ANTIBACTERIAL ACTIVITIES OF CRUDE *EUDRILUS EUGENIAE* EXTRACT - MEDIATED SILVER NANOPARTICLES ON ISOLATES FROM DIABETIC PATIENTS WITH FOOT INFECTIONS

BY

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ABSTRACT

Diabetic foot infection pose a significant challenge to health care system because of its tremendous utilization of resources.The antibacterial activities of crude extract of *Eudrilus eugeniae* and extract-mediated silver nanoparticles were investigated against isolates from patients with diabetic foot infection using agar well diffusion method. Saponins, proteins, terpenoids, phenols and steroids were detected in the crude extract and fractions. Crude ethanol, ethyl-acetate and aqueous fractions at 4mg and 8mg showed very weak inhibitory zone in diameter (mm) on *Psuedomonas aeruginosa*, *Staphylococcus aureus*, *Streptococcus pyogenes*, *Klebsiella pneumoniae* and *Escherichia coli*. Dark brown aqueous extract-mediated silver nanoparticles (AAgNPs) was formed which gave maximum absorbance at 408nm. The synthesized particles were spherical, less aggregated and contained alkanes, esters and alcohol functional groups. The zones of inhibition produced by AAgNPs (at 1:4 dilution) were 15.33±0.58 mm and 17.67±0.58 mm against *E. coli* and *P aeruginosa* while at 1:5 dilution, 12.33±2.08 mm, 14.67±0.58 mm and 15.33±1.16 mm were zones of inhibition produced against *E. coli*, *K. pneumoniae* and *P aeruginosa* respectively. The minimum inhibitory concentration (MIC) of AAgNPs against *P. aeruginosa* was at 1.25mg/ml while it was at 2.5mg/ml against *E. coli* and *K. pneumoniae.* The extract was bacteriostatic on the test isolates. Significant ($P<0.05$) wound closure was observed in rats from groups 1-6, which were topically treated with AAgNPs from Day 0 (1.00±0.44mm to 1.20±0.25 mm) to Day 14 (0.30±0.00 mm to 0.73±0.25 mm) compared to group 7 (1.21±0.10mm to 1.47±0.21mm). The administration of ointment of AAgNPs on the rats did not show skin irritation, rashes and other allergic reactions. The results of the finding showed the potential of AAgNPs as a safe topical therapeutic agent.

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ABBREVIATIONS

A	-	Aqueous extract
AAgNPs	-	Aqueous fraction mediated silver nanoparticles
ABS	-	Absorbance
AF	-	Aqueous fraction
$AgNO_3$	-	Silver nitrate
AgNPs	-	Silver nanoparticles
ANOVA	-	Analysis of variance
BA	-	Blood agar
BW	-	Body weight
CG	-	Coagulase
CI	-	Citrate
CT	-	Catalase
DFI	-	Diabetic foot infection
DMRT	-	Duncan's multiple range test
E	-	Ethanol extract
EC	-	Ethyl acetate extract
EDS	-	Energy dispersive spectroscopy
ESC	-	Extract Sterility Control
FTIR	-	Fourier transformed infrared spectroscopy
G	-	Glucose
H_2O	-	Water
H_2O_2	-	Hydrogen peroxide
H_2S	-	Hydrogen sulphide
H_2SO_4	-	Tetraoxosulphate (iv) acid
IN	-	Indole
KIA	-	Kligler iron agar
L	-	Lactose
MBC	-	Minimum bactericidal concentration
MCA	-	MacConkey agar
Mg/dl	-	milligram per deciliter

Mg/ml	-	Milligram per milliliter
MIC	-	Minimum inhibitory concentration
MR	-	Methyl red
MSC	-	Medium sterility control
NaOH	-	Sodium hydroxide
ND	-	Not detected
NH	-	N- Hexane extract
NIPRD	-	National Institute for Pharmaceutical Research and Development
O_2	-	Oxygen molecule
^oC	-	Degree Celsius
OPX	-	Distyrene plasticizer xylene
OVC	-	Organism viability control
OX	-	Oxidase
pH	-	Hydroxyl ions
SEM	-	Scanning electron microscopy
SPSS	-	Statistical package for social sciences
SSC	-	Silver nitrate solution sterility control
UR	-	Urease
UV-Vis	-	Ultra violet -visible
VP	-	Vogues-Proskauer
XRD	-	X-ray diffraction
α	-	Alpha
β	-	Beta
γ	-	Gamma

APPENDIX

CHAPTER ONE

1.0 INTRODUCTION

1.1 Background to the Study

Diabetes mellitus is a chronic metabolic disorder imparting loss in health and economic burden on the patients (Alok *et al.*, 2018; Kavya and Alain, 2020). According to International Diabetes Federation, in 2017 about 451 million people were diabetic and figure is expected to increase to 693 million people in 2045 (Cho and Malanda, 2018; Pouya *et al*., 2020). The risk for developing foot ulcer is 25 % high in patients with diabetes and it was reported that in every 30 seconds one lower limb amputation in diabetic patients occurs around the world. A meta-analysis study published recently showed that the world wide prevalence rate of diabetic foot infection is 6.3 % (Alok *et al.*, 2018; Pouya *et al.*, 2020).

Diabetic foot infection (DFI) represents a severe complication of long-standing diabetes mellitus. It is an open wound that occurs in approximately 15 percent of patients with diabetes, commonly located at the lower limb precisely the foot. Inadequate treatment of diabetic foot infection could lead to the spread of the infection from foot ulcer to the bone, thus causing osteomyelitis that exposes the patient to the risk of amputation, bacteremia and death (Lauri *et al*., 2020).

Patients with diabetic foot infection are often infected with multidrug resistant organisms due to chronic course of the wound, inappropriate antibiotic treatment, frequent hospital admission, neuropathy, nephropathy and peripheral vascular disease (Priya *et al.*, 2019; Vanessa *et al.*, 2020). For example methicillin-resistant *Staphylococcus aureus* infections delay wound healing time, increase hospitalization

stay, increase the need for surgical procedures and result in treatment failure (Vanessa *et al.,* 2020). Some of the microorganisms responsible for diabetic foot infection include *Pseudomonas aeruginosa, Staphylococcus aureus, Streptococcus* spp. *Escherichia coli, Enterobacteriaceae, Acinetobacter* species *and Klebsiella pneumoniae* (Priya *et al.,* 2019; Vanessa *et al.,* 2020). Treatment of diabetic foot infection results to devastating economic crisis for the patient's families and society at large (Alok *et al.,* 2018; Bus *et al*., 2020). Furthermore, deep soft-tissue infections in diabetic persons can be associated with gas- producing, Gram negative bacilli (Michael, 2018; Lauri *et al.,* 2020).

Some factors contributing to development of the diabetic foot infection include walking barefooted, delay in reporting to the medical center for clinical assessment, impaired sweating, dry and cracked skin, toenail infections and foot abnormalities such as Charcot foot. Diabetes cause impairment in the body production of insulin (a hormone that enable the cells to obtain and utilize glucose from the blood stream for energy generation) and this disruption to insulin production makes it more difficult for the body to manage blood glucose levels. When blood glucose remains permanently high, it impairs the function of white blood cells (WBCs). The white blood cells are central to the role of immune system. When WBCs are unable to function properly, the body is less able to fight bacteria and heal wounds. Also uncontrolled diabetes may develop poor circulation. As circulation slows down, blood moves slowly, this makes it more difficult for body to deliver nutrients to wounds site. As a result, the injuries heal slowly or may not heal (Patrick, 2019). Prolonged treatment of infectious diseases such as diabetic foot infection with synthetic antimicrobial agents had led to resistance of microorganisms to the antibiotics. Also due to socioeconomic effects of the existing

drug, it becomes vital to source for a well-tolerated more effective and affordable antimicrobial agents.

Eudrilus eugeniae is a tube like, segmented worm found in the phylum Annelida. It belongs to the Family*: Eudrilidae*, Genus*: Eudrilus*, Species*: eugeniae* (Sethulakshmi *et al.*,2018). *Eudrilus eugeniae* is called 'Earthworm' in English, 'Ogbu mmiri' in Igbo, 'Tana' in Hausa and 'Ekolo' in Yoruba, 'Egwongi' in Nupe and 'Mbuombulo' in Gwari. Earthworms are commonly found in soil and play a major role in the proper functioning of the soil ecosystem.

The medical value of earthworms had been known for centuries. The extracts prepared from earthworm tissues have been used for the treatment of numerous diseases since they are valuable source of proteins, peptides, enzymes and physiologically active substances. Earthworm has been recognized in oriental medicine as anti- inflammatory, analgesic and antipyretic agent. Earthworm surface excreta were found to have potent antimicrobial activity. It also has anticoagulatory or fibrinolytic activity which results in the facilitation of blood circulation. Earthworms were used as a traditional Chinese medicine and have been applied pharmacologically and clinically. It has healing effects on wounds. It decreases the wound healing time and reduced ill- effects of inflammation as determined by macroscopy, histopathology, hematology and immunohistochemistry parameters (Zhen-hen *et al.,* 2018). To further enhance the antimicrobial activities of the earthworm extract, silver nanoparticle was synthesized from it. Silver nanoparticles is a carrier, owing to its high targeting potentials and efficient delivery, it moves the antimicrobial agent to the targeted site of the cells of the microorganisms (Chengzhu *et al*., 2020).

Silver nanoparticles are nanoparticles of silver between 1 nm and 100 nm in size. While frequently described as being silver, some are composed of a large percentage of silver

oxide due to their large ratio of surface to bulk silver atoms. Numerous shapes of nanoparticles can be constructed depending on the application at hand. Commonly used silver nanoparticles are spherical in shapes but diamond, octagonal and thin sheet are also popular. The green synthesis of silver nanoparticles (AgNP) is considered an eco-friendly technology leading to a reduction in the generation of hazardous substances. Silver ions and silver based compounds are highly toxic to microorganisms, including important species of pathogenic bacteria (Johnston *et al.*, 2016). Silver nanoparticles have emerged with diverse medical applications including silver-based dressings and silver-coated medicinal devices, such as nano-gels and nano-lotions. All bacteria use an enzyme as a form of chemical lung in order to metabolize oxygen. Silver ions cripple the enzyme and stop the uptake of oxygen. This effectively inhibits the growth of the bacteria, killing it within 6 minutes and leaving the surrounding tissue or material unaffected (Johnston *et al.*, 2016). These potentials of silver nanoparticles necessitated the synthesis using earthworm extracts as both function in synergy in inhibiting the growth of microorganisms.

1.2 Statement of the Research Problem

Diabetic foot infection is one of the most common health problems throughout the globe, imparting loss in health and economic burden on patients, families and society at large. Diabetic foot ulcer is often infected with multidrug resistant organisms due to inappropriate antibiotic treatment, chronic cause of the wound and frequent hospital admission. This result to amputation of the lower limb. Example of such multidrug resistant organisms include; methicillin resistant *Staphylococcus aureus,* carbapenem resistant *Enterobacteriaceae, Pseudomonas* and *Acinetobacter* species.

1.3 Justification for the Study

The failure in acquiring new compounds of therapeutics benefits from microbial sources, side effects from residue leaving synthetic drugs and the need for alternative source to combat these problems has necessitated the search for new antimicrobial agent. Also due to socioeconomic effects of the existing drug and resistance issue, it becomes vital to source for a novel, well tolerated, more effective and affordable antimicrobial agents. Great efforts have been made in identifying the new antimicrobial agents from natural resources as alternative approach but research is still needed to be done especially on that of animal origin. The field of examining the potential health benefits of consuming earthworms owes its increasing interest to bioprospecting. Data generated from this research will add to the existing data on diabetic foot infection.

1.4 Aim and Objectives of the Study

1.4.1 Aim of study

To determine the antibacterial activities of crude *Eudrilus eugeniae* extract- mediated silver nanoparticles on isolates from diabetic patients with foot infections

1.4.2 Objectives

The objectives of the study were to:

i screen crude extract and fractions of *Eudrilus eugeniae* for the presence of various bioactive components

ii determine the antibacterial potentials of crude extracts of *Eudrilus eugeniae* and extract-mediated silver nanoparticles against isolates from patients with diabetic foot infection

iii determine the minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC)

iv characterize extract- mediated silver nanoparticles of *Eudrilus eugeniae*

v carry out toxicological study in rats

CHAPTER TWO

2.0 LITERATURE REVIEW

2.1 Biology of Earthworm

Eudrilus eugeniae (Earthworms) are tube-shaped segmented worms which have no skeleton. Table 2.1 shows the scientific classification of *Eudrilus eugeniae.* An earthworm's digestive system runs through the length of its body. From evolutionary point of view, the earthworms are very old species. They survived over a million years due to their ability to adjust to different environmental conditions (Sahjahan, 2018). The body of earthworm is covered with small fluffs, which is important in environmental adjustment and for search of food in the soil. Waste products enrich the soil with nutritive substances, which stimulate the growth of plants. However, the earthworms are very important source of diet for numerous animals in the soil. They are hermaphrodites, this characteristic also contributes to good environmental adjustment because the worms reproduce very easily (Sahjahan, 2018). The eggs hatched in soil and are protected with capsule, which arises from the secretion from *clitellum*. The capsule protects the young worms until complete maturation. Their living place is damp soil enriched with organic substances (Ali and Kashem, 2018). .

They breath through the skin and they are very sensitive to changes in temperature, light and on touch. During the winter they burrow in deeper layer to protect from low temperature and during the summer and dryness to protect from dehydration.. It has a double transport system composed of coelomic fluid that moves within the fluid-filled coelom and a simple, close blood circulatory system. It has a central and a peripheral nervous system (Sethulakshmi *et al.,* 2018). The central nervous system consist of two ganglia above the mouth, one on either side connected to a nerve cord running back along its length to motor neuron and sensory cells in each segment. Large number of

chemoreceptors are concentrated near its mouth. Circumferential and longitudinal muscles on the peripheral of each segment enable the worm to move. Similar sets of muscle line the gut and their action moves the digesting food toward the worm's anus. The ventral conducting system of pharynx serves as passage for the ingested materials. The worms maintain their structure with fluid- filled coelom chambers that function as a hydrostatic skeleton (Priyajankar and Debnath, 2020)

Eudrilus eugeniae, also called African Nightcrawler is an earthworm species native to tropical West Africa and now widespread in warm regions under vermicompost. Their growth, maturation and reproduction are significantly greater at 25 ^{o}C. The greatest number of cocoons per week and the number of hatchlings per cocoon are obtained at 25 ^{o}C. Cocoons of *E. eugeniae* hatches in 12 days at 25 ^{o}C and the worms reach sexual maturity in 35 days after hatching. The African Nightcrawler has a uniform purple-grey sheen and the posterior segments evenly tapered to point (Ali and Kashem, 2018).

