DETECTION OF BIOACTIVE COMPOUNDS BY BIOAUTOGRAPHY OF *DIODIA SCANDENS* ON *STAPHYLOCOCCUS AUREUS* AND COAGULASE NEGATIVE *STAPHYLOCOCCUS*

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CERTIFICATION

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DEDICATION

This project work is dedicated to God almighty for his provision, strength, wisdom and the knowledge he gave me to successfully complete this work.

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ABSTRACT

Plant samples collected within and around Federal University Oye-Ekiti was air dried, pulverized and subjected to hot (soxhlet) extraction and cold extraction using three (3) extractive solvent (ethyl acetate, methanol, n-hexane). The extracts were seperated from the solvent using rotary evaporator while the crude extracts were subjected to thin layer chromatography (TLC) using different solvent ratio mixtures on a silica gel coated TLC plates. The antibacterial activity of active constituents of each crude extracts were determined using Bioautography (agar-overlay) method by spraying the TLC plates with tetrazolium salt and incubated for one hour. Zones of inhibition were observed for each extract on the chromatogram. The result revealed varying retention factor (R*f*) of different extract ranging from 0.05-0.72 while the total number of active bands range between 1 to 6. the zones of growth inhibition recorded among the three different solvent of extraction on cold and hot extraction range between 14mm-34mm. This results show that there are bioactive constituent present in *Diodia scandens* and Bioautography (agar-overlay) method can be used in detecting them.

CHAPTER ONE

1.0 INTRODUCTION

Medicinal plants would be the best source to obtain a variety of newer herbal drugs. For centuries plants have provided mankind with useful, sometimes life saving drugs (Asha *et al*., 2013). Over the years, plants and their extracts have been applied as herbal remedies for diverse human ailments. Presently, plant is still being utilized by numerous developing countries as sources of therapeutic agents because they believe that medicinal plants are readily available, affordable and potent with relatively lower incidences of adverse reactions compared to modern conventional drugs (Ojo *et al*., 2013).

Diodia scandens SW (Rubaeceae) is a straggling perennial herb with slender angular stem up to 3m high, with opposite to alternate ovate lanceolate leaves and white clustered flowers (Ogu *et al*., 2011). Traditionally, different parts of the plants-sap, leaf, stem and root, are used for various medicinal purposes. In Western Africa, it is used as antidotes (venomous stings, bites and pain killers, venereal disease), In Nigeria, the leaf extracts or sap are used for curing eczema, stop bleeding, treat bruises and minor cuts and ear problems, and also as antiabortifacient. According to Essiett *et al*. (2010), tannins, saponins, and cardiac glycosides were identified as the phytochemical constituents of *D. scandens* leaf extract that are active for the treatment of the above mention infections.

Staphylococci have emerged as a predominant organism causing infections. Bacteria in the genus *Staphylococcus* are pathogens of man and mammals. They are gram-positive cocci about 0.5-1.0μm in diameter. They grow in clusters, pairs and occasionally in short chains. Since the introduction of antimicrobials, bacteria have developed mechanism for resisting the effect of antibiotics and the level of antibiotic resistant infections in developing world have increased (Ojo *et al*., 2013).

Based on the growing knowledge of the potency of traditional medicinal plants and coupled with the fact that Staphylococci are becoming resistant to synthetic drugs, researchers all over the world have intensified the screening of the active lignans in these plants in other to provide documented scientific backing and ultimately recommend them as novel sources of future antimicrobial agents (Ojo *et al*., 2013). Bioautography is a highly efficacious assay for the detection of antimicrobial compounds because it allows localization of activity even in a complex matrix, and therefore facilitates the target-directed isolation of the active constituents (Suleiman *et al*., 2009).

RESEARCH PROBLEM

Inadequate assay and pious knowledge has limited the use of various natures' flora (medicinal plants) that is abundant in our environment.

AIM OF STUDY

This research work is aimed at the detection of bioactive constituents present in *Diodia scandens* using Bioautographic (agar-overlay) method of Thin Layer Chromatography against *S. aureus* and Coagulase negative Staphylococcus strains.

OBJECTIVES OF STUDY

1. To perform a confirmatory test on the reference strains using culturing and biochemical method.
2. To carry out hot (soxhlet) and cold crude extraction method in different solvent.
3. To determine the various active bands in *Diodia scandens* using varying solvent systems.
4. To detect the antimicrobial activities of the various active bands of different solvents against *S. aureus* and Coagulase Negative Staphylococcus strain.

2.0 LITERATURE REVIEW

2.1 Medicinal Plants

Plants are endowed with various phytochemical molecules such as vitamins, terpenoids, phenolic acids, lignins, stilbenes, tannins, flavonoids, quinones, coumarins, alkaloids, amines, betalains, and other metabolites, which are rich in antioxidant activity. Studies have shown that many of these antioxidant compounds possess anti-inflammatory, antiatherosclerotic, antitumor, antimutagenic, anticarcinogenic, antibacterial, and antiviral activities (Sanjay *et al.*, 2013). The ingestion of natural antioxidants has been associated with reduced risks of cancer, cardiovascular disease, diabetes, and other diseases associated with ageing and in recent years, there has been a worldwide trend towards the use of the natural phytochemicals present in berry crops, teas, herbs, oilseeds, beans, fruits and vegetables (Sanjay *et al.*, 2013). Plants have been used by man to cure diseases and heal injuries since time immemorial. The universal role of plants in the treatment of disease is exemplified by their employment in all major systems of medicine irrespective of the underlying philosophical premise. Plants have been used throughout the world in folk medicine and as local cures for common ailments. Folk medicine gave rise to traditional systems of medicine in various diseases. World Health Organisation reported that of 119 plants derived pharmaceutical medicine, about 75% are used in modern medicine in ways that correlated directly with the traditional uses as plant medicine by native culture (Essiett *et al.*, 2010). Several plants are used in the production of different arrays of medicines that are commercially available today. The extracts from this plant contains lignans with antimicrobial properties and some of this have been used in treating fungal and bacterial infections in man. Medicinal plants are plants which contain substances that could be used for therapeutic purposes and precursors for the synthesis of useful drugs (Abolaji *et al.*, 2011).

