ANTIBACTERIAL ACTIVITIES OF LEECH SALIVARY EXTRACT-SILVER NANOPARTICLES AGAINST SOME PATHOGENIC BACTERIA

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MTech/SLS/2017/7437

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A THESIS SUBMITTED TO THE POSTGRADUATE SCHOOL FEDERAL UNIVERSITY OF TECHNOLOGY, MINNA, NIGERIA IN PARTIAL FULFILMENT OF THE REQUIREMENTS FOR THE AWARD OF THE DEGREE OF MASTER OF TECHNOLOGY (MTech) IN MICROBIOLOGY (PHARMACEUTICAL MICROBIOLOGY)

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ABSTRACT

Leeches are blood sucking annelid worms that lives in fresh water habitat. Leech saliva and silver nanoparticles have both been reported to possess antibacterial activities against bacteria pathogens. The aim of this study was to determine the antibacterial activities of leech salivary extract silver nanoparticles against some pathogenic bacteria. Crude Leech salivary extract (LSE) and leech salivary extract silver nanoparticles (LSE-AgNp) were screened for antimicrobial activity against *Pseudomonas aeruginosa*, *Escherichia coli*, *Staphylococcus aureus* and *Klebsiella* species using agar diffusion method and was compared with Augmentin (8 µg/ml). The minimum inhibitory concentration (MIC) and minimum bacteriocidal concentration (MBC) of LSE-AgNp were assayed using micro broth dilution method with tetrazolium dye as indicator. The LSE-AgNp was synthesized and then characterized by UV-Vis spectroscopy and a Zeta nanosizer. The acute and sub-acute toxicity of the LSE-AgNp were determined in Wister rats using Lorke's method. The crude neat LSE was inactive on all test isolates while the LSE-AgNp was active against *P. aeruginosa* with zones of inhibition of 8.3 ± 0.88 mm and 12.3 ± 0.88 mm at 100 µL and 200 µL respectively. The zones of inhibition for *Klebsiella* spp were 12 ± 0.57 mm and 12.3 ± 0.33 mm at 100 µL and 200 µL respectively. The MIC and MBC of LSE-AgNp for *P. aeruginosa* and *Klebsiella* spp were 100 µL and 200 µL respectively. The wavelength of the LSE-Ag was at 456 nm while the size was 98.04 nm. The nanoparticles were bactericidal on the isolates. The lethal dose (LD_{50}) of the LSE-AgNp in rats was above 5000 mg/kgbw. Oral administration of the LSE-AgNp to rats at 25 mg/kgbw, 50 mg/kgbw and 100 mg/kgbw for 21 days produced no significant ($P > 0.05$) change in the body weight, red blood cell indices, differential blood counts, renal function indices and liver biomarkers. The LSE-AgNp at 100 mg/kgbw increased the relative spleen weight and decreased the feed intake of treated rats when compared with the control ($AgNO_3$ and normal saline). The results obtained suggest that the LSE-AgNp could be used in stabilizing the immune system and treating infectious diseases caused by *P. aeruginosa* and *Klebsiella* species.

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LIST OF ABBREVIATIONS

Abbreviation	Meaning
AGDE	Australian Government Department of the Environment
AgNPs	Silver nanoparticles
ANOVA	Analysis of variance
AUG	Augmentin
CA	Catalase
CFU	Colony forming unit
CI	Citrate
CO	Coagulase
DMRT	Duncan multiple range test
DNA	Deoxyribonucleic acid
EDC	Endocrine-disrupting compounds
F	Fructose
FTIR	Fourier transmission infrared spectroscopy
G	Glucose
GIT	Gastrointestinal tract
GR	Gram's reaction
h	Hour(s)
Hb	Haemoglobin
IUPAC	International union of pure and applied chemistry
L	Lactose
LD_{50}	Lethal dose 50
LSE	Leech salivary extract

