to *Diodia scandens* extracts using ethyl acetate and hexane for the crude extraction, which resulted in an increased immunity of the rats. The survival of the animals after 7 days of treatment revealed that both ethyl acetate and hexane extracts at the concentration of 50mg/kg and 100mg/kg has effectively inhibit the growth of *Staphylococcus epidermidis*

6.0 CONCLUSION AND RECOMMENDATION

6.1 CONCLUSION

The protection offered by *Diodia scandens* after organism challenged test has proven that the medicinal plant is potent as an antibacterial agent, effective at the different concentrations used and has boosted the immunity of the rats against *Staphylococcus epidermidis* used. It has been cogitated that the 50mg/kg and 100mg/kg of *Diodia scandens* used during pretreatment affirm that it was effective and not toxic on the rats. Therefore the skepticism often encountered in the use of herbal medicine for treatment of various pathological and physiological diseases owing to the undetermined toxic level of such herbal medicine should be annulled.

6.2 RECOMMENDATION

Further research work should be done on this study using other antibiotic resistant microorganisms.

6.3 CONTRIBUTION TO KNOWLEDGE

I. The concentration of *Diodia scandens* extracts in pre-treatment on swiss albino rats did not cause any inflammation, weakness, and any feverish conditions at the different concentrations used.

II. *Diodia scandens* offered a prophylactic activity on the treated groups after being challenged with *Staphylococcus epidermidis* at the various concentrations used (50mg/kg and 100mg/kg).

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Sugars used were lactose, sucrose, fructose and maltose. 1g of each sugar was weighed into different beakers; peptone water was added and made up to 100mls with distilled water. Exactly 0.01g phenol red was added as an indicator.

PROCEDURE

2ml each of sugar solution was dispensed into different test tubes, cotton wool and aluminum foil paper was used to close the test tube and sterilized in an autoclave for 10mins at 121°C. Tubes were allowed to cool before inoculation. The sugar solution in test tubes were then inoculated with test organism and incubated at 36°C for 24 hours.

RESULTS

A change in the color to yellow indicates acid production while bubble in Durham tube indicates gas production.

- **CATALASE**

This test is used to differentiate those bacteria that produce the enzyme catalase such as staphylococci. Catalase acts as catalyst in the breaking down of oxygen and water. A colony of the test organism was picked and placed in a drop of 3% hydrogen peroxide on a clean glass slide bubbles was checked for immediately and result was recorded.

RESULTS

Effervescence caused by the liberation of oxygen as gas bubbles indicate the production of catalase by the organism.

- **COAGULASE**

Materials used include: Blood plasma, normal saline

The test is used to identify *S. epidermidis* which does not produce the enzyme coagulase. Coagulase causes plasma to clot by converting fibrinogen to fibrin. A colony of test organism was emulsified in normal saline on a clean glass slide and an equal volume of plasma was added and mixed together.

RESULTS

Negative coagulase test shows no agglutination.

- **CITRATE AGAR**

This is a defined medium used to determine if an organism can use citrate as its sole carbon source. In organisms capable of utilizing citrate as a carbon source, the enzyme citrase hydrolyzes citrate into oxaoloacetic acid and acetic acid. The oxaloacetic acid is then hydrolyzed into pyruvic acid and CO_2. If CO_2 is produced, it reacts with components of the medium to produce an alkaline compound

RESULT

The alkaline pH turns the pH indicator (bromthymol blue) from green to blue. This is a positive result.

MANNITOL SALT AGAR (MSA)

This type of medium is both selective and differential. The MSA will select for organisms such as *Staphylococcus* species which can live in areas of high salt. The differential ingredient in MSA is the sugar mannitol. Organisms capable of using mannitol as a food source will produce acidic byproducts of fermentation that will lower the pH of the media.

RESULT

The acidity of the media will cause the pH indicator, phenol red, to turn yellow. *Staphylococcus aureus* is capable of fermenting mannitol while *Staphylococcus epidermidis* is not. *Staphylococcus epidermidis* grows on MSA, but does not ferment mannitol (media remains light pink in colour and colonies are colorless).

- **ANIMAL STUDY**

The Grouping of the Swiss albino rats was done according to their weight

Grp I: 118-135g

Grp II: 144-156g

Grp III: 173-188g

Control IV: > 190g

To calculate for the average weight of the rats

For grp I: 118+135=253÷2= 126.5g

For grp II: 144+156g=300÷2= 150g

For grp III: 173+188= 361÷2=180.5g

For grp IV: 190g.

- PREPARATION OF EXTRACT CONCENTRATION

For 25mg/kg body weight

Average weight = 126.5g= $\frac{25\times126.5}{1000}$ =3.16mg

To give 0.5ml × 5 animals × 7 days = 17.5ml

$\frac{3.16}{1000}$ = 0.00316