Medicinal plants are of great importance to the health of individuals and communities in general.

Traditional medicine is an important part of African cultures and local medicinal systems vary between different cultural groups and regions. In developing countries of the world, a vast number of people live in extreme poverty and some are suffering and dying for want of safe water and medicine they have no alternative for primary health care. Therefore, the need to use medicinal plants as alternatives to orthodox medicines in the provision of primary health care cannot be over-emphasized (Suleiman *et al.*, 2009).

Herbal medicines have received much attention as sources of lead compounds since they are considered as time tested and relatively safe for both human use and environment friendly. They are also cheap, easily available and affordable (Ojo *et al.*, 2013). The medicinal value of plants lies in some chemical substances that produce a definite physiological action on the human body. There is therefore the need to look inwards to search for herbal medicinal plants with the aim of validating the ethno-medicinal use and characterization of compounds which will be added to the potential lists of drugs (Omale and Emmanuel, 2010). Medicinal plants play a significant role in providing primary health care services to rural people and are used by about 80% of the marginal communities in the world. Each medicinal plant species has its own nutrient composition besides housing pharmacologically important phytochemicals. These nutrients are essential for the physiological functions of the human body. Such nutrients and biochemicals like carbohydrates, fats and proteins play an important role in satisfying human wants and needs for energy and other life processes. Many medicinal plants are used by marginal communities to cure various diseases. As various medicinal plant species are used either in the form of extract or decoction by the local people in different regions, therefore, evaluating their marginal significance can help to understand the worth of these plants species in different ecological conditions. Some of these medicinal plants serve as both food and medicine. (Omale and Emmanuel, 2010).

2.2 Natural Antibiotic Properties of Plant Metabolites

The plant constituents are classified as primary or secondary metabolites.

a. **Primary metabolites:** Primary metabolites are widely distributed in nature, occurring in one form or another in virtually all organisms. In higher plants, such compounds are often concentrated in seeds and vegetative storage organs and are needed for physiological development because of their role in basic cell metabolism. Primary metabolites obtained from higher plants for commercial use are high volume-low value bulk chemicals (e.g. vegetable oils, fatty acids, carbohydrates etc.) (Gullo *et al*., 2006).

b. **Secondary metabolites:** Plants generally produce many secondary metabolites which are biosynthetically derived from primary metabolites and constitute an important source of microbicides, pesticides and many pharmaceutical drugs. For a long period of time medicinal plants or their secondary metabolites have been directly or indirectly playing an important role in the human society to combat diseases (Wink *et al*., 2005). Secondary metabolites are frequently accumulated by plants in smaller quantities than the primary metabolites (Karuppusamy et al., 2009). Secondary metabolites (compounds) have no apparent function in a plant's primary metabolism, but often have an ecological role like being pollinator attractants, representing chemical adaptations to environmental stresses, or serving as chemical defence against microorganisms, insects, higher predators and even other plants (allelochemics) (Karuppusamy et al., 2009).

In contrast to primary metabolites, secondary metabolites are synthesized in specialized cell types and at distinct developmental stages, making their extraction and purification difficult. As a result, secondary metabolites that are used commercially as biologically active compounds, are generally high value-low volume products than the primary metabolites (e.g. steroids, quinines, alkaloids, terpenoids and flavonoids), which are used in drug manufacture by the pharmaceutical industries (Gullo *et* al., 2006). These are generally obtained from plant materials by steam distillation or by extraction with organic or aqueous solvents and the molecular weights are generally less than 2000. Some biologically active plant compounds have found application as drug entities or as model compounds for drug synthesis and semi-synthesis. A survey of current pharmaceutical use revealed that, of the total prescription drugs dispensed, 25% are plant derived. Plant compounds are highly varied in structure; many are aromatic substances most of which are phenols or their oxygen-substituted derivatives, others are aliphatic substances.

However increased attention has been given to extracts and biologically active compounds isolated from plant species used in herbal medicine because synthetic drugs are seen to have side effects and there is more antimicrobial resistance from pathogenic microorganisms against synthetic antibiotics. Of the various Pharmaceuticals used in modern medicine, aspirin, atropine, ephedrine, digoxin, morphine, quinine, reserpine and tubocurarine serve as examples of drugs discovered through observations of indigenous medical practices (Gilani and Atta-ur-Rahman, 2005). Plant constituents may be isolated and used directly as therapeutic agents or as starting materials for drug synthesis or they may serve as models for pharmacologically active compounds in drug synthesis. The general research methods includes proper selection of medicinal plants, preparation of crude extracts, biological screening, detailed chemo pharmacological investigations, toxicological and clinical studies, standardization and use of active moiety as the lead molecule for drug design (Wink *et al.*, 2005).

2.2.1 Types of Secondary Metabolites

I. Alkaloids

Alkaloids are naturally occurring chemical compounds containing basic nitrogen atoms and are produced by a large variety of organisms including bacteria, fungi, plants, and animals. Many alkaloids are toxic and often have a pharmacological effect, which makes them to be used as medications and recreational drugs (Gilani and Atta-ur-Rahman, 2005).