LSE-AgNp	Leech salivary extract silver nanoparticles
M	Maltose
MBC	Minimum bactericidal concentration
Mch	Mean corpuscular haemoglobin
Min	Minutes
Mchc	Mean corpuscular haemoglobin concentration
Mcv	Mean cell volume
MIC	Minimum inhibitory concentration
MO	Motility
MR	Methy red
NADH	Nicotinamide adenine dinucleotide hydrogen
NADPH	Nicotinamide adenine dinucleotide phosphate
NCBI	National centre for biotechnology information
NCDC	Nigerian centre for disease control and prevention
NIPRD	National institute of pharmaceutical research development
NPs	Nanoparticles
OX	Oxidase
Pcv	Packed cell volume
Rbc	Red blood cell
S	Sucrose
SEM	Scanning electron microscopy
SH	Starch hydrolysis
sp	Specie
SPSS	Statistical package for social science

TEM	Transmission electron microscopy
UR	Urease
Wbc	White blood cells
WHO	World health organization
XPS	X-ray photo electron spectroscopy
XRD	X-ray diffractometry

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CHAPTER ONE

1.0 INTRODUCTION

1.1 Background to the Study

Pathogenic microorganisms that cause diseases especially bacteria have always been a major threat to human lives and hospitalized patients (Sarmah *et al*., 2018). It is one of the cogent challenges faced by medical professionals. Early and accurate diagnosis and treatment of infectious diseases can be challenging due to the toxic nature of some chemically synthesized drugs and the resistance shown by pathogenic strains against conventional mode of antibiotics. Pathogenic organisms use different techniques to evade conventional drugs and these techniques may include: biofilms and/or capsule formation, the use of proton efflux pump to mention but a few. These in turn add up to the virulence of an organism (Pigłowski, 2019).

In spite of the progress accomplished in recent decades, the rate of morbidity and mortality due to infectious disease has been on the rise. The Nigeria Centre for Disease Control (NCDC) in 2019 stated that infectious diseases are the leading cause of hospitalisation and death in Nigeria. World health organisation's (WHO) annual report established that out of 48 million cases of diarrhea (one of the infectious diseases) 128,000 cases led to hospitalisation and 3000 deaths (Njidda *et al*., 2018).

The high rate of infectious diseases in Nigeria and Africa at large is on the increase, and the rate of antibiotic resistance, toxic nature of some chemically synthesized drug as well as high cost of drug in relation to poverty in the continent of Africa have contributed immensely to the ineffectiveness in the treatment of infectious disease (Chattu and Yaya,

2020). Attention is now being turned to nature, in sourcing for safe, cheap and reliable drugs in combating infectious diseases.

Leeches belongs to the phylum Annelida, order '*Arhynchobdellida'* and family '*Hirudinidae'* with prominent examples such as *Hirudo medicinalis, H. verbena* and *H. orientalis* (Wollina *et al*., 2016). Leeches are hermaphroditic worms in nature that sucks blood. More than 600 species have been identified and fifteen of those have been classified as medicinal. They are locally called 'Mosasaku' in Hausa, 'Mujemuje' in Yoruba, 'Tuturu' in Nupe and 'Idu' in Igbo. Other species of Leeches have been studied and a number of proteins and peptides have been found in their salivary extract. Leeches have been used for treatment of a wide range of diseases such as dental infections, skin diseases; wound healing, nervous system abnormalities, problem of urinary and reproductive system, and inflammation (Ghawi *et al*., 2012).

Nanoparticles (NPs) are particles that range in size from 1 to 100 nanometers (nm). They are synthesized mostly from transition metals in the periodic table. A wide variety of approaches (such as chemical, physical and biological or biogenic) have been used for the synthesis of nanoparticles (Patra and Baek, 2014). However, biogenic reduction of metal precursors in producing its corresponding NPs has proven to be eco-friendly, less expensive and free of chemical contaminants. Natural products such as plant extract or animal secretions that are proteineous in constituent, and embedded with natural stabilizing, growth terminating and capping compounds have been a good source of biogenic reduction of metallic particles to nanoparticles. Metallic nanoparticles produced by biogenic reduction such as silver nanoparticles (AgNPs) have been revealed to have antimicrobial properties against some pathogenic organisms (Nadaroğlu *et al*., 2017). Its application also extends into biomedical sciences for bio-imaging, drug transport, cancer treatment, medical

diagnosis and sensor construction due to their unique properties, structure and size (Nadaroğlu *et al*., 2017).