Earthworms are commonly found living in soil, feeding on live and dead organic matter (Priyajankar and Debnath, 2020). While earthworms, the largest of the Oligochaeta, have medicinal properties, they are also related to various other species, such as leeches, that have been shown to exhibit therapeutic benefit (Vasanthi *et al*., 2016). Earthworms are the major decomposers of dead and decomposing organic matter and acquire their nutrition from the bacteria and fungi that grow upon these materials. They decompose the organic matter and make the major contributions in recycling the containing nutrients. The earthworms are found in the warmest soils and many tropical soils. They are divided into 23 families, more than 700 genera and over 7,000 species. Their size ranges from an inch to two yards, and are found seasonally at all depths of the soil (Ali and Kashem, 2018). Specifically, earthworms have been vital to investigations of developmental organizations of the nervous, immune and endocrine systems.

Earthworms are not only important in understanding biological processes of development (Sethulakshmi *et al.,* 2018) but also their well known significance to ecosystems, especially their ability to improve soil fertility. Earthworms have been utilized as a form of nutrition as well as treatment for specific conditions as traditional forms of medicine such as those in Asia (Sethulakshmi *et al.,* 2018). Several studies have shown that the earthworm extracts contain different macromolecules which exhibited the variety of activities, such as antioxidant, antibacterial, anti-inflammatory, anticancer etc. In some countries the earthworms are used as food. They have very high nutritive value because their bodies contain high percentage of various proteins. Besides the human food, the earthworms are used in the feeding of animals (example, fish, chicken, etc.).

The field of examining the potential health benefits of consuming earthworms owes its increasing interest to bioprospecting (Vasanthi *et al*., 2016). Researchers have discovered that in addition to ecological benefit, specifically soil preservation, the long neglected earthworm innate immune system holds the key to these observed medicinal qualities (Yadav and Shukia, 2021).

2.2 Coelomic Fluid of Earthworm

The coelomic cavity of earthworm is filled with fluid derived from mesenchymal lining which is known as the coelomic fluid. The coelomic fluid consists of watery matrix, the plasma and a large number of coelomocytes having wide variety of functions, which plays a very important role in building innate immunity. The coelomic fluid is generally secreted by the earthworms for maintaining moisture in the body to help their physiological activities such as respiration and burrowing. It is generally expelled through the dorsal pores at the time of stress in response to mechanical and chemical irritation. The fluid is circulated by mesothelial cilia or by contraction of muscles in the

body wall. It also contains a complex mixture of small- molecule matabolites including aromatic metabolites. Among other functions, it allows free movement and growth of internal organs, acts as hydroskeleton, and serve for transport of gases, nutrients and waste products around the body. It equally acts as reservoir for waste (Simone and Jecob, 2017). Coelomic fluid has many biological properties such as cytotoxic, proteolytic, anti-microbial, haemolytic, haemo-agglutinating and mitogenic activities. Several bioactive compounds have also been found in the coelomic fluid. Coelomocytes located in the coelomic fluid is responsible for both innate cellular immune functions such as phagocytosis and encapsulation against pathogenic microorganisms. The transport of coelomic fluid between neighboring segments is ensured by channels comprised of sphincters within the septa.

Each segment of the coelomic cavity is opened to the outer environment by paired nephridia and by one dorsal pore through which soluble metabolites and corpuscular materials respectively can be expelled out. Studies on the composition of coelomic fluid has shown the presence of haemolytic, proteolytic and cytotoxic enzyme which are active against foreign cells and peptides. The coelomic fluid is generally secreted by the earthworms for maintaining moisture to help their physiological activities such as respiration and burrowing activities. These coelomocytes play a very important role in building innate immunity of earthworms. They are differentiated into four different types of immune cells such as amoebocytes, mucocytes, circular cells and chloragogen cells, which have different shape, size and have wide variety of functions.

Amoebocytes: These cells number are usually more, which are large in size and spherical in shape and helps mostly in removing harmful bacteria from earthworms. They are also known as granulocytes or phagocytes.

Mucocytes: These cells are elongated and its narrow end bears nucleus, they secretes mucous so as to keep the skin always moist for respiratory and other physiological functions.

Circular cells: These cells are nucleated, circular in shape and the functions of these cells are unknown.

Chloragogen cells: These cells are found in large number; functionally have been described as trophocytes, which are taking part in upliftment of nutrients such as proteins, lipids and glycogens to different cells and organs of the body through coelomic fluid by circulation (Soumya and Pulikeshi, 2017).

All these coelomocytes present in the coelomic fluid have their own functions which include pressuring, desiccation, promoting cutaneous respiration or producing protective measures against predators, regeneration, in circulating nutrients and mainly in defensive mechanism both in cell mediated and humoral immunity for the protection of earthworms **(**Soumya and Pulikeshi, 2017).

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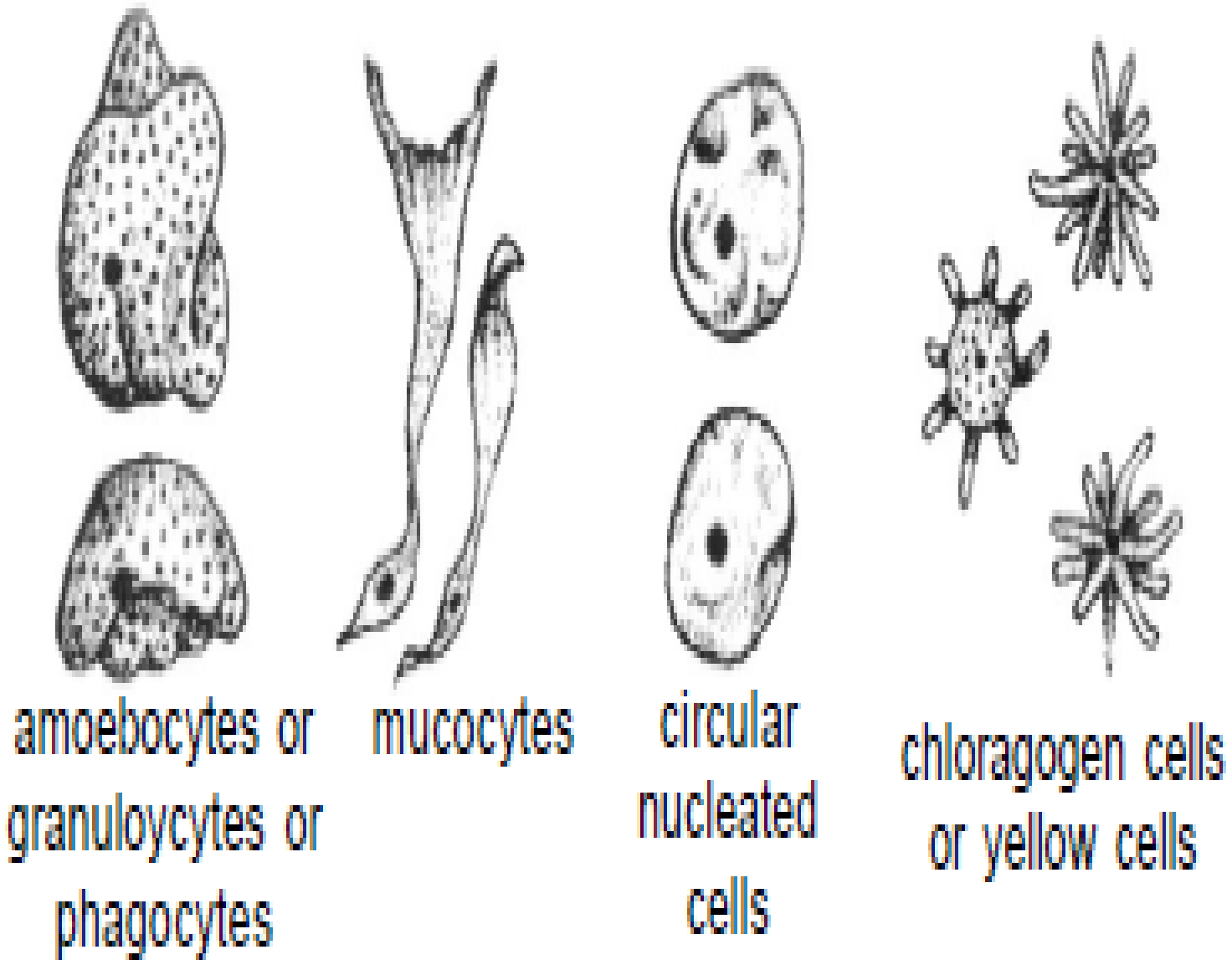


Plate I: Coelomocytea

Source: Soumya and Pulikeshi (2017)

Table 2.1: Taxonomy of *Eudrilus eugeniae*

Taxonomic group	Animal
Kingdom	Animalia
Phylum	Annelida
Class	Citellata
Sub-Class	Oligochaeta
Order	Haplotaxida
Family	Eudrilidae
Genus	*Eudrilus*
Species	*Eugeniae*

Source: Hmar *et al.* (2018)

2.3 Silver Nanoparticles

Nanotechnology is a multidisciplinary field with huge applications in many areas including medicine and pharmacology industries. It deals with the study of materials at nano scale. The increasing development of multidrug resistant microorganisms has become one of the most common problems in medicine worldwide (Bryan *et al*., 2018). Great advances in nanotechnology have provided a solid foundation which makes it possible to curb with the issue of multidrug resistant microorganism using nanoparticles. Nanoparticles serves as a strong bridge between the bulk material and atomic or molecular . A bulk material has constant physical properties regardless of the shape and size but at the nanoscale, the size, morphological substructure of the substance and shape are major driving factors for changing their biological, chemical and physical properties (Neelu, 2018). The three main methods of nanoparticle synthesis are physical, chemical and biological (green synthesis).

The chemical and physical methods are costly, they involve the use of higher amount of energy, toxic solvents and hazardous reagents. Plant, animal and microbes are used in green synthesis of nanoparticles. Green synthesis is the utilization of a set of principles that will help reduce the use and generation of hazardous substance during the manufacturing process (Michael *et al*., 2017).

The green synthesis of silver nanoparticles has less financial implication, energy efficient, use of nontoxic solvents, environmental friendly and wide areas of application. The synthesis and activities of different types of nanoparticles has been reported but silver nanoparticle (AgNPs) is considered to be more significant because of its great antimicrobial activities which makes it suitable for many biotechnology application. Authors have reported the synthesis of AgNPs using microorganisms and extract from plants (Adelere, 2017) but not much is reported of the synthesis using animal extract. Therefore, there is every need to harness the biodiversity of animal such as earthworm and their potential secondary metabolites.

2.4 Diabetic Foot Infection

Diabetic foot infection (DFI) is a soft tissue or bone infection that is often associated with neuropathy or peripheral arterial disease in diabetic patients. Diabetic foot ulcer is a common problem among people living with diabetes. It is often an unpleasant experience due to its chronicity, unsightly nature and some degree of negative psychological feeling associated with having a chronic wound. Mobility may also become an issue at a point which further compounds the problem. Closely related is the major sequela of limb amputation. Amputation leads to permanent disability with inability to perform some daily activities. As the diabetes epidemic continues to spread, it is logic to anticipate a rise in complications like DFU in the absence of well articulate strategies that are executed at all levels. Hence, particular attention to feet care should

be a central focus in educating and managing patients with diabetes to ensure that DFU is either prevented or noticed early enough (Amin, 2018).

2.4.1 Epidemiology

Diabetes mellitus is a pandemic affecting over 400 million people worldwide, which is approximately 9 % of the adult population. The prevalence of diabetic foot infection (DFI) varies, ranging from 3-13 %, with a global average of 6.4 %. Prevalence data from the International Diabetes Federation estimates that DFI develop in 9.1-26.2 million people with diabetes per year worldwide. The annual incidence is around 3 %. The geographical variation in the prevalence is related to the prevalence of diabetes as well as sociocultural factors that enhance the occurrence. Also important are the socioeconomic standard and access to quality health care. DFU has been identified as the leading reason for hospitalization among patients with diabetes. It is estimated to account for 25 % of all hospital admissions in patients with diabetes (Amin, 2018).

2.4.2 Causes of diabetic foot infection

Ulcers in people with diabetes are commonly caused by;

1 Poor circulation of blood; this is a form of vascular disease in which blood flow to the feet is inefficient. It makes it difficult for the wound to heal

2 High glucose level; it slows healing process of infected foot ulcer

3 Nerve damage; is a long-term effect and can lead to a loss of feeling on the feet. It reduces sensitivity to wound pain and result to painless wound (Michelle and Robison, 2021).

2.5 Medical Importance of Test Isolates

2.5.1 *Staphylococcus aureus*

Staphylococcus aureus is a Gram positive cocci bacterium from the phylum Firmicutes and a member of microbiota of the body, frequently found in the upper respiratory tract and on the skin. The organism acts as a commensal of human microbiota but can also become an opportunistic pathogen (Lindsay, 2017). It is a common cause of skin infections such as abscesses and respiratory infections such as sinusitis. The skin infection can progress to impetigo (a crusting of the skin) or cellulites (inflammation of the deeper layers of the skin and connective tissue under the skin, leading to swelling and redness of the area). Diabetic foot infections (DFI) are mainly polymicrobial and . *S. aureus* is often isolated from diabetic foot infections. The pathogenesis of *S. aureus* in DFI is classical and corresponds to the physiopathology of skin and soft tissue infection.

The first defense against *S. aureus* infection is the neutrophil response. When *S. aureus* enters injured skin, neutrophils and macrophages migrates to the site of infection. It evades this response using different methods (example, blocking sequestering host antibodies, chemotaxis of leukocytes, hiding from detection via capsule or biofilm formation and resisting destruction after ingestion by phagocyte). It equally cause food poisoning, pimples, boils, folliculitis, carbuncles, scalded skin syndrome and life threatening disease such as pneumonia, meningitis and osteomyelitis (Gordon *et al*., 2021). Pathogenic strains often promote infections by virulence factors such as potent protein toxins and the expression of a cell- surface protein that binds and inactivates antibodies (Wollina, 2017).

2.5.2 *Pseudomonas aeruginosa*

Pseudomonas aeruginosa belongs to the phylum – Proteobacteria and family-Pseudomonadaceae. It is a common encapsulated, Gram-negative, rod-shaped bacterium that can cause disease in plants and animals including humans. It is a facultative anaerobe which proliferates in conditions of partial or total oxygen depletion and is found in soil, water, skin flora and on surfaces. It colonizes many natural and artificial environment (Bryan *et al.,* 2018*). Pseudomonas* has become an important cause of Gram-negative infection, especially in patients with compromised host defense mechanisms. It is the most common pathogen isolated from patients who have been hospitalized longer than one week and it is a frequent cause of nosocomial infections. Pseudomonal infections are complicated and can be life-threatening. It is association with serious illnesses. The bacterium, because it thrives in moist surfaces, can be found on and in medical equipment causing cross-infections in hospitals and clinics (Bryan *et al.,* 2018). It affects urinary tract, airway, wounds and burns (Gerard, 2016; Kansaa *et al*., 2021).

2.5.3 *Streptococcus pyogenes*

Streptococcus pyogenes is a Gram positive cocci, aerotolerant bacterium in the genus *Streptococcus*. These bacteria are extracellular, made up of non-motile and non- sporing cocci. It is clinically important for humans. It is an infrequent but usually pathogenic, part of the skin microbiota. It is the predominant species harboring the Lancefield group A antigen. It is the cause of many human diseases, ranging from mild superficial skin to severe invasive infection. It has several virulence factors that enable it to attach to the host tissue, evade the immune response and spread by penetrating host tissue layers (Avire *et al*., 2021).

2.5.4 *Escherichia coli*

Escherichia coli is a type of bacterium that lives mostly in intestines, is a Gram negative bacilli that exist singly or in pairs. It is facultatively anaerobic with a type of metabolism that is both fermentative and respiratory. It is also found in the gut of some animals. Most strains of *E. coli* are normal flora and help in keeping the digestive tract clean but some strains can cause diarrhea when contaminated food or drink is taken. It can cause food poisoning, pneumonia, urinary tract and wound infections among others (Martinez, 2021).

2.5.5 *Klebsiella pneumoniae*

Klebsiella pneumoniae is a Gram negative, non-motile, encapsulated, lactose-fermenting facultative anaerobic, rod- shaped bacterium. It appears as a mucoid lactose fermenter on MacConkey agar. It belongs to the phylum- Proteobacteria and family- Enterobacteriaceae. Although found in the normal flora of mouth, skin and intestine, it can cause destructive changes to human and animal lungs if aspirated, specifically to the alveoli resulting in the bloody, brownish or yellow colored jelly like sputum. In the clinical setting, it is the most significant member of the genus *klebsiella* of the Enterobacteriaceae. *Klebsiella* species have become important pathogens in nosocomial infections. Those with health challenges such as cancer, kidney failure, diabetes, wound, alcoholism, liver problems among others are easily infected by the bacteria. Also taking antibiotic for a very long time can raise the chances for a *klebsiella* infection (Wioletta *et al*., 2021).

2.6 Local Medicinal Uses of *Eudrilus eugeniae*

Earthworm has been used as drug for centuries. It was used in improving blood circulation, treatment of wound and as antitumor agent (Sabahat and Aslaaf, 2020).

Powdered form of the earthworm was used in the treatment of tooth related illness. Earthworms were considered as potent and effective remedy in Iranian culture. In this culture, earthworms were baked and eaten with bread to reduce bladder stones which are expelled after the meal. Furthermore, earthworms were dried and eaten to cure yellow skin in patients with jaundice. Concerning alopecia or hair loss, powdered earthworm applied on the scalp with rose oil, assist the regrowth of hair. It was used to treat smallpox by soaking in water which the patient will bath with. The worms were then roasted, powdered, mixed with coconut water and given to the patient with smallpox to drink (Sabahat and Aslaaf, 2020). Lumbrokinase is an organic compound derived from earthworm which is used as dietary supplement for reduction of inflammation as a result of hyper coagulation in other diseases.

In Chinese medicine, the earthworm have been used to improve blood circulation, treat apoplectic stroke and as antipyretic and diuretic agents. In Burma, their primary use is in the treatment of pyorrhea disease. The worms are prepared by heating in a closed pot until reduced to ashes. These ashes are used either alone as a tooth powder or combined with roasted tamarind seeds and betel nuts. Also when a woman feels postpartal weakness and is unable to nurse her infant, earthworms are boiled in water with salt and onions. The clear fluid is decanted and mixed with the patient's food. In Indian, earthworms are well known remedy for the pain of rheumatism (Zhenjun, 2016). Earthworm extract have antiulcer, antioxidant, anti-inflammatory and antipyretic properties. Proteins, peptides, enzymes and physiologically active substances are found in earthworm tissue. Earthworms, like other types of complex invertebrates possess several types of leukocytes which synthesize and secrete a variety of immune protective molecules. The immune protective system is involve in phagocytosis, encapsulation, agglutination, opsonization, clotting and lysis of foreign components. The mitogenic,

antibacterial, haemostatic and antioxidative properties present in earthworm have a major influence on wound healing and epithelization. It shortened the healing time by increasing the epithelization, granulation and synthesis of collagen (Zhenjun, 2016).

CHAPTER THREE

3.0 MATERIALS AND METHODS

3.1 Study Area

Sample collection for the research was done in Bosso Local Government area of Niger State, with its headquarter in Maikunkele. It has an area of 1,592 km and a population of 203,134 as projected in 2019 using the national population census figure of 2006 with 2.5 % annual growth.

3.1.1 Materials

Olympus Microscope, Vertical pressure steam sterilizer, Culturingbox- biochemical, Shp 200, Whatman filter paper (England), Weighing balance, ottans pioneer, MacConkey Agar, Nutrient Agar, Nutrient broth, Peptone water, Eosine methylene blue (Titan Biotech Limited, Rajasthan, India), Ethanol, N-Haxane, Ethyl acetate, plain sample bottles (JHD, China), Glass wares - UK, cotton wool, foil paper, wire loop, JSM needle and syringe, Amitch swab stick (PACPRO Industries limited Lagos, Nigeria), Hand gloves- unigloves (Thailand), Ferric chloride, sodium hydroxide, normal saline, tryptone broth, hydrochloric acid, Dragen droff''s reagent, sulphuric acid, chloroform, acetic anhydride, Nitric acid (sigma product, JHD, China), Hydrogen peroxide, normal saline, distilled water, Kovac's reagent, citrate agar, oxidase reagent, methyl red reagent, vogues-proskauer medium, kligler iron agar, phenol red lactose, sucrose and fructose broth, glucose fermentation medium and liquid paraffin (Titan Biotech Limited, Rajasthan, India), UV- visible spectrophotometer, Scanning electron microscopy, Energy dispersive spectrum, fourier transformed infrared spectrum and X-ray diffraction spectrum (LUCIDEON, Materials Development and Commercialization, USA.)

3.1.2 Collection and identification of earthworm

Matured earthworms were collected in the month of July, 2019. The earthworms were collected by digging the soil and hand sorting. The earthworms were kept in a dark container which was half filled with soil to create a familiar environment and covered with a perforated lid. The worms were identified by a zoologist, Dr K. A. Adeniyi, in the Department of Animal Biology, Federal University of Technology, Minna, as *Eudrilus eugeniae.* Plate II shows the picture of *Eudrilus eugeniae*

Plate II: *Eudrilus eugeniae*

Source; Field work

3. 2 Preparation and Extraction of Earthworm

The earthworms were washed under running tap water to remove soil and mucus from the surface of the body. They were rewashed with sterile distilled water to further remove all the dirt. The worms, weighing 300 g were freeze dried in FGJ-18 Freeze dryer at -20 to -28 °C. The dried worms were pulverized to obtain the powdered form

using ES-242 eurosonic electric blender. It was stored in a sterile container until required for use. The pulverized worms were successively and exhaustively extracted using 70 % ethanol (1.5 Liter), ethylacetate (1 Liter), distilled water (1 Liter) and n-hexane (1 Liter) by cold maceration method. The powdered worm (300 g) was soaked in 1.5 Liter of 70 % ethanol in a conical flask for 72 hours. The mixture was filtered using Whatman filter paper number 1 and half of the filtrate was evaporated to dryness using rotary evaporator at 50 °C and freeze dryer at -28 °C to obtain crude ethanol extract (E). The remaining filtrate was dispensed into a separating funnel and One liter of ethyl acetate was intermittently added and left stand for 24 hours. The soluble ethyl acetate extract was released into a beaker by adjusting the regulating knob of the separating funnel, evaporated to dryness and labeled EC. The crude ethanol extract was re-extracted in 1 Liter of n-hexane for another 24 hours and soluble n-hexane portion (NH) was removed and evaporated to dryness. Finally, the crude ethanol extract was extracted with 1 Liter of sterile distilled water for 24 hours to obtain the aqueous portion (A), (Samatra *et al.*, 2017).

3. 3 Biochemical Screening of Crude Extract and Fractions *Eudrilus eugeniae*

Quantitative and Qualitative biochemical screening was carried out on all the four crude extract and fractions of *E. eugeniae* to detect the presence or absence of various secondary metabolites (steroids, saponins, flavonoids, alkaloids, phenols, terpenoids, tannins and proteins) using standard methods (Roghini and Vijayalakshmi, 2018)

3.3.1 Test for saponins

The extracts (0.2 g) were diluted each with 20mL distilled water and they were shaken vigorously in test tubes for 15 minutes. A layer of foam was formed which indicated the presence of saponins.

3.3.2 Test for phenols

Extracts were (0.2 g) treated in test tubes with 3 drops of ferric chloride solution each. Formation of bluish black colour indicated the presence of phenols.

3.3.3 Test for flavonoids

Extracts (0.2 g) were treated with few drops of 10 % NaOH solution and two drops of HCL, formation of intense yellow colour indicated the presence of flavonoids.

3.3.4 Test for alkaloids

Small quantity of each extract was taken into different test tubes, 1 mL of diluted HCl was added and the mixture was heated gently for 20 minutes. It was cooled, filtered and 2 drops of Dragen droff's reagent was added to the filtrate. The development of a creamy precipitate indicated the presence of alkaloids.

3.3.5 Test for tannin

Extract (0.2 g) was added to 2 mL of water. Two drops of diluted ferric chloride solution was added. A dark green or blue green colouration indicated the presence of tannins.

3.3.6 Test for steroids

In 2 mL of extract, 2 mL of chloroform was added. Equal volume of concentrated sulphuric acid (H_2SO_4) was added by sides of the test tube. Chloroform layer appeared red and acid layer greenish yellow fluorescent. This confirmed the presence of sterols.

3.3.7 Test for terpenoids

Two milliliter of extract was added to 2 mL of acetic anhydride and 3 mL of concentrated H_2SO_4 was carefully added to form a layer. A reddish brown colouration at the interface was formed to show positive result for the presence of terpenoids.

3.3.8 Test for protein

The extracts were treated with few drops of concentrated nitric acid solution. Formation of yellow colour indicated the presence of proteins.

3.4 Collection and Identification of Test Organisms

Samples were collected using sterile cotton swabs from patients with diabetic foot infection attending IBB Specialist Hospital, Minna, Niger State after obtaining an Ethical Clearance in the same hospital. The samples were stored in sterile normal saline containers and were transported to Vaccine laboratory, Center for Genetic Engineer and Biotechnology, Federal University of Technology, Minna within one hour of collection. The isolates were identified using cultural techniques. The swabs were immediately inoculated on nutrient agar and incubated at 37 ^{o}C for 24 hours. Subsequently, the isolates were subcultured on blood agar (BA) and MacConkey agar (MCA). The resulting colonies were Gram stained and further characterized using standard biochemical tests such as catalase, coagulase, indole, hydrogen sulphide production, haemolysis production, sugar fermentation, methyl red test, starch hydrolysis, citrate test and Voges-Proskauer using the method by Cheesebrough, 2018. The organisms were maintained on nutrient agar slants at 4 ^{o}C until required for use.

3.4.1 Gram staining and microscopy

A thin smear of each of the pure 24 hours old culture was prepared on a glass grease-free slides, air dried and were gently passed through flame to heat-fix. The slides were stained by addition of 2drops of crystal violet solution for 60 seconds and rinsed with water. They were flooded with Gram's iodine for 30 seconds and rinsed with water. The slides were decolourised with 70 % alcohol for 15 seconds and rinsed with distilled water. The smear were counterstained with 2 drops of Safranin for 60 seconds, rinsed with water and allowed to air dry. The slides were mounted on microscope and

observed under oil immersion objective lens. Gram negative cells appeared pink while Gram positive organisms appeared purple.

3.4.2 Catalase test

Catalase is an enzyme produced by some specific group of bacteria known as aerobic bacteria and this enzyme is able to breakdown hydrogen peroxide (H_2O_2) to oxygen (O_2) and water (H_2O).This test was performed by placing the test organism on a drop of hydrogen peroxide, the production of effervescence or gas bubbles indicated that the organism is a catalase producer.

3.4.3 Coagulase test

Coagulase is an enzyme capable of coagulating certain blood plasma, notably human and rabbit plasma. This test differentiates pathogenic from non-pathogenic *Staphylococcus* spp. The test was carried out using 24 hours old culture. A loopful of isolate was emulsified with normal saline solution on a microscope slide. A drop of undiluted plasma was added to the suspension and stirred for five seconds. A coagulase positive result was indicated by clumping of colonies.

3.4.4 Indole test

This test was employed to determine the ability of some isolates to produce the enzyme tryptophan which breakdown the amino acid tryptophan into indole, pyruvic acid and ammonia. Tryptone broth (5 mL) was placed each into different test tubes and the tubes were inoculated with a loopful of the bacterial isolate, leaving one of the tubes uninoculated to serve as control. The tubes were incubated at 37 ^{o}C for 48 hours. After incubation, 0.5 mL of Kovac's reagent was added and shaken gently. It was allowed to stand for 20 minutes to permit the reagent to rise. Red or red-violet colour at the top surface of the tube indicated a positive result while yellow colour indicated a negative result.

3.4.5. Citrate test

About 2.4 g of citrate agar was dissolved in 100 mL of distilled water. Ten milliliter (10 mL) of citrate medium was dispensed into each test tube and covered, then sterilized and allowed to cool in a slanted position. The tubes were inoculated by streaking the organisms once across the surface and were incubated at 37 ^{o}C for 4 days. A change from green to blue colour indicated utilization of the citrate.

3.4.6 Oxidase test

Colonies from 24 hours cultures were placed on filter papers and a drop of oxidase reagent was added onto each filter paper and examined within 10 seconds. Oxidase-positive bacteria developed bluish-purple colour

3.4.7 Urease test

The surface of urea agar slant was streaked with 24 hours broth cultures. The cap of the tubes was left on loosely and incubated at 37 ^{o}C for 48 hours then examined for colour change. Urease positive-bacteria developed a magenta to bright pink colour in 24 hours while there was no colour change for urease-negative bacteria.

3.4.8 Methyl red test

A broth culture of 48 hours was used for this test. One milliliter of methyl red reagent was added to 10 mL of the broth in a test tube. Production of red colour indicated a positive result while yellow colour indicated negative result.

3.4.9 Vogues–Proskauer test

The Vogues-Proskauer (VP) medium was allowed to equilibrate to room temperature. Inocula from 24 hours bacterial cultures were inoculated in the tubes containing VP medium. The tubes were incubated at 37 ^{o}C for 24 hours. Following 24 hours of incubation, 1 mL of the broth was dispensed into each clean test tube. The remaining

broth were reincubated for an additional 24 hours. Six drops of 5 % alpha-naphthol were added to each aliquot and homogenized. Two drops of 40 % potassium hydroxide were added to each aliquot and agitated. The tubes were agitated vigorously for 30 minutes and observed for colour change. A pink-red colour at the surface of the tubes indicated a positive reaction while absence of pink-red colour at the surface of the tubes indicated a negative reaction.

3.4.10 Hydrogen sulphide production test

Inocula picked from colonies of 24 hours bacterial cultures were inoculated in tubes containing Kligler iron agar (KIA) by straight stabbing to a depth of 2 mm. The tubes were incubated at 37 ^{o}C for 48 hours and were observed for colour change for H_2S production. Bacteria that produced hydrogen sulphide (H_2S) turned the medium black while there was no colour change for H_2S negative tubes.

3.4.11 Sugar fermentation test

i. Lactose fermentation

Inocula from 24 hours bacterial cultures were transferred aseptically to sterile tubes of phenol red lactose broth. The inoculated tubes were incubated at 37 ^{o}C for 24 hours and observed for colour change. A colour change from red to yellow indicated a positive reaction.

ii. Sucrose fermentation

Inocula from 24 hours bacterial cultures were transferred aseptically to sterile tubes of phenol red sucrose broth. The inoculated tubes were incubated at 37 ^{o}C for 24 hours and observed for colour change. A colour change from red to yellow indicated a positive reaction.

iii. Glucose fermentation

Tubes of glucose fermentation medium were inoculated with inocula from 24 hours bacterial cultures using a straight wire by stabbing half way to the bottom of the tubes. One tube of each pair was covered with 1 cm layer of sterile mineral oil or liquid paraffin (creates anaerobic condition in the tube by preventing diffusion of oxygen). The other tubes were left open. All tubes were incubated at 37 ^{o}C for 48 hours, up to 4 days and observed for colour change in the medium. Acid production was detected in the medium by colour change from green to yellow, which indicated glucose fermentation while no colour change indicated a non-glucose fermentation.

iv. Fructose fermentation

Inocula from 24 hours bacterial cultures were transferred aseptically to sterile tubes of phenol red fructose broth. The inoculated tubes were incubated at 37 ^{o}C for 24 hours and observed for colour change. A colour change from red to yellow indicated a positive reaction (Baydaa, 2018).

3.4.12 Haemolysis production

Inocula from 24 hours bacterial cultures were inoculated on blood agar plates. The plates were incubated at 37 ^{o}C for 24 hours and observed for the presence of haemolysis. Beta-haemolysis showed complete lysis of red blood cells surrounding the colonies. Alpha-haemolysis showed greenish discolouration of red blood cells surrounding the colonies while gamma-haemolysis showed slight discolouration in the medium (Fernando, 2020)

3. 5 Standardization of Bacteria

The method of National Institute for Pharmaceutical Research and Development (NIPRD) (2018) was employed in standardizing the bacteria. Five milliliter of sterile nutrient broth was dispensed into test tubes and the test tubes were inoculated with

different isolates. They were incubated for 24 hours at 37 ^{o}C. A measured quantity (0.2 mL) of the overnight culture of each bacterium was dispensed into 20 mL of sterile nutrient broth and incubated for 5 hours at 37 ^{o}C to standardize the culture to 10^{6} cfu/mL. The absorbance of 5 hours culture was determined using spectrophotometer. The range of the readings includes 0.07 to 0.10 at a wavelength of 600 nm. The standardized culture was used for antibacterial assay.

3.6 Antibacterial Screening of Crude Extract and Fractions

The antimicrobial activity of the extract and fractions (ethanol (E) extract, n-hexane (NH), ethyl acetate (EC) and aqueous (A)) of *Eudrilus eugeniae* at 40 mg/mL were assayed on the standard test organisms using agar diffusion method as described by Clinical and Laboratory Standard Institute, (2016)**.** Mueller Hinton agar was prepared according to manufacturer's description, sterilized, dispensed on petri dishes and allowed to solidify. Forty milligrams per milliliter (40 mg/mL) was obtained by reconstituting 0.2 g of each extract in 5 mL of sterile distilled water for the polar extract (aqueous) while for the non-polar and mid polar extracts (hexane, ethyl acetate and ethanol) the extracts were first homogenized with 1 m/L of its solvent and then added to 4 mL of sterile distilled water. For homogeneity, the extracts were stirred and agitated vigorously. The standardized test bacteria were seeded on the surface of Mueller Hinton plates, each on different plate aseptically using sterile inoculating loop. Using sterile cork borer (6 mm radius), 4 wells were bored on each plate aseptically. The reconstituted extracts (0.2 mL each, containing 8mg of the extract) were dispensed into the wells, each per well in a plate aseptically using sterile syringe and needle. The plates were prepared in triplicates. Amoxicillin, Clavulanate Potassium (0.05 mg/mL) was used *as* the positive control and the solvents were used as negative controls on different plates. The plates were incubated at 37 °C, for 24 hours. The antibacterial activity was

determined by measuring the diameter of zone of inhibition around the wells in millimeter. Control plates which include organism viability control (OVC), medium sterility control (MSC), extract sterility control (ESC) were made in parallel and observed. The antibacterial activity of the earthworm extracts were compared with that of the Amoxicillin and Clavulanate Potassium (0.05 mg/mL). The negative control plate was observed.

3.7 Biosynthesis of Silver Nanoparticle Using Extract and Fractions of *Eudrilus eugeniae*

Aqueous solution of silver nitrate (2 mM) was prepared by dissolving 0.0358 g of silver nitrate ($AgNO_3$) in 100 mL of sterile distilled water. One gram of ethanol extract was dissolve in 100 mL of distilled water (10 mg/mL). Forty milliliters of the $AgNO_3$ solution was added to 10 mL of ethanol extract solution of *E. eugeniae*. Fifty milliliters of silver nitrate solution was added to 10 mL of ethanol extract solution and were stirred thoroughly using magnetic stirrer at room temperature. The pH of the solution was adjusted to 12 using NaOH solution and the mixture was exposed to direct sunlight for 15 minutes. The same procedure was carried out for the synthesis of silver nanoparticles using ethyl acetate, n-hexane and aqueous fractions respectively (Jaganathan *et al.*, 2016).

3.7.1 Characterization of the synthesized silver nanoparticles

Characterization techniques were adopted from Juan *et al.* (2020). The silver nanoparticle was characterized as follows;

(I) UV-Visible (UV-Vis) Spectrometry was used to identify, monitor the stability, characterize and analyze the synthesized AgNPs. It uses a wavelength between 200 to 800 nm and is able to identify nanoparticle with size range of 2 to100 nm. It is done by placing the cuvette containing the sample on the spectrophotometer and measuring the

intensity of light that passes through the sample with respect to the intensity of light through a reference sample or blank

(ii) Scanning Electron Microscopy (SEM) allow surface and morphological characterization at both nanometer and micrometer scale. It was used to determine the shape and size of the synthesized AgNPs. A little quantity of AgNPs was used in this experiment. Using one gloved hand, the sample was loaded on a sample holder which held the sample in rigid manner to avoid vibrations. It was positioned in a way that the top of the sample was on the same level with the top of the sample holder. The sample was secured in the holder using set screws. The vacuum chamber was vented by letting air through it. Then the sample holder was inserted into the microscope. The scanning electron microscope relied on the detection of high energy electrons emitted from the surface of the sample after being exposed to a highly focused beam of electrons from an electron gun. These beams of electrons were focused on the small spot on the sample surface using SEM objective lens. The electrons in the beam interacted with the sample, producing various signals that were used to obtain information about the surface topography and composition of the sample. Variables like the accelerating voltage used, size of aperture employed and the distance between the specimen and the electron gun were optimized to achieve the best quality image

(iii) X-ray diffraction (XRD) analysis is a technique used to determine the crystallographic structure of the silver nanoparticles. It was done by irradiating the sample with incident x-rays and then measuring the intensities and scattering angles of the x-rays that leave the sample (Armr *et al.*, 2020)

(iv) Fourier transformed Infrared Spectroscopy (FTIR) allowed the identification of all the organic functional groups and determined which of them are attached to AgNPs'

surface. The procedure involves placing AgNPs sample in the FTIR spectrometer. The spectrometer directs beams of IR at the sample and measures the quantity of the beam and the frequencies the sample absorbs the infrared light.

(v) Energy dispersive x-ray spectroscopy (EDS) is a chemical microanalysis technique used in conjunction with scanning electron microscope (SEM). Energy dispersive x-ray spectroscopy detector determines different elements within the sample.

Procedure; The prepared sample with thickness of 0.1µ, flat and polished was kept in dry environment to lessen any built up contamination on the surface before the analysis. A qualitative analysis was carried out to determine different elements within the sample and quantitative analysis was conducted by measuring x-ray intensities of each element in the sample

3.7.2 Antibacterial screening of synthesized silver nanoparticles

The antimicrobial activity of the synthesized AgNPs was determined by an agar-diffusion method (Clinical and Laboratory Standards Institute, 2016). Mueller Hinton agar was prepared according to manufacturer's description, sterilized, dispensed on petri dishes and allowed to solidify. The standardized test organisms were seeded on the surface of the Mueller Hinton plates, each on one plate using sterile inoculating loop. Using sterile cork borer, one well was bored on each plate aseptically (6 mm in diameter). The synthesized silver nanoparticle (0.2 mL) was dispensed into each well using sterile syringe and needle. The plates were prepared in triplicates Amoxicillin, Clavulanate Potassium (0.05 mg/mL) was used *as* the positive control and silver nitrate solution was used as negative control on different plate. The plates were incubated at 37 °C, for 24 hours. The antibacterial activity was determined by measuring the diameter of zone of inhibition around the wells in millimeter. Control plates which include organism viability control (OVC), medium sterility control (MSC), silver nitrate

solution sterility control (SSC) and extract sterility control (ESC) were made in parallel and observed. The antibacterial activity of AgNPs was compared with that of the Amoxicillin**,** Clavulanate Potassium (0.05 mg/mL). The negative control plate was observed.

3.7.3 Determination of minimum inhibitory concentration of the synthesized silver nanoparticles

The minimum inhibitory concentration (MIC) of the synthesized silver nanoparticle (AgNP) was determined against the test organisms according to the method of So-Yeon *et al.* (2018). It was carried out by varying the concentration of the synthesized AgNPs by means of double fold serial dilution (40 mg/mL, 20 mg/mL, 10 mg/mL, 5 mg/mL, 2.5 mg/mL, 1.25 mg/mL). Five milliliter of synthesized AgNPs was dispensed into test tubes containing 5 mL of sterile nutrient broth each using sterile syringe and needle, the test tubes were serially diluted with 10 mL of sterile distilled water. They were inoculated with a loopful of the standardized test organism using a sterile wire loop. The dilution was carried out for all the standardized test organisms. Incubation was done at 37 ^{o}C for 24 hours. The MIC of the extract was determined by observing the lowest concentration of the extract that inhibited growth of each organism and was incubated at 37 ^{o}C for 24 hours. This procedure was carried out for all the test organisms. Presence of growth after the period of incubation was interpreted as bacteriostatic while the absence of growth is bactericidal.

3.8 Evaluation of Wound Healing Activity of Synthesized Silver Nanoparticles

The method by Wubant *et al*., (2018) was used for the topical toxicological studies.

3.8.1 Experimental animals

A total of fifty (50) healthy Wister rats of both sexes, weighing 100 to 150 g were obtained from animal farm in llorin, Kwara State, Nigeria and were used for this study.

The rats were housed in plastic cages bedded with dry clean wood shaving, maintained at a temperature of (25±2) ^{o}C and observed under 12 hours light/dark cycle of the prevailing time period in a well-ventilated room and allowed to acclimatize for 2 weeks before the commencement of the experiment. They were fed with standard commercial diet (rat pellet) and water *ad- libitum*. The animal house was cleaned and disinfected regularly. The soiled base wood shavings were replaced often. The feed and water containers were washed regularly. The animals were cared for in accordance with the guidelines for the care and the use of laboratory animals of the Institute for Laboratory Animal Research Council, National Research Council, USA (2011).

3.8.2 Ointment formulation

The simple ointment used for treating the experimental animals was produced by mixing the synthesized silver nanoparticles (5 mL) with petroleum jelly (1 g) and 5 g of white soft paraffin until an even mixture was obtained (Dons and Soosairaj, 2018).

3.8.3 Experimental design

After an acclimatized period of two weeks, the rats were divided into ten (10) groups each consisting of five (5) animals in accordance with the treatment model and the experiment was undertaken for a period of 14 days (two weeks) with the following groups of animals.

Group1:- Diabetes + Wound + *Streptococcus pyogenes* + Ointment

Group2:- Diabetes + Wound + *Escherichia coli* + Ointment

Group3:- Diabetes + Wound + *Klebsiella pneumoniae* + Ointment

Group 4:- Diabetes + Wound + *Staphylococcus aureus* + Ointment

Group 5:- Diabetes + Wound + *Pseudomonas aeruginosa* + Ointment

Group 6:- Diabetes + Wound + Mixed organisms + Ointment

Group 7:- Diabetes + Wound (Negative control)

Group 8:- Wound only (Positive control)

Group 9:- Wound + Ointment

Group10:- Shaved skin + Ointment

3.8.4 Diabetes induction

Alloxan monohydrate is a diabetogenic agent. The required dose of alloxan monohydrate to induce experimental diabetes intraperitoneally in the experimental rat at a standard dose of 150 mg/kg was ascertained by using the formula shown below

Required dose = $\frac{Weight\ of\ animals\ (g)}{1000\ g}$ x Standard dose (mg) (3.1)

Groups 1 to 7 were starved overnight, blood glucose level taken and injected with alloxan monohydrate via intraperitoneal route. After 3 days of injecting the rats, symptoms such as fatigue, blurred vision, frequent urination which attracted flies to the cages and high blood glucose level (>250 mg/dL) indicated that the rats are diabetic.

3.8.5 Wound induction model

The animals (groups1-10) were anesthetized using 3 % isoflurane before wound induction. After wound area sterilization with 70 % alcohol, the dorsal fur of the animals were shaved using shaving blades and the anticipated area of the wound to be created was outlined on the back of the animals on the dorsal thoracic region 1cm away from the vertebral column . Full thickness circular excision wounds were created along the markings using toothed forceps, scalpel, blades and scissors (in groups 1-9). Hemostasis was achieved by blotting the wound with cotton swab soaked in normal saline and the entire wounds were left open. The wound was allowed to establish for 24 hours. Subsequent infection of the wound with the test organisms was done for groups 1 to 5 while group six (6) was infected with a mixture of the test isolates. Groups 7 and 8 were the negative and positive control respectively while group 9 served as the extract control. Group 10 (shaved skin and ointment) is to check the toxicity of the ointment on

the skin of the rats. The wounds were left for four days for the infection to establish after which physical observations such as smelly liquid draining from the wound, swollen wound and rapidly spreading of wound indicated the wound rotting or necrotic phase. After 48 hours of infection with the respective organisms, treatment of infected wounds (groups 1-6) commenced with ointment. Animals in group 9 were also treated while the ointment was applied on the skin of animals in group 10. Wound contractions were measured for 14 days at interval of 2 days. The wound contraction was measured using a white thread placed on the wound area measuring from one end to the other end of wound, then placed on a meter rule to determine the size of wound closure.

3.8.6 Wound contraction measurement

Wound contraction was calculated as a percentage of the original wound size throughout the experiment. The wound closure rate was assessed by tracing the wound on days 2, 4, 6, 8, 10, 12, and 14 using a meter rule. The evaluated surface area was used to calculate the percentage of wound contraction, taking initial size of the wound as 100 % as shown below:

$$\% \text{ Wound closure} = \frac{(\text{wound area on 1st day} - \text{Wound area on day (n)})}{\text{Wound area on 1st day}} \times 100 \qquad (3.2)$$

Where n is number of days

3. 8 .7 Histopathological Studies

The parts of the skin that were treated were collected for histopathological studies after sacrificing the rats. The skin was washed in normal saline and fixed immediately in 10 % formol saline for a period of 24 hours. Grossing was achieved by selection of the skin to be processed. The skin was placed on tissue cassette alongside the identification number. The selected skin tissues were processed using automatic tissue processor,

which involved four major stages; fixation, dehydration, clearing and impregnation. An embedding machine was used to dispense wax into an embedding mould, unto which the processed tissues and tissue cassettes are placed and allowed to solidify. The solidified tissue in the cassette was placed in microtome and tiny sections were cut at 5 microns. The tissue sections were flooded on a heated water bath maintained at 3 ^{o}C below melting point of the wax. Tissue sections were picked using microscopic slides angled at about 45 ^{o}C for water to drain and dry. The slide was then placed on hot plate and allowed to fix at maintained temperature of 3 ^{o}C above the melting point of wax. This was done to ensure bond between the tissue and slides and allowed fixing for minimum of 30 minutes. Thereafter, the slides were stained using Harri's haematoxylin and eosin method and allowed to air dry . The dried slides were mounted with Distyrene Plasticizer Xylene (DPX) mountant and cover slips and examined under the microscope. Any alterations compared to the normal structures were registered (Amany and Mona, 2021).

3.9 Statistical Analysis and Data Evaluation

All numeric data generated were expressed as the mean ± standard error of mean (SEM). Comparisons between different groups were performed using analysis of variance (ANOVA Test). The significant difference between control and experimental groups were assessed using Duncan's multiple range test (DMRT) using SPSS version 19.

CHAPTER FOUR

4.0 RESULTS AND DISCUSSION

4.1 Results

4.1.1 Description of extract and fractions of *Eudrilus eugeniae*

Table 4.1 shows the physical appearance and percentage recovery of *Eudrilus eugenia* crude ethanol extract (E), ethyl acetate (EC), n-hexane (NH), and aqueous (A) fractions. Aqueous fraction had the highest percentage recovery (51.56 %) followed by ethanol crude extract (21 %), ethyl acetate extract (19.69 %) while n-hexane extract had the least percentage recovery (14.38 %).

Table 4.1: Physical Characteristics of Crude Extract and Fractions of *Eudrilus. eugeniae*

Extract/fractions	Code	Colour	Appearance	Weight (g)	% Recovery
Ethanol	E	Deep brown	Gummy mix	32	21
Ethylacetate	EC	Deep brown	Gummy mix	6.3	19.69
N-Hexane	NH	Brown	Sticky	4.6	14.38
Aqueous	A	Brown	Sticky	16.5	51.56

E: Ethanol extract EC:Ethylacetate fraction

NH: N-hexane fraction , A:Aqueous residual fraction, %::Percentage

$$\textbf{Percentage recovery} = \frac{\textbf{weight of the extract obtained}}{\textbf{Weight of the pulverized } E.\ eugeniae} \times \frac{100}{1} \qquad (4.1)$$

4.1.2 Bioactive components of crude *Eudrilus eugeniae* extract and fractions

4.1.2.1 *Qualitative bioactive components of crude extract and fractions of Eudrilus eugeniae*

The bioactive components present in the crude ethanol extract (E), ethyl acetate (EC), n-hexane (NH) and aqueous (A) fractions are shown in Table 4.2. Steroids and proteins were present in all the extract and fractions of *E. eugeniae* while alkaloids, tannins and flavonoids were absent. Saponins were present in crude ethanol and aqueous fraction while phenols were present only in aqueous fraction. Also, terpenoids were present only in ethanol extract.

Table 4.2: Qualitative Bioactive Components of *Eudrilus. eugeniae*

Bioactive components	Extracts			
	E	EC	NH	A
Saponins	+	ND	ND	++
Alkaloids	ND	ND	ND	ND
Phenols	ND	ND	ND	+
Tannins	ND	ND	ND	ND
Flavonoids	ND	ND	ND	ND
Steroids	+	+	+	+
Proteins	+	+	+	+
Terpenoids	+	ND	ND	ND

ND: Not detected, +: Present NH: N-Hexane fraction
EC: Ethyl acetate fraction, E; Ethanol extract
A: Aqueous residual fraction

4.1.2.2 *Quantitative bioactive components of Eudrilus. eugeniae*

The quantitative bioactive components of *E. eugeniae* are shown in Table 4.3. The results revealed higher concentration (mg/%) of cyanides (2975.800), phytates (2471.364), moderate amount of phenols (158.084), saponins (100.992) and very low concentration (mg/%) of oxalates (0.3955).

Table 4.3: Quantitative Bioactive Components of *Eudrilus eugeniae*

Components	**Quantity (mg/100g)**
Phenols	158.084
Flavonoids	20.986
Alkaloids	19.198
Cyanide	2975.800
Phytates	2471.364
Oxalates	0.396
Tannins	21.110
Saponins	100.992

4.1.3 Morphological and biochemical identification of bacterial isolates

The identities of the test organisms are summarized in Table 4.4. The organisms isolated were *E. coli, S. aureus, S. pyogenes, K. pneumoniae and P. aeruginosa.*

Table 4.4: Morphological and Biochemical Identification of Isolates

GR	SHAPE	OX	CG	CT	IN	MR	VP	CI	UR	H_2S	Sugar Fermentation				Haemolysis			Suspected Organisms
											L	S	G	F	A	B	Γ	
G-	Rod	-	-	+	+	+	-	-	-	-	+	+	+	-	-	-	-	*Escherichia coli*
G+	Cocci	-	+	+	-	+	+	+	+	-	+	+	+	+	-	+	-	*Staphylococcus aureus*
G+	Cocci	-	-	-	+	+	-	-	-	-	+	+	+	+	-	+	-	*Streptococcus pyogenes*
G-	Rod	-	-	+	-	-	+	+	+	-	+	+	+	+	-	-	-	*Klebsiella pneumonia*
G-	Rod	+	-	+	-	-	-	+	-	-	-	-	-	-	-	-	+	*Pseudomonas aerugenosa*

GR: Gram's reaction, G+: Gram positive, G-: Gram negative, OX: Oxidase, CG: Coagulase, CT: Catalase, IN: Indole, MR: Methyl red, Vogues-Proskauer, CI: Citrate utilization, UR: Urease, H_2S: Hydrogen sulphide production, , L: Lactose sugar fermentation, S: Sucrose sugar fermentation, G: Glucose sugar fermentation, F: Fructose sugar fermentation, α: Alpha haemolysis production, β: Beta heamolysis production, γ: Gamma haemolysis production.

4.1.4 Antibacterial activity of crude *Eudrilus eugeniae* extract and fractions against test isolates

The antibacterial activity of the crude *Eudrilus eugeniae* extract and fractions against test isolates at 20 mg/mL of the extract, are shown in Table 4.5. The zones of inhibition produced by ethanol extract were between 1.67±1.52 mm and 3.00±1.00 mm against *K. pneumoniae, P. aeruginosa, S. pyogenes*, *S. aureus* and *E. coli* respectively while that of ethyl acetate fraction were between 1.33±0.58 mm and 3.67±0.58 mm against *K. pneumoniae S. aureus*, *S. pyogenes, E. coli* and *S. aureus* respectively. The zones of inhibition produced by aqueous residual fraction were between 1.00±1.00 mm and 5.00±1.00 mm against *K. pneumoniae, S. pyogenes, S. aureus, E. coli* respectively. The n-hexane (NH) fraction zones of inhibition were between 0.33±0.58 mm and 2.33±0.58 mm against *P. aeruginosa, S. pyogenes, S. aureus* and *E. coli* respectively

Table 4.5: Antibacterial Activities of Crude Extract and Fractions of *Eudrilus eugeniae* at 20 mg/mL

	Zone of inhibition (mm)				
Extract	*Escherichia coli*	*Staphylococcus aureus*	*Streptococcus pyogenes*	*Pseudomonas aeruginosa*	*Klebsiella pneumoniae*
E	3.00±1.00^{ab}	2.67±0.58^{a}	2.33±2.08^{a}	2.33±1.53^{a}	1.67±1.53^{a}
EC	3.00±1.00^{ab}	3.67±0.58^{ab}	3.33±0.58^{ab}	2.33±0.58^{a}	1.33±0.58^{a}
A	5.00±1.00^{a}	2.33±0.58^{a}	2.00±1.00^{a}	2.33±1.53^{a}	1.00±1.00^{a}
NH	2.33±0.58^{b}	0.67±0.57^{b}	0.33±0.58^{b}	0.33±0.58^{ab}	0.00±0.00 ^{ab}
Control	20.00±1.00^{c}	21.00±1.50^{c}	21.00±1.50^{c}	20.00±1.10^{c}	20.00±0.00^{b}

Values are zones of inhibition mean ± standard error of mean of triplicate determinations. Values with the same superscript in the same column are not significantly different at $p \leq 0.05$. E; Ethanol extract, EC: Ethyl acetate fraction, A: aqueous residual fraction, NH: N-hexane fraction, control: Amoxicillin and Clavulanate Potassium, Mg/ml: milligram per milliliter

4.1. 5 Characteristics of *Eudrilus eugeniae* extract mediated silver nanoparticles

Silver nanoparticles in the solution appeared as dark brown colour (plate III). The UV-visible spectra of biosynthesized silver nanoparticle (AgNPs) are shown in Fig. 4.1 and indicated the absorbance peak at 408 nm. The SEM micrograph of the particle (Fig.4. 2)

showed spherical and less aggregated morphology with size ranging from 10-50 nm. The EDX (Fig. 4. 3) showed the elemental composition of the particle and their atomic numbers while the XRD (Fig.4. 4) displayed the crystalline nature of the AgNPs. The FTIR (Fig.4. 5) showed the organic functional groups present in the extract. The transmission peaks of the particles were at 3488, 2922, 2858, 1736, 1640.1461, 1349, 1297, 1248, 1092, 946, 846 and 723 cm^{-1} respectively. The distinct peak at 2858 cm^{-1} corresponded to the bonding vibration of alkanes (C-H) while the peak at 1736 cm^{-1} is an indicative of C=O stretch of esters. Furthermore, the bend at 1092 cm^{-1} resulted from the C-O bending vibration of alcohol functional group.

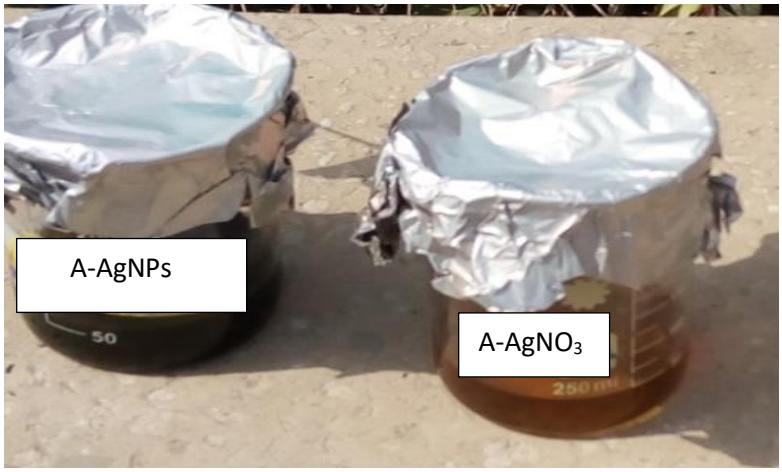
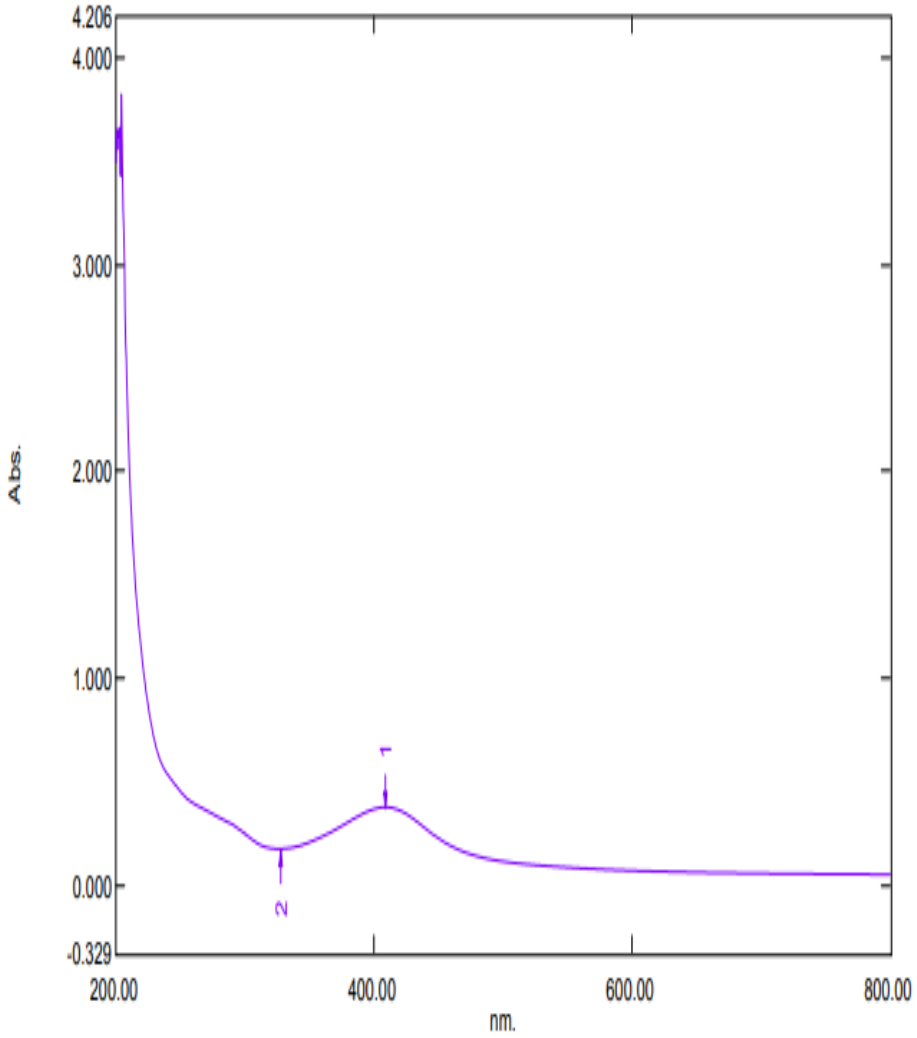


Plate III: Synthesized Silver Nanoparticles



Wave length (nm)

Figure 4. 1: Ultra Violet-Visible Spectra of Aqueous Residual Fraction Mediated Silver Nanoparticles

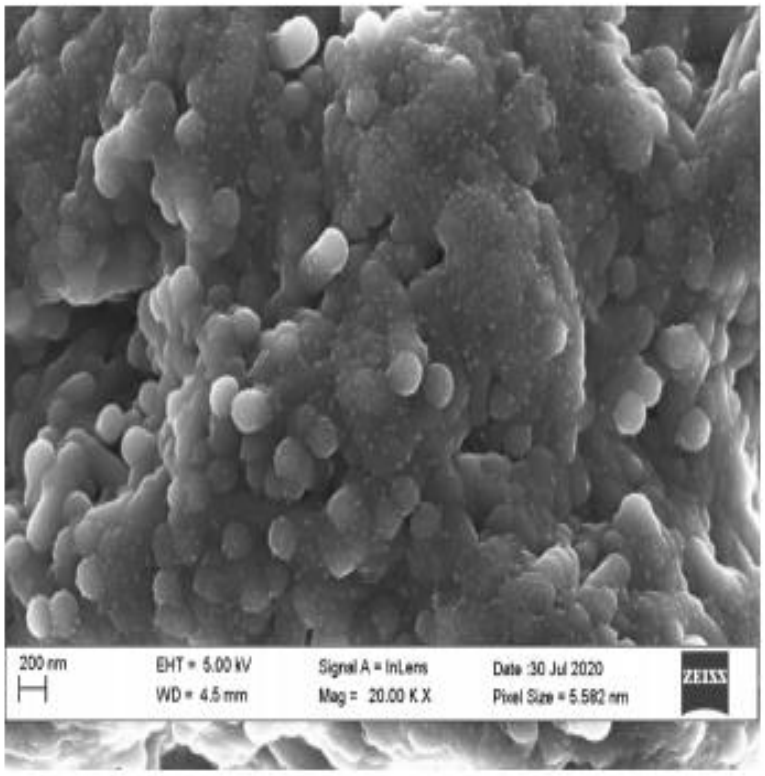


Figure **4.2: Scanning Electron Micrograph of *Eudrilus eugeniae* Aqueous Residue Fraction Mediated- Silver Nanoparticles**

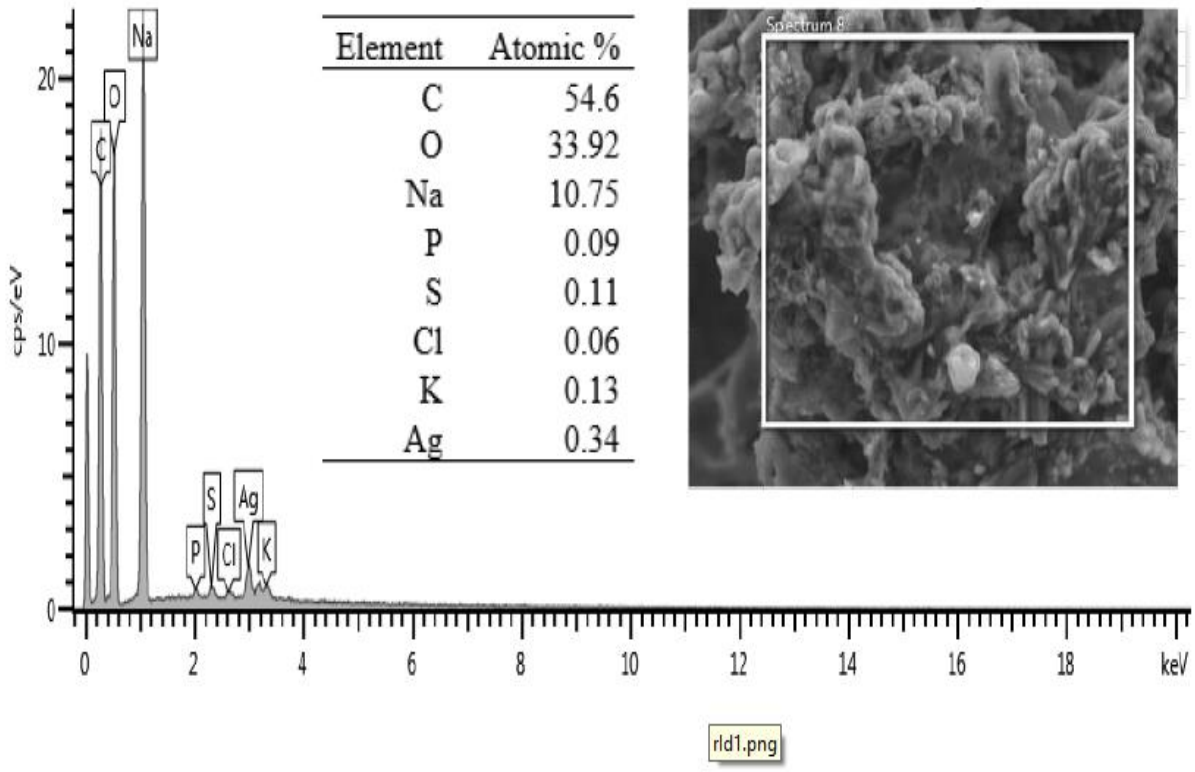


Figure 4.3: Energy Dispersive Pattern of *Eudrilus eugeniae* Aqueous Residual Fraction Mediated Silver Nanoparticle

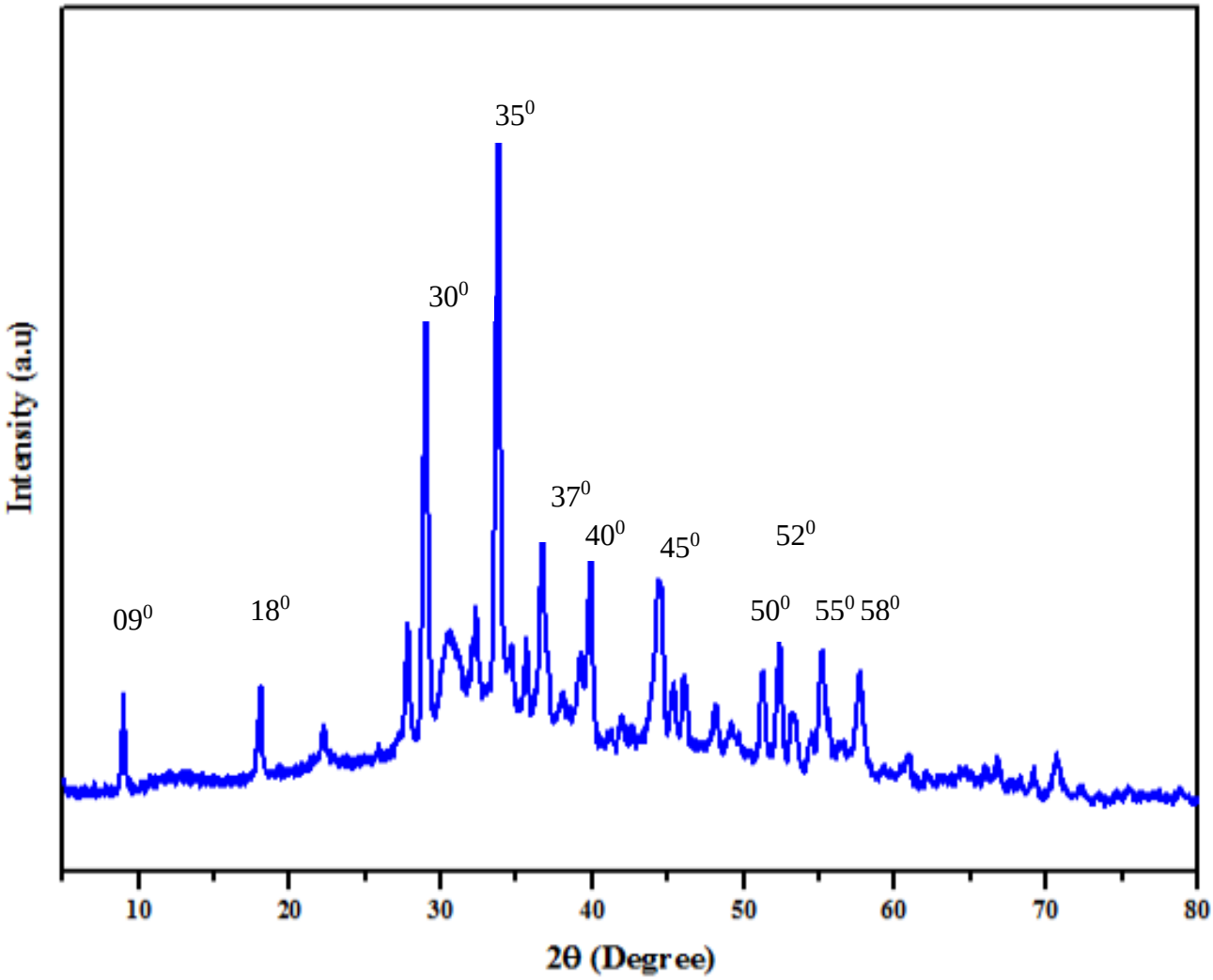


Figure 4.4: X-ray Diffraction Pattern of *Eudrilus eugeniae* Aqueous Fraction Mediated Silver Nanoparticles

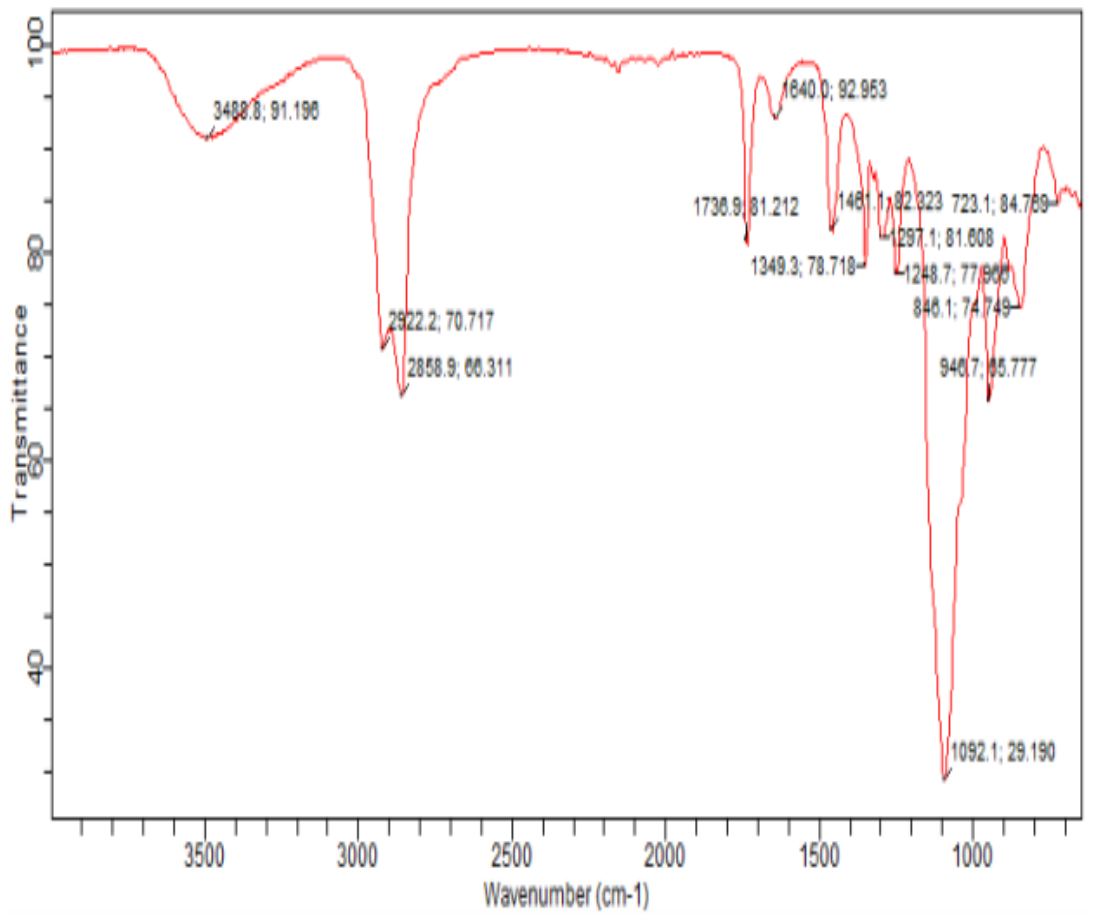


Figure 4.5: Fourier-Transform Infrared Spectrum of Aqueous Residual Fraction Mediated Silver Nanoparticles

4.1.6 Antibacterial activity of *Eudrilus eugeniae crude* extract, fractions and extract mediated silver nanoparticles against test isolates

Table 4.6 shows the antibacterial activity of *Eudrilus eugeniae* crude extract and fractions against test isolates at 40 mg/mL. The zones of inhibition produced by crude ethanol extract were between 0.33±0.58 mm and 2.00±1.00 mm against *S. pyogenes, K. pneumonia*e, *S. aureus* and *E. coli.* Zones of inhibition produced by ethyl acetate faction were between 1.00±1.00 mm and 3.33±1.16 mm against *S. pyogenes,, E. coli, P. aeruginosa, K. pneumonia*e and *S. aureus* while the zones of inhibition produced by aqueous residual fraction were 0.67±1.16 mm and 2.67±1.53 mm against *S. pyogenes, K. pneumoniae*, *S. aureus, P. aeruginosa,* and *E. coli.* Zone of inhibition produced by n-hexane was 0.67±0.58 mm against *S. aureus*. The antibacterial activity of *Eudilus eugeniae* aqueous residual fraction mediated silver nanoparticles (AAgNPs) against test isolates at 8 mg/mL (at 1:4 and 1:5 dilution) are shown in Table 4.6. The zones of inhibition produced by AAgNPs (at 1:4dilution) were between 6.00±1.00 mm and 17.67±0.58 mm against *Streptococcus pyogenes, Staphylocccus aureus, Klebsiella pneumoniae, Escherichia coli and Psuedomonas aeruginosa* respectively while at 1.5 dilution the zones of inhibition were between 7.67±1.52 mm and 15.33.±1.16 mm against *S. aureus,, S. pyogenes E. coli, K. pneumoniae and P. aeruginosa* respectively.

Table 4.6: Antibacterial activities of crude extract and fractions of *Eudrilus eugeniae* and extract- mediated silver nanoparticles at 40 mg/mL

	Zone of inhibition (mm)				
Extract	***Escherichia coli***	***Staphylococcus aureus***	***Streptococcus pyogenes***	***Pseudomonas aeruginosa***	***Klebsiella pneumonia***
E	2.00 ± 1.00^{a}	2.00 ± 1.00^{a}	0.33 ± 0.58^{a}	0.00 ± 0.00^{a}	1.33 ± 1.16^{a}
EC	2.00 ± 1.00^{a}	3.33 ± 1.16^{ab}	1.00 ± 1.00^{ab}	2.67 ± 0.58^{ab}	2.67 ± 1.53^{ab}
A	2.67 ± 1.53^{a}	2.00 ± 1.00^{a}	0.67 ± 1.16^{a}	2.00 ± 1.73^{ab}	1.00 ± 1.00^{a}
NH	0.00 ± 0.00^{ab}	0.67 ± 0.58^{b}	0.00 ± 0.00^{a}	0.00 ± 0.00^{a}	0.00 ± 0.00^{a}
(AF1:4 $AgNO_3$)	15.33 ± 0.58^{b}	7.33 ± 1.53^{c}	6.00 ± 1.00^{c}	17.67 ± 0.58^{b}	7.667 ± 1.53^{b}
(AF1:5AgNO3)	12.33 ± 2.08^{c}	7.67 ± 1.52^{c}	9.33 ± 2.52^{b}	15.33 ± 1.16^{c}	14.67 ± 0.58^{c}
Control	21.50 ± 1.00^{d}	22.00 ± 1.50^{d}	21.00 ± 0.10^{d}	19.00 ± 0.00^{d}	22.00 ± 0.10^{d}

Values are zones of inhibition mean ± standard error of mean of triplicate determinations. Values with the same superscript in the same column are not significantly different at $p\leq0.05$. E; Ethanol extract, EC: Ethyl acetate fraction, A: aqueous residual fraction, NH: N-hexane, Mg/mL: milligram per milliliter, AF1:4 $AgNO_3$: 10 mL of aqueous residual fraction to 40 mL of silver nitrate, AF1:5 $AgNO_3$: 10 mL of aqueous residual fraction to 50 mL of silver nitrate

4.1.7 Minimum inhibitory concentration of synthesized silver nanoparticles against isolates

The minimum inhibitory concentrations (MIC) of synthesized silver nanoparticle against test organisms are shown in Table 4.8. The MIC of the synthesized nanoparticles against *P. aeruginosa, S. pyrogenes* and *S. aureus were at* 1.25 mg/mL, 5 mg/mL and 10 mg/mL respectively while for *E. coli and K. pneumonia*e was at 2.5 mg/mL .

Table 4.7: Minimum inhibitory Concentration of Synthesized Silver Nanoparticles

Isolates	**Concentrations (mg/mL)**						**MIC**
	40	**20**	**10**	**5**	**2.5**	**1.25**	
Escherichia coli	+	+	+	+	-	-	2.5
Staphylococcus aureus	+	+	-	-	-	-	10
Streptococcus pyrogenes	+	+	+	-	-	-	5.0
Pseudomonas aeruginosa	+	+	+	+	+	-	1.25
Klebsiella pneumoniae	+	+	+	+	-	-	2.5

+: Activity, -: No activity

, mg/mL: milligram per milliliter

MIC- Minimum inhibitory concentration

4.1.8 Wound healing activity of synthesized silver nanoparticles

4.1.8.1 *Diabetes mellitus in rats*

Rats induced with 150 mg/kg body weight of alloxan became diabetic after 3 days with blood sugar level ≥250 mg/dL. The diabetic rats were fatigued and urinated often which attracted flies to the cages.

4.1.8.2 *Wound healing activity of synthesized silver nanoparticles in rats*

The infected wound on the skin of animals were found to be smelly, rapidly spreading and swollen with discharges. The initial diameter of the wound taken for all the 9 groups (Table 4.9) were between 1.00± 0.44 mm and 1.23±0.15 mm at Day 0. Treatment of the wound with AgNPs ointment reduced the diameter of the wound to 0.20±0.1 and 1.47±0.21 mm from Day 8 to 14. There was total wound closure at day 14 in all the treatment groups as compared to the negative group (diabetes + wound). There was significant ($p<0.05$) wound closure observed from day 8 to 14 in all the treated groups (grroups1-6) as compared to group 7 (wound + diabetes) which had the highest wound size. Groups 9 (wound + extract) and 8 (wound only) had the lowest size of the wound when compared to the treated groups.

4.1.8.3 *Microbial growth in mixed group*

There was heavy growth on plate with mixed group of organisms characterized by different colonies similar to those cultured earlier from patients with diabetic foot infection. The Gram reactions showed Gram negative rod and Gram positive cocci, characteristics similar to *E. coli, S. aureus,, S. pyogenes, P. aeruginosa and K. pneumoniae.*

4.1.8.4 *Safety of the Eudrilus eugeniae extract mediated silver nanoparticles*

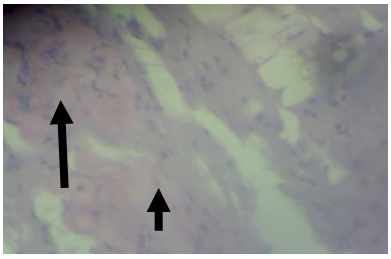
The application of the ointment on the skin of the rats in group 10 did not show any sign of inflammation and edema. There was also neither mortality nor any sign of toxicity (irritation, allergies and rashes) observed on the skin of the rats.

Table 4.8: Wound Healing Activity of Extract Mediated Silver Nanoparticles

Groups	Diameter of (mm) measured over various time (Days) ±SD							
	0	**2**	**4**	**6**	**8**	**10**	**12**	**14**
1	1.20±0.25[a]	1.20±0.15[a]	1.21±0.25[a]	0.93±0.23[a]	0.91±0.25[a]	0.90±0.36[a]	0.73±0.25[a]	0.77[a]±0.55[a]
2	1.13±0.20[a]	1.20±0.27[a]	1.0±0.25[a]	0.93±0.21[a]	0.77±0.15[a]	0.37±0.06[d]	0.30±0.00[d]	0.30±0.10[e]
3	1.20±0.04[a]	1.17±0.40[a]	1.13±0.46[a]	0.93±0.55[a]	0.80±0.35[b]	0.63±0.21[c]	0.57±0.23[b]	0.43±0.25[c]
4	1.03±0.31[a]	1.01±0.35[a]	0.87±0.23[a]	0.87±0.23[a]	0.70±0.30[c]	0.40±0.17[d]	0.40±0.10[c]	0.30±0.10[d]
5	1.10±0.20[a]	1.07±0.15[a]	0.80±0.20[a]	0.73±0.25[a]	0.70±0.27[c]	0.57±0.12[d]	0.40±0.17[c]	0.33±0.15[d]
6	1.03±0.42[a]	0.90±0.36[a]	0.83±0.40[a]	0.67±0.46[a]	0.65±0.38[a]	0.57±0.46[d]	0.33±0.32[d]	0.33±0.32[d]
7	1.23±0.15[a]	1.23±0.10[a]	1.21±0.10[a]	1.31±0.23[b]	1.33±0.23[c]	1.35±0.29[b]	1.36±0.23[b]	1.47±0.21[b]
8	1.00±0.44[a]	0.93±0.49[a]	0.83±0.49[a]	0.60±0.35[a]	0.57±0.46[c]	0.500±0.35[c]	0.40±0.27[c]	0.27±0.21[d]
9	1.10±0.30[a]	0.97±0.25[a]	0.97±0.25[a]	0.87±0.15[a]	0.63±0.12[c]	0.61±0.15[c]	0.43±0.12[c]	0.20±0.10[d]

4.1.8.5 *Effect of Eudrilus eugeniae extract - mediated silver nanoparticles in rats*

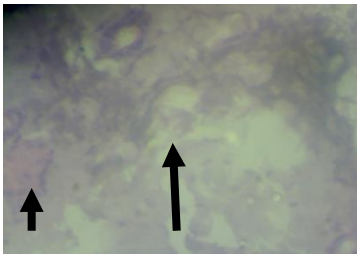
Plates IV and V showed the histology of the skin of the control rats and those exposed to AgNPs for 14days. The results obtained from the study revealed that all the tissues possessed fibroblast, collagen and inflammatory cells while blood vessel was present in all the plates except plates g and b. All the plates possessed epithelial cells except f and h plates.



Wound + Diabetes

(a)

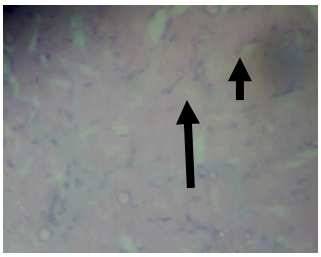
Thinner epithelial layer with less and loosely packed collagen, fibro blast, blood vessels and high infl amatory cells



Wound + Ointment

(b)

Complete epithelialization of of the treated tissue, with regu larly arranged collagen, more fibroblast and blood vessels with less inflammatory cells.



Wound only

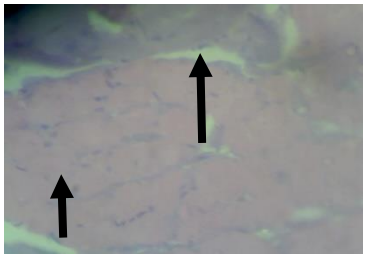
(c)

Loosely packed colla gen, fibroblast with with irregular epithelializ ation and mild inflamma tory cells.

Diabetes + wound + EC + ointment

(d)

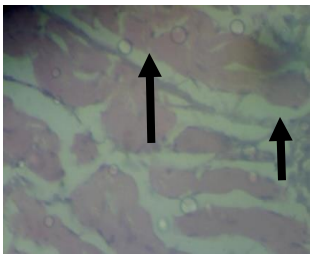
Complete epithelialization of the treated tissue with regularly arra nged collagen, more fibroblast and blood vessels with less inflammatory cells



Diabetes + wound + SP + oint

(e)

New blood vessel forma tion with dense collagen deposition, fibroblast and blood vessels with mild inflammatory cells



Diabetes + wound + KP +oint

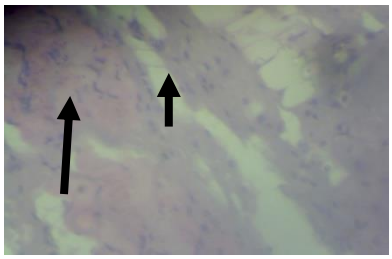
(f)

Complete epithelialization of the treated tissue with regularly arranged collag agen, more fibroblast and blood vessels with less inflammatory cells

EC: *Escherichia coli,* SP: *Streptococcus pyogenes* KP: *Klebsiella pneumoniae*

Oint: Ointment, Long arrow: Connective tissue, Short arrow: Fibroblast.

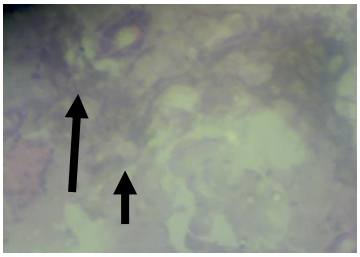
Plate V: Photomicograph of the Skin Section of Infected and Uninfected Rats WoundsTreated with *Eudrilus eugeniae* Extract-mediated Silver Nanoparticles



Wound + Diabetes

(a)

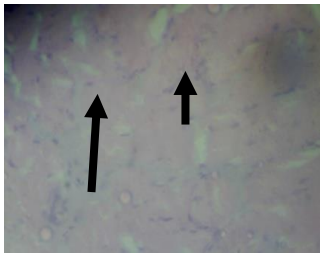
Thinner epithelial layer with less and loosely packed collagen, fibroblast, blood vessels and and high inflammatory cells



Wound + Ointment

(b)

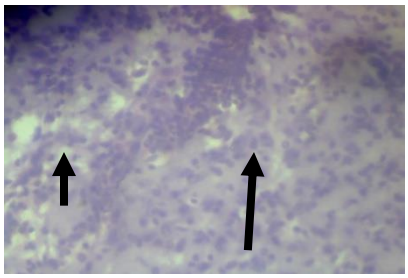
Complete epithelialization of the treated tissue, with regularly arranged collagen, more fibroblast and blood vessel with less inflammatory cells



Wound only

(c)

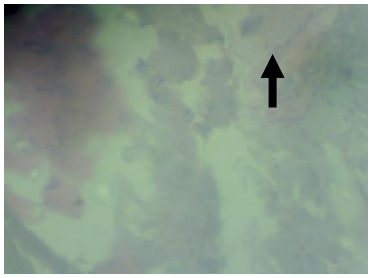
Loosely packed collagen fibroblast with irregular epithelialization and mild inflammatory cells



Diabetes + wound + SA + ointment + mixed+ oint

(g)

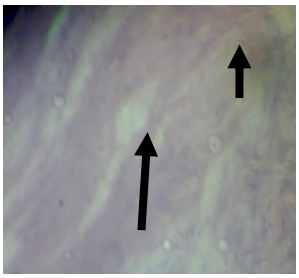
Loosly packed collagen, fibrobl ast with irregular epithelialization and mild inflammatory cells.



Diabetes + wound + PA + oint

(h)

Granulation tissue contains mild collagen, fibroblast, blood capillaries and mild inflammatory cells.



Diabetes + wound

(i)

Thinner epithelial layer with less and loosely packed collagen, fibrob last, blood vessels and high inflammatory cells.

SA: *Staphylococcus aureus,* PA: *Pseudomonas aeruginosa,* Mixed: Mixed organisms, Oint: Ointment, Long arrow: Connective tissue, Short arrow: Fibroblast.

Plate VI: Photomicograph of the Skin Section of Infected and Uninfected Rats Wounds Treated with *Eudrilus eugeniae* Extract-mediated Silver Nanoparticles

4.2 Discussion

In the present study, the investigation revealed that most of the test organisms were oxidase negative (*E. coli, S. aureus, S. pyogenes and K. pneumoniae)*, coagulase negative (*E. coli, P. aeruginosa, S. pyogenes and K. pneumoniae*) and catalase positive (*E. coli, S. aureus, P. aeruginosa and K.pneumoniae*) (Table 4:3). This is similar with the findings of Muhammad *et al*. (2020) who identified similar organisms using the same biochemical tests. Gram staining of test isolates revealed Gram-positive cocci (*S. aureus, and S. Pyogenes*) and Gram negative rods (*E. coli, K. Pneumoniae and P. aeruginosa*). This is similar with the report of Muhammad *et al*. (2020) who used Gram staining to classify the same organisms.

It was observed that saponins, phenols, steroids, proteins and terpenoids were present in the crude extract and fractions of *E. eugeniae* (Table 4.2a). The results coincided with the findings of Mizanur *et al*. (2016), Justin and Njonka (2017) on species of *Amorphophallus* and *Lophira* containing alkaloids, saponins, flavonoids, steroids, terpenoids, proteins, carbohydrates and tannins. In the present study, high concentration of cyanides (2975.800) and phytates (2471.364) (Table 4.3) were observed in dried *Eudrilus eugeniae.* This result contradicts the findings by Ekpa and Sani, (2019) that observed low content of phytates in fruits and James *et al.* (2018) that reported low amount of cyanides in plants. Phytates inhibit the growth of microorganisms by damaging their cell membrane (Zhou *et al.,* 2019) while cyanides in combination with hydrogen inhibit the growth of microorganisms (Abhishek *et al*., 2020). The high concentration of cyanides and phytates in earthworm may be useful for its defense and protection against pathogens in the soil.

Crude ethanol extract (E), ethylacetate (EH) and aqueous (A) fractions of *E. eugeniae* at 20 mg/mL and 40 mg/mL respectively displayed very weak inhibitory effect on *E. coli, S. aureus, S, pyogenes, P. aeruginosa and k. pneumoniae.* The n-hexane fraction was unable to inhibit the growth of all the test isolates at 40 mg/mL. The weak activity of the extracts could be as a result of low concentration of antibacterial components in the extracts and extraction capacity of the solvents. This is in agreement with the report of Ashraf and Bakri. (2018). The production of silver nanoparticles using *Eudrilus eugeniae* aqueous (A) fraction was accompanied by colour change from brown to dark brown (Plate I). Similarly, Jaganathan *et al.* (2016) reported that the silver nanoparticles exhibited colour change from yellow to dark brown. Silver nanoparticles showed dark brown in aqueous solution due to excitation of surface plasmon resonance (Shakeel *et al.,* 2016). The maximum absorbance peak for *E. eugeniae* aqueous fraction mediated silver nanoparticles (AAgNPs) was seen at 408 nm (Fig.4.1) which falls within the range of 391 to 460 nm absorbance characteristics of silver nanoparticles earlier reported by Adelere *et al.* (2017). Concentration and pH that had been identified as factors affecting the yield of silver nanoparticles were optimized. Fourie transform infrared (FTIR) pattern (Fig 4.5) confirmed the bioreduction of Ag^{+} ions to silver nanoparticle which may be due to the reduction by capping material of *E. eugeniae* aqueous extract and revealed the presence of biomolecules which include alkanes, alcohol and ester functional groups. Similarly, Hossam *et al.* (2015) reported that earthworm is rich in protein which can bind to the silver nanoparticles through amino or carboxylic groups.

In the scanning electron (SEM) micrograph (Fig 4.2), spherical and less aggregated morphology of nanoparticles ranging from 10 to 50 nm was observed. Adelere *et al*. (2017) reported the spherical shape of silver nanoparticle while Lateef *et al.* (2016) reported the

synthesis of spherical AgNps in the size range of 3 to50 nm. The XRD peaks (Fig 4.5) were observed at 2θ (30^o, 35^0, 37^0,40^0,45^0,50^0,52^0,55^0 and 58^0) respectively which indicated the crystalline nature of the AgNps. These findings is similar to the observation by Mehta *et al.* (2017) who reported the strong diffraction peaks of AgNps at 2θ value of 32.35^0, 38.29^0, 46.38^0, 54.66^0 and 64.82^0 respectively. The sharpness of the diffraction peaks indicated the crystalline nature of nanoparticles. In this findings, energy dispersive x-ray (EDX) spectrum exhibited signals of silver and other elements from the synthesized nanoparticle. The EDX spectrum indicated weak signals of chlorine, silver, phosphorus Sulphur and potassium. This may be due to the biomolecules in the extract bound to the surface of biosynthesized AgNPs. However, the high presence of signal of carbon, oxygen and sodium may be due to environmental interference during sample preparation on a glass substrate. This finding differs from that of Roua and Sabah, (2021) who reported high presence of silver with moderate amount of carbon and chlorine.

Silver nanoparticles obtained from *E eugeniae* aqueous extract at 40 mg/mL (Table4.7) was able to inhibit the growth *K. pneumoniae, E. coli and P aeruginosa* significantly ($p \leq 0.05$) as against the crude counterpart. This result is in line with the study of Swarnali *et al. (*2020) who reported the antimicrobial effects of biosynthesized silver nanoparticles on Gram negative and Gram positive bacteria.

In the present study, rats induced with diabetes became diabetic after 3days with high blood sugar level (≥250 mg/dL), fatigued and urinated often. Similarly, Shaimaa *et al*., (2021) reported high blood glucose level in diabetic rats. In this study, group 6 of albino rats infected with mixed culture of organisms had confluent growth of organisms that include *S. aureus, K. pneumoniae, E. coli, S. pyogenes and P. aeruginosa* after four days of infection

which were similar to those isolated from patients with diabetic foot infection. This is in line with the observation by Tianhang *et al.,* (2011) who reported the isolation of *S. aureus* and *P. aeruginosa* from dead mice that were wounded and infected with *S. aureus* and *P. aeruginosa.*

In the present study, significant ($P<0.05$) wound closure was observed from day 8-14 in all the treated groups (1-6) (Table 4.9). Group 9 which was treated with ointment only, had the lowest diameter of wound closure and variation was statistically significant ($P\leq0.05$). Shaimaa et al., (2021) made similar observation on the fast wound healing of non diabetic group of rats as compared to the diabetic group. Histology of the treated rats indicated the presence of regularly arranged collagen, fibroblast, new blood vessel,, mild inflammatory cells, dense collagen deposition and complete epithelialization (Plates i-ix). *Eudrilus eugeniae* enhanced formation of granulation tissue deposition of collagen, remodeling of tissue and contraction of wound. Zhen-hen, (2018) made similar observation in the ability of earthworm extract to enhance collagen formation, fibroblast proliferation and antimicrobial activities.

The ointment of *E. eugeniae* aqueous extract mediated silver nanoparticles showed significant ($P< 0.05$) wound healing ability as demonstrated in rats infected with test isolates (*P. aeruginosa, S. aureus*, *S. pyogenes*, *K. pneumoniae* and *E. coli*). This infiltration, blister formation, swollen and exudates exhibited in wounds of rats before treatment disappeared in all the treated groups (1-6) except group 7 (diabetes + wound). Groups treated with extract showed faster rate of wound concentration than group 7. This finding revealed the wound healing ability of *E. eugeniae* aqueous extract mediated silver nanoparticles. This is similar to the observation by Wubante *et al*., (2018) who reported

wound healing ability of the crude extract of leaves of *Acanthus polystachyus* on wounded rats.

The eradication of the colonizing organisms from the infected wounds created a suitable environment for wound healing to take place, thus, the antimicrobial activity showed by the *E. eugeniae* aqueous extract mediated silver nanoparticles in infected wound model revealed the promising potentials of *E. eugeniae* in the management of wound.

The presence of bioactive components (proteins, steroids, terpenoids, phenols and saponins) in aqueous extract of *E. eugeniae* could singly or synergistically contribute to its wound healing activity. Zhen-hen *et al.* (2018) made similar observations in activity of leaves of *Leucaena leucocephala* and concluded that the plant phytoconstituents may be responsible for the wound healing capabilities. The lack of irritation, allergies and rashes as well as mortality in group 10 rats treated with ointment alone indicated that the ointment is relatively safe for topical application.

CHAPTER FIVE

5.0 CONCLUSION AND RECOMMENDATIONS

5.1 Conclusion

Bioactive constituents such as protein steroids, terpenoids, and phenols were present in the crude extract and fractions of *E. eugeniae*. Very high quantities of cyanides and (2975.800) and phytates (2471.364) were detected in the *E. engeniae* powder. The results of the study revealed that crude ethanol, ethyl acetate and aqueous fractions of *E. eugeniae* at 20 mg/mL and 40 mg/mL respectively showed very mild inhibitory effect on the test isolates. Aqueous fraction mediated silver nanoparticles (AAgNPs) at 1.4 dilution displayed marked inhibitory effect against *P. aeruginosa* and *E. coli* while at 1;5 dilution, significant ($p \leq 0.05$) inhibitory effect was displayed on *K. pneumoniae* which was earlier insensitive.

The MIC of AAgNPs against *P. aeruginosa* was at 1.25 mg/mL while it was at 2.5 mg/mL for *K. pneumoniae* and *E. coli*. The extract showed bacteriostatic effect against the test isolates.

Silver nanopanticles using *E. eugeniae* aqueous extract was synthesized by colour change from brown to dark brown. The maximum absorbance was at 408mm. Fourier-transform infrared spectrum (FTIR) indicated the presence of biomolecules such as alkane, alcohol and ester functional groups. The scanning electron micrograph (SEM) of aqueous extract mediated silver nanoparticles revealed spherical and lass aggregated morphology while the XRD peaks were observed at 2Ɵ (30^0, 35^0, 37^0, 40^0, 45^0, 50^0, 52^0, 55^0 and 58^0) respectively, which indicated the crystalline nature of the AgNps.

The extract displayed significant ($P < 0.05$) wound healing activity from day 8-14 in diabetic and normal rats. The histology study revealed the presence of fibroblast, collagen, blood vessels, epithelial and inflammatory cells in groups 1-6 while group 7 (the negative group) showed the presence of thinner epithelial layer with less and loosely packed collagen, fibroblast, blood vessels and high inflammatory cells. The aqueous extract mediated silver nanoparticle is relatively safe for topical application in experimental rats.

5.2 Recommendations

i *Eudrilus eugeniae* mediated silver nanoparticles has wound healing properties. This validates the traditional claims of wound healing property of *Eudrilus eugeniae.*

ii The investigation revealed antibacterial effectiveness of extract-mediated silver nanoparticles. This could serve as a combination therapy for the treatment of diabetic infected ulcer.

iii Further studies should be carried out on the mechanism of action of the antimicrobial compounds present in extract mediated silver nanoparticles.

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