II. Flavonoids

Flavonoids are derived from 2-phenylchromen-4-one (2-phenyl-1-4-benzopyrone) and are commonly known for their antioxidant activities. Flavonoids, which are widely distributed in plants, fulfill many functions including producing yellow, red or blue pigmentation in flowers and protection from attacks by microbes and insects. Compared to other active plant compounds, they are low in toxicity. They show anti-allergic, anti-inflammatory, antimicrobial and anticancer activity Flavonoids are referred to as nature's biological response modifiers because of their inherent ability to modify the body's reaction to allergens, viruses and carcinogens (Spencer, 2008).

III. Saponins

Saponins are the glycosides of 27 carbon atom steroids, or 30 carbon atom triterpenes in plants. They are found in various plant parts; leaves, stems roots, bulbs, flowers and fruits. They are used medically as expectorant, emetic and for the treatment of excessive salivation, epilepsy, chlorosis and migraines. They are used in Ayurvedic medicine as a treatment for eczema, psoriasis and for removing freckles. Saponins are believed to be useful in the human diet for controlling cholesterol. Saponins also inhibit cancer tumour growth in animals, particularly, lung and blood cancers, without killing normal cells. Saponins are the plant's immune system acting as an antibiotic to protect the plant against microbes and fungus (Oleszek, 2002).

IV. Cardiac Glycosides

Cardiac glycosides are drugs used in the treatment of congestive heart failure and cardiac arrhythmia. These glycosides are found as secondary metabolites in several plants and in some animals. Some of these compounds are used as arrowhead poisons in hunting (Filippos *et al*., 2007).

V. TANNINS

Tannins are astringents, bitter plant polyphenols that either bind and precipitate or shrink proteins. The astringency from the tannins is that which causes the dry and puckering feeling in the mouth following the consumption of red wine, strong tea or unripened fruits. They may be employed medicinally in antidiarrhoeal, homeostatic and antihaemorrhoidal compounds. The anti-inflammatory effects of tannins help control all indications of gastritis, esophagitis, enteritis and irritating bowel disorders. It also controls irritation in the small intestine (Praveen and Kumud, 2012).

2.3 *Diodia scandens*

Diodia scandens Sw (Rubiaceae) is an evergreen perennial herb, which has an alternate leaf arrangement, petiole is present. It has compound leaves, ovate to lanceolate in shape, reticulate venation, entire in margin, its apex is acute, its base is cuneate, it has glabrous surface and its texture is charteceous. *Diodia scandens* Sw has a dark green coloration, tasteless, odourless and has solitary inflorescence. It is a straggling herb, which has been in use in the Western African system of medicine (Ogu *et al*., 2011). It has enormous usefulness and importance; whole parts of the plants are useful in curing various ailments such as dysentery, diarrhoea, asthma, convulsion, epilepsy, oedema, gout, swelling and it is said to be anti-abortifacient, antidotes, antimicrobial, anti-inflammatory in Nigeria and other countries. The phytochemistry of the leaf extract revealed the presence of saponins, tannins, cardiac glycosides and absence of flavenoids, phlobatannins, alkaloids and anthraquinones (Essiett *et al.*, 2010).

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

2.4 Bioautography

Bioautography has enabled rapid progress for quick detection of new antimicrobial compounds from plants and other natural products. This technique allows the localization of antimicrobial activity directly on a chromatographic plate where the organism is applied (Suleiman et al., 2009). Bioautography screening methods are based on the biological activities, e.g. antibacterial, antifungal, antitumor, and antiprotozoan of the tested substances. This detection method can be successfully combined with layer liquid chromatography techniques, such as thin-layer chromatography (TLC), high- performance thin-layer chromatography (HPTLC), over pressured-layer chromatography (OPLC) and planar electrochromatography (PEC) (Choma and Grzelak, 2011).

A number of bioautographic assays have been developed, which can be divided into three groups. These include direct bioautography; where the microorganisms grow directly on thin-layer chromatography (TLC) plates, contact bioautography; where the antimicrobial compounds are transferred from the TLC plate to an inoculated agar plate through direct contact, and agar overlay or immersion bioautography; where a seeded agar medium is applied onto the TLC plate. The latter technique can be considered as a hybrid of direct and contact bioautography. For direct bioautography, the principle is that a developed TLC plate is dipped in a suspension of microorganisms growing in a proper broth and then incubated in a humid atmosphere. A silica surface of the TLC plate covered with the broth medium becomes a source of nutrients and enables growth of the microorganisms directly on it. However, in the places where antimicrobial agents were spotted, the inhibition zones of the microorganism growth are formed. In the contact bioautography, the TLC plate or paper chromatograms are placed on the inoculated agar surface for some minutes or hours to allow diffusion. Next, the plate is removed and the agar layer is incubated. The zones of inhibition growth appear in the places, where the antimicrobial compounds were in contact with the agar layer. In the immersion (agar-overlay) bioautography, the plate is first immersed in or cover with agar medium, which after solidification is seeded with the tested microorganisms and then incubated. Visualization of these zones is usually carried out using dehydrogenase activity-detecting reagents and the most common are tetrazolium salts. The dehydrogenase of living microorganisms converts tetrazolium salt into intensely colored formazan. As a result, cream-white spots appear against a purple background on the TLC plate surface, pointing the presence of antibacterial agents (Choma and Grzelak, 2011).

2.5 Antimicrobial Agents

A systematic survey of antimicrobial agents shows that they are general nomenclature for all drugs or chemical substances that act on microorganisms either to kill or suppress their growth (Laport *et al.*, 2009). Among the antimicrobial agents are antibacterial drugs, antiviral agents, antifungal agents and antiparasitic drugs. Agents that kill microorganisms are called 'cidal agents' while those that inhibit their growth are known as 'static agents' (Laport *et al.*, 2009)
They can be further categorized based on their target specificity: narrow-spectrum and broad spectrum. This antimicrobial agents target specific types of bacteria and a wide range of bacteria respectively (Laport *et al.*, 2009).

Li and Vederas (2009) and Gullo *et al.* (2006) described three main basic sources of antimicrobial agents which include:

Natural Sources: Few antimicrobial agents occur naturally, they can be gotten or extracted from animals or plants and can be produced by microorganism's biochemical pathways (Gullo *et* al., 2006). An example is antibiotic like Polymyxin gotten from *Streptomyces* species of bacteria.

Synthetic Sources: So many antimicrobial agents have been produced synthetically. They include antibiotics like chloramphenicol and chemical preservatives, as well as other chemotherapeutic drugs used in treating various diseases.

Semi-synthetic Sources: These antimicrobial agents can be produced naturally but still requires synthetic processes to finish up its production. These chemical processes enhance/ modify the products (Li and Vederas, 2009).

2.5.1 Factors that Influence the Rate and Extent of Antimicrobial Action of a Substance

I. **Temperature**: An increase in the temperature at which the chemical acts often enhances its activity, as long as it is within its range and does not denature it (Sivonen, 2001).

II. **pH of the Environment**: The changes in pH may affect the activities of the antimicrobial agents by affecting the rate of growth of microbial cells and physiochemical states of their surfaces (Sivonen, 2001).

III. **Concentration or Intensity of an Antimicrobial Agent**: Often, but not always, the more concentrated a chemical agent or intense a physical agent, the more rapidly micro-organisms are destroyed.

IV. **Duration of Exposure**: The longer populations of micro-organisms are exposed to a microbial agent, the more organisms are killed.

V **Local Environment**: Depending on what situation or what atmosphere you are in, will depend on how effective your agent will be. This affects the efficacy of the drugs. For example, antimicrobials that can kill a micro-organism in vitro may only inhibit its growth invivo (using the body for example) (Sivonen, 2001)

2.5.2 Mechanism of Action of Antimicrobial Agents

There are five main mechanisms by which antimicrobial agents act. These include:

I. **Inhibition of Cell Metabolism**: Antimicrobial agents involved in the inhibition of cell metabolism are known as 'Antimetabolites'. These compounds inhibit the metabolism of a micro-organism, but not the metabolism of the host. They do this by inhibiting an enzyme-catalyzed reaction which is present in the bacterial cell, but not in animal cells. The best known examples of antimicrobial agents acting in this way are the sulfonamides (Dibrov et al., 2002).

II. **Inhibition of Bacteria Cell Wall Synthesis**: The inhibition of cell wall synthesis in bacteria leads to cell lysis and death. Agents operating in this way include penicillin and cephalosporins (Dibrov *et al.*, 2002).

III. **Interactions with the Plasma Membrane**: Some antimicrobial agents interact with the plasma membrane of bacteria cells affecting membrane permeability. This brings about fatal results for the cell. Polymyxins and tyrothricin operate in this way.

IV. **Disruption of Protein Synthesis**: Disruption of protein synthesis means that essential enzymes required for the cells survival can no longer be made. Agents which disrupt protein synthesis include the rifamycins, aminoglycosides, tetracyclines and chloramphenicol (Dibrov *et al.*, 2002).

V. **Inhibition of Nucleic Acid Transcription and Replication**: The inhibition of nucleic acid function prevents cell division and/or the synthesis of essential enzymes. Agents acting in this way include nalidixic acid and proflavin (Dibrov *et al.*, 2002).

2.6 Description of *Staphylococcus aureus*

Staphylococcus aureus belongs to the family *Micrococcaceae* and is part of the genus *Staphylococcus*, which contains more than 30 species such as *S. epidermidis*, *S. saprophyticus* and *S. haemolyticus*. Among the staphylococcal species, *S. aureus* is by far the most virulent and pathogenic for humans (Helen and Ralph, 2008). *S. aureus* is a 1µm, Gram-positive cell that in the laboratory may be observed as single cells, in pairs or as grape-like irregular clusters. It is characterized as coagulase- and catalase positive, non-motile, non-spore-forming and as facultative anaerobic. It grows in yellow colonies on nutrient rich media and is referred to as the yellow Staphylococci

S. aureus was discovered in 1880 by the surgeon Sir Alexander Ogston. He observed grape-like clusters of bacteria when examining a purulent discharge from patients with post-operative wounds during microscopy. He named them staphylé, the Greek expression for a bunch of grapes. In 1884, Rosenbach succeeded in isolating yellow bacterial colonies from abscesses and named them *Staphylococcus aureus*, "aureus" from the Latin word for golden. *S. aureus* has the ability to adapt to different environments and it may colonize the human skin, nails, nares and mucus membranes and may thereby disseminate among recipient host populations via physical contact and aerosols. Staphylococci have emerged as a prominent organism causing infections. Since the introduction of antimicrobials, this bacterium have developed mechanism for resisting the effects of antibiotics and the levels of antibiotic resistant infections in developing world have increased (Ojo *et al.*, 2013).

2.6.1 Epidemiology of *Staphylococcus aureus*

The frequency of *Staphylococcus aureus* infections continues to grow in hospital associated settings and, more recently, in community settings globally. The increase in the incidence of infections due to *S. aureus* is partially a consequence of advances in patient care and also of the pathogen's ability to adapt to a changing environment (Helen and Ralph, 2008). Infection due to *S. aureus* imposes a high and increasing burden on health care resources. A growing concern is the emergence of MRSA infections in patients with no apparent risk factors. MRSA infection in community settings involves considerable morbidity and mortality, as does nosocomial MRSA infection. For community-associated MRSA, person-to-person transmission has been reported, and several factors have been shown to predict disease. *Staphylococcus aureus* is an opportunistic pathogen often carried asymptomatically on the human body. Methicillin-resistant *S. aureus* (MRSA) strains have acquired a gene that makes them resistant to all beta-lactam antibiotics. Hospital-associated strains of this organism are serious nosocomial pathogens that have become resistant to most common antibiotics, and treatment can be challenging (Pallab *et* al., 2011). Community-associated MRSA strains occur in people who have not been hospitalized or recently had invasive procedures. They first appeared in high- risk populations (e.g., intravenous drug users, people with chronic illnesses), but are now found even in healthy children. Until recently, community-associated strains were susceptible to many antibiotics other than beta-lactams; however, resistance seems to be increasing, and multiple antibiotic resistant strains have started to emerge. MRSA are usually transmitted by direct contact, often via the hands, with colonized or infected people. Humans remain infectious as long as the carrier state persists or the clinical lesions remain active. MRSA can also be disseminated on fomites (including food that has been contaminated by human carriers) and in aerosols. *S. aureus* (and presumably MRSA) can be transmitted from the mother to her infant during delivery.

2.6.2 Pathogenesis of *S. aureus*

Various virulence factors contribute to the ability of *S. aureus* to cause infection and they include enzymes, toxins, adhesion proteins, cell-surface proteins, factors that help the bacteria to evade the innate immune defense, and antibiotic resistance mediate survival of the bacteria and tissue invasion at the site of infection (Rachel and Franklin, 2008). Moreover, certain toxins cause specific disease entities. In the case of severe *S. aureus* disease, the infection may not be explained by the action of a single virulence factor, and it is likely that a number of different factors operating together in the pathogenic process. This assumption is supported by studies in animal models where the infection caused by a mutant isolate, deficient in a single virulence determinant, is compared with the infection caused by the wild type strain (Pallab *et al.*, 2011).

2.6.3 Clinical Manifestation of *S. aureus*

Staphylococci are capable of producing a wide variety of clinical syndromes, including syndromes with high mortality rates, as was reviewed almost 50 years ago. Clinical manifestations of *S.aureus* include; Bacteremia and Sepsis, Toxic Shock Syndrome, Endocarditis, Infections of the Intestinal Tract, Food poisoning, chest pain, prostethic joint (Helen and Ralph, 2008).

2.6.4 Diagnosis of *S. aureus*

Diagnosis has most often been made by direct culture of the infected tissues or abscesses if the disease is focal or by cultures of blood, urine, or cerebrospinal fluid on selective media like the mannitol salt agar (Rachel and Franklin, 2008).

2.6.5 Prevention of *S. aureus*

Major efforts to prevent staphylococcal infections rather than being required to treat them, are of great value. Staphylococci may be spread through fomites; overcrowding of people may increase the risk of colonization and the potential for disease. In an outbreak situation, attempts to control the spread of staphylococci through remediation of overcrowding and isolation of infected or colonized patients have been shown to be effective in helping to curtail the outbreak (Larson, 1998). Proper hand hygiene among nursery health care providers is a fundamental factor in reducing colonization rates. Larson. (1998), achieved a reduction in colonization from 92% to 53% by insisting that attendants wash their hands. Proper education and monitoring of hand hygiene practices are critical to the effectiveness of this intervention. Hands must be cleaned before and after patient contact or contact with equipment that is used for patient care.

2.7 Antibiotic Resistance

At first, penicillin was used to treat *S. aureus* infections. Soon afterwards, resistance emerged when strains acquired a genetic element coding for β-lactamase production, and today over 80 % of all *S. aureus* strains are resistant to penicillin. The next drug to be introduced for treating infections with *S. aureus* was the semisynthetic, penicillinase-resistant penicillin named oxacillin or methicillin, but shortly after its introduction the first isolate with resistance was detected. With the emergence of resistance to the penicillinase-resistant penicillins, the glycopeptide agent vancomycin became the treatment of choice for infections with MRSA, and in 1996 the first isolate with intermediate vancomycin resistance was detected in Japan. So far, this has not emerged to be a major concern, but the resistance has been detected in different parts of the world and needs to be monitored (Pallab *et al.*, 2011).

CHAPTER THREE

3.0 MATERIALS AND METHODS

3.1 Collection of Test Organisms

Referenced pure culture of antibiotic resistant *Staphylococcus aureus* (SA241-OJO) and Coagulase Negative *Staphylococcus* (CoNS 10b-OJO) were obtained from Drug Discovery and Developmental Research (DDDR) unit of the Department of Microbiology, Federal University Oye Ekiti, Ekiti State, Nigeria. They were subcultured into freshly prepared nutrient agar plates and incubated at 37°C for 24hours.

3.2 Confirmation of Test Organisms

Colonies growing on mannitol salt agar were streaked onto freshly prepared nutrient agar plates and incubated again at 35°C. Primary characterization of isolates was based on Gram staining, sugar fermentation (lactose, sucrose, fructose and maltose), citrate utilization test, coagulase test and catalase test as described by Ojo et al., (2013)

3.3 Collection of Plant Sample-*Diodia scandens*

Fresh plants of *Diodia scandens* were collected from farmland in Federal University Oye Ekiti, Ekiti State, Nigeria and its environs. The plant was thoroughly washed with running water and air-dried to crispiness on the laboratory workbench for two weeks. The dried materials were reduced to coarse form using clean pestle and mortar and further pulverized to very fine particles with an electric blender (Nakai magic blender S1-889BD). The powered plant obtained were then properly stored in a container until needed for analysis.

3.4 Extraction of Plant

3.4.1 Soxhlet Extraction Method

Seventy-five grams (75g) of powdered plant sample was weighed and poured into a clean handkerchief and placed into a thimble. 500ml of the solvent was poured into the round bottom extraction flask and placed in a water bath. The thimble containing the powdered plant sample was fixed into the extraction chamber, and connected to the condenser in a vertical position. The extraction was performed in a water bath at 65°C for six (6) hours. The solvent was evaporated under pressure using rotary evaporator (Senco Technology Co. Ltd, model no: R205, SN 13605) for 15mins at 39-40rpm. This procedure was carried out using ethyl-acetate, n-hexane and methanol as solvent of extraction.

3.4.2 Cold Extraction Method

Seventy-five grams (75g) of plant sample was soaked in 500ml of solvent (ethyl-acetate, n-hexane and methanol) and agitated manually. It was then allowed to extract for 48hours before each extract was filtered using Whatmann No 1 filter paper. The solvent was evaporated using rotary evaporator (Senco Technology Co. Ltd, model no: R205, SN 13605) under pressure for 15mins at 39-40rpm. The extract was stored until needed.

3.4.3 Thin Layer Chromatograpy (TLC)

Different mobile phase systems were developed by mixing the different solvents in different ratios. Methanol:n-hexane:ethyl-acetate (40:30:30), ethyl-acetate:n-hexane (80:20) and ethyl-acetate:n-hexane (70:30). TLC was performed on a silica gel glass plate (Jinotech Instruments, GF254, Lot No: 121220, 20x20cm) for chromatographic separation of the extracted plant material. Development of the chromatograms was done in a closed tank containing eluent in which the atmosphere had been saturated by the eluent vapour. The plates were then dried under a stream of air to remove excess solvent. The cold and soxhlet extract of ethyl-acetate, n-hexane and methanol were dissolved in few drops of chloroform. A capillary tube was used through capillary action to spot the baseline of the TLC chromatogram before being placed in the closed tank. The Retention factor values were then calculated.

3.4.4 Bioautography (Agar-Overlay Method)

A bioautography technique was employed to define the active constituents. Bioautographic method is basically to localize the antibacterial compound from crude extract in the chromatogram. The agar overlay technique was used to conduct the bioautographic assay. Overnight broth culture of 10^6cfu/ml of *S. aureus* and CoNS was introduced into the molten Mueller Hinton agar. The developed TLC plates were placed in Petri dishes and overlaid with thin layer of the molten agar seeded with the organisms. It was allowed to solidify and incubated at 35°C for 24hours. Tetrazolium salt of 1g was then prepared in 9ml of distilled water and sprayed on the incubated TLC plates in the Petri dish. This was then incubated again at 35°C for 1hour.

CHAPTER FOUR

4.0 RESULTS

Table 1 shows Gram staining reaction on the organisms with a positive result indicating that they are Gram positive cocci. The organisms were catalase positive, SA241-OJO was positive to coagulase while CoNS 10b-OJO was negative. SA241-OJO was able to ferment mannitol while CoNS 10b-OJO was negative. Production of acid and gas was observed in CoNS 10b-OJO when exposed to lactose, sucrose, fructose and maltose while SA241-OJO produced acid and gas in sucrose and fructose but acid alone in lactose and maltose.

Table 2 shows the active bands of *Diodia scandens* extract and the Retention factors (R*f*) of different eluents in TLC plates. Methanol cold and soxhlet extract showed one active band each in a solvent ratio 40:30:30 (methanol:n-hexane:ethyl-acetate) with both having a retention factor value of 0.36. Ethyl-acetate cold extract had a five active bands with retention factor (R*f*) values of 0.05, 0,18, 0.43, 0.63 and 0.70. The soxhlet extract of ethyl-acetate and n-hexane in a solvent ratio of 80:20 had four active bands with retention factor (R*f*) values of 0.20, 0.33, 0.43 and 0.30 while the cold extract had five active bands. Cold extract of ethyl-acetate:n-hexane (70:30) had six active bands with retention factor (R*f*) values of 0.07, 0.12, 0.62, 0.68, 0.72, 0.78, while the soxhlet extract had four active bands with retention factor (R*f*) values of 0.15, 0.23, 0.63 and 0.68.

The zones of growth inhibition on bioautographic TLC plates by *Diodia scandens* extract as revealed in Table 3 showed that zones of inhibition of cold extract of ethyl-acetate on *S. aureus* was 14mm while the soxhlet extract was 21mm. Cold extract of n-hexane had an inhibition size of 33.7mm while the soxhlet extract had 25mm. The methanol extract showed no zone of inhibition on *S. aureus*.

On CoNS, ethyl acetate cold extract had zone size of 19.5mm while soxhlet extract showed no zones of inhibition. The cold and soxhlet extracts of n-hexane had zone sizes of 34.0mm respectively. Methanol cold extract had zone size of 28.5mm while soxhlet extract had 27.5mm.

Table 1. Morphological and Biochemical Confirmation of test organisms

						Sugar Fermentation				
organism	**Shape**	**Gram reaction**	**Catalase**	**Coagulase**	**Citrate Test**	**Mannitol**	**Lactose**	**Sucrose**	**Fructose**	**Maltose**
SA241-OJO	Cocci	+	+	+	+	+	A	AG	AG	A
CoNS 10b-OJO	Cocci	-+	+	-	+	-	AG	AG	AG	AG

KEY: + Positive, - Negative, A= Acid, AG= Acid and Gas, SA= *Staphylococcus aureus*, CoNS= Coagulase Negtive *Staphylococcus*

Table 2. Retention factor (R*f*) of *Diodia scandens* extract on TLC plates

Extract	Total number of band	Total number of active bands	R*f* values	Solvent ratio
Methanol (C)	1	1	0.36	M:N:E 40:30:30
Methanol (S)	1	1	0.36	
Ethyl-acetate (C)	-	5	0.05, 0.18, 0.43, 0.63, 0.70	
Ethyl-acetate (S)	4	4	0.20, 0.33, 0.43, 0.30	
n-hexane (C)		6	0.07, 0.12, 0.62, 0.68, 0.72, 0.78	
n-hexane (S)	4	4	0.15, 0.23, 0.63, 0.68	

KEY: (C) = Cold, (S) = Soxhlet, M= Methanol, N= n-hexane, E= Ethyl-acetate

Table 3. Zones of Growth Inhibition on Bioautographic TLC Plates by *Diodia scandens* Extract.

Organisms	Ethyl-acetate C/H	n-hexane C/H	Methanol C/H
S. aureus	14mm/21mm	33.7mm/25mm	nil/nil
CoNS	19.5mm/nil	34.0mm/34.0mm	28.5mm/27.5mm

KEY: C/H= Cold/Hot, SA= *Staphylococcus aureus,* CoNS= Coagulase Negative *Staphylococcus*, nil= no zones of inhibition

Plate 2. **Photograph showing different active bands of *Diodia scandens* on ethyl-acetate and n-hexane extracts (ratio 80:20)**

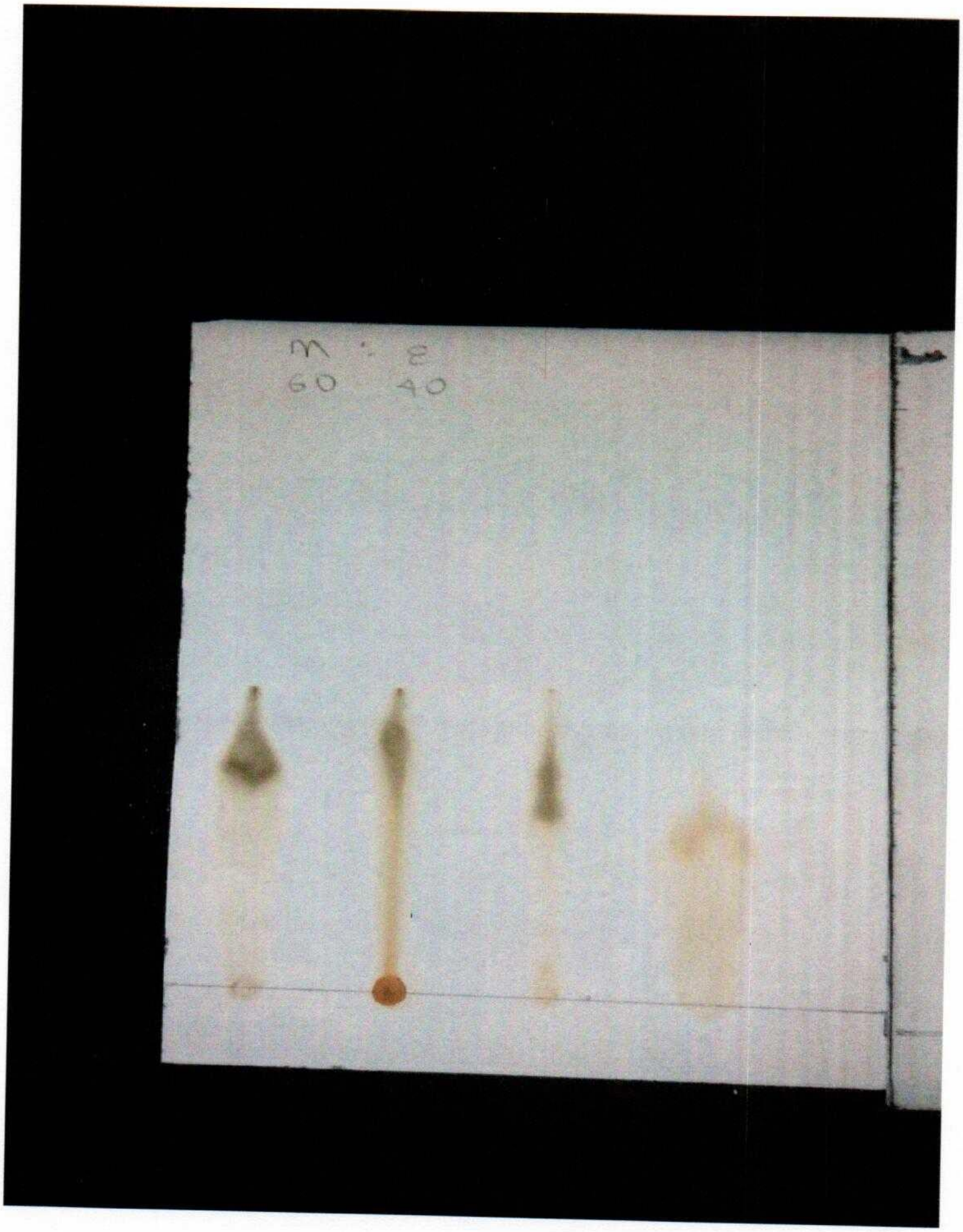

Plate 3. Photograph showing active band of *Diodia scandens* on methanol and n-hexane extract. (ratio 60:40)

Plate 4. Photograph showing zones of inhibition of *Staphylococcus aureus* in *Diodia scandens* extract.

Plate 5. Photogragh showing zones of inhibition of CoNS on *Diodia scandens* extract.

CHAPTER FIVE

5.0 DISCUSSION

The universal role of plants in the treatment of diseases is exemplified by their employment in all major systems of medicine (Okeniyi *et al*., 2007).

The viability of reference strains were confirmed from the result in Table 1 showing Gram positive cocci, coagulase positive and fermentation on mannitol which indicate *S. aureus* while strain showing Gram positive cocci, coagulase negative and non fermentation on mannitol, indicate coagulase negative *Staphylococci,* which was similar to a report by Ojo *et al.* (2013).

Cold extracts from this study was shown to have more active bands of phytochemical constituents than soxhlet crude extract thus indicating that more active phytochemicals are better extracted from cold percolation method than soxhlet extraction method, which was corroborated by Abah and Egwari (2011), where cold extract showed more activity than soxhlet extract. This result however differs with the result of Aderiye and David (2014) where cold extact showed lower active bands than hot extract. However this was on a different plant produce.

The development of mobile phase system for the TLC showed different variations in the extracting ability of the solvent as typified by the number of active bands this findings corroborate with Christianah and Roland (2012), where different solvent combination (chloroform/ethyl acetate/formic acid) and (benzene/ethanol/ammonium hydroxide) showed variations in their extracting ability with the former solvent combination showing more active bands than the latter.

Table 3 shows zones of inhibition of plant extract on *Staphylococcus aureus* and CoNS. N-hexane cold extraction showed highest zone of inhibition (33.7mm and 34.0mm respectively) while methanol extraction had no zone of inhibition on *Staphylococcus aureus,* which was similar to a report by Abah and Egwari (2011), that wider zones of inhibition were obtained with cold extraction than soxhlet extraction with certain organism capable of being resistant to soxhlet extract. This study confirms the antimicrobial activity of *Diodia scandens* extract on *Staphylococcus aureus* and CoNS however some constituents might have been lost due to heat.

6.0 CONCLUSION AND RECOMMENDATION

6.1 CONCLUSION

This study reveals that there are various bioactive constituents present in *Diodia scandens* extracts, which exhibited antibacterial activity against *S.aureus* and CoNS. The cold extract of the plant tends to show more zones of inhibition than the hot extract which could be that some constituent present in plant are susceptible to high heat. Solvent systems and ratio also played its part in the number of bands that may be visible during TLC.

6.2 RECOMMENDATION

This study shows that the bioactive constituents present in *Diodia scandens* extract exhibit antibacterial activity. Therefore further research should be carried out to identify the specific constituent. Other Bioautographic methods should also be tested for comparism with more solvent system variations. Toxicity test can also be carried out to reveal side effect of the extract.

6.3 CONTRIBUTION TO KNOWLEDGE

1. Active band sites on the TLC plates of *Diodia scandens* extracts was high in ethyl-acetate and n-hexane (4-6) than methanol with one
2. Zones of inhibition ranged between 14mm and 34mm on the test organisms.

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APPENDIX

Morphological Test

a. **Gram staining**

Materials

Crystal violet, iodine, ethanol, saffranin, Bunsen burner, inoculating loop, clean glass slide, microscope.

Procedure

A thin smear of cell suspension was made on a clean glass slide and then heat fixed by passing slide over a blue flame. The smear was flooded with crystal violet for one minute and mildly rinsed with water. Some drops of iodine was added for one minute (the iodine decreases the solubility of the purple dye forming dye-iodine complexes), which was then rinsed with water. The purple dye-iodine complex where then decolorized with 95% ethanol for fifteen seconds and rinsed with water. The smear was then counter stained with saffranin for thirty seconds. The excess stain was rinsed off with water. Smeared slide was then allowed to air dry. A drop of oil immersion was then added to it before examination under the microscope.

Result

Purple colour observed under the microscope indicate a Gram positive result while pin colour indicate a Gram negative result

Biochemical Test

a. **Catalase Test**

Materials

3% hydrogen peroxide, inoculating loop, clean glass slide, Bunsen burner.

Procedure

A colony of the organism was picked and placed in a drop of 3% hydrogen peroxide on a clean glass slide. Effervescence caused by the liberation of oxygen as gas bubbles indicates the production of catalase by the organism while an absence of gas bubbles indicates a negative result.

b. Coagulase Test

Materials

Blood plasma, normal saline, glass slide, inoculating loop, Bunsen burner.

Procedure

A colony of the organism was emulsified in normal saline on a clean glass slide and an equal volume of plasma was added and mixed together. Clumps in the mixture indicate a positive coagulase test.

c. Sugar Fermentation Test

Sugar fermentation is used to test for the ability of organism to different sugars by breaking them down to alcohol. Sugar fermentation coupled with acid production which can be formed during the reaction and it is detected by the use of Durham tube in inverted position in a given test tube.

Procedure

Sugars used were mannitol, lactose, sucrose, fructose, and maltose. 1g of each sugar was weighed into different conical flask and labelled accordingly. Into each flask, 1g of peptone water was added and made up to 100ml with distilled water. Exactly 0.01g phenol red was added as an added indicator. About 5ml each of 100ml sugar solution was dispensed into different test tubes with Durhams' tube inserted into each tube in an inverted position. The tubes were properly labelled and covered tightly with cotton wool and aluminium foil paper and sterilized. The sugar solution in test tubes were then inoculated with the isolate and incubated at 35°C for 24hrs. A change in the colour to yellow indicates acid production while bubbles in Durham tubes indicate gas production.

d. Citrate Utilization Test

A colony of sample organism was picked and inoculated into Simmons citrate agar and incubated at 35°C. Growth in the medium indicates a positive result with a colour change from green to blue.