1.2 Statement of the Research Problem

Pathogenic microorganisms have been a constant threat to human lives and hospitalised patients. Their ability to develop resistances against conventional antibiotics has posed a major challenge in the treatment of infections. Likewise, the toxic nature of chemically synthesized drugs, and the high cost of drugs and the increased level of poverty have singly and synergistically contributed to the ineffectiveness in the treatment of infectious diseases. The crude saliva from leeches has been tested against some pathogenic with low activity recorded.

1.3 Justification for the Study

The desire for the acquisition of novel antimicrobials from natural products of plant and animal metabolites is one of the recent trends in drug discovery and development. Leeches are valuable medicinal annelids found in major continents of Asia, Europe and Africa. Their salivary gland secretes more than hundreds of substances that have antimicrobial and therapeutic potency. Since the crude saliva from Leeches has been tested against some pathogenic organisms, with very low activity recorded. Therefore, the antimicrobial potency of the salivary extract of leeches would be enhanced by treating it with silver nitrate and testing it against pathogenic microorganisms. Data generated would contribute to the knowledge of antimicrobial potency of leeches.

1.4 Aim and Objectives of the study

The aim of this study was to determine the antibacterial activities of leech salivary extract silver nanoparticles against some pathogenic bacteria.

The specific objectives of this study were to:

i. determine the antibacterial activity of the Leech salivary extract (LSE) and leech salivary extract nanoparticles (LSE-AgNp) on the test isolates.
ii. determine the minimum inhibitory and minimum bactericidal concentrations of the LSE-AgNp.
iii. characterise the LSE-AgNp.
iv. determine the safety of the LSE- AgNp in rats.

CHAPTER TWO

2.0 LITERATURE REVIEW

2.1 Description of Leeches

Leeches are bisexual (hermaphrodites) invertebrate worms with more than 600 species known worldwide. Leeches belongs to the phylum Annelida which comprises of both the predacious invertebrate worms and the sanguivorous invertebrate worms. The predacious leeches are predators of many invertebrates while the sanguivorous leeches are ectoparasites that feed on the blood of vertebrate including humans. The life cycle of these leeches out rightly hinges on their feeding pattern and habitat. Out of the known six hundred (600) species of leeches identified till date, only fifteen (15) of them has been classified as medicinal leeches (leeches that is usable and suitable for medical practices). *Hirudo medicinalis* amidst the other medicinal leeches have been the most exploited and accounted for in both ancient and contemporary medicine (Atalayin *et al*., 2017; Rehman, 2021).

2.2 Description of *Hirudo medicinalis*

Hirudo medicinalis is a sanguivorous, segmented invertebrate worm. It belongs to a phylum of specialised annelids and of the sub-class 'Hirudinea.' It is triploblastic (it has three primary gem layer), protastomes with a coelom, closed circulatory system and true segmentation. Their coelomic space is usually filled with a fluid generally referred to as coelomic fluid, which contains tissues and it is reduced to a system of narrow canals. The body of *H. Medicinalis* is made up of roughly thirty-three to thirty-four (33 to 34) segments which is usually a fix number. These body segments are divided into pre-oral non-

metameric segmes and 32 post-oral metameres (somites). The somites are further sub-sectioned into two to sixteen external annuli, and the annulation pattern is considered as a dianostic feature for both leeches' genus and species. Sensory structures such as the eyes, oculiform spots, papillae and sensilla are also used by taxonomists to identify some leeches into their respective genus and species (Upshaw and O'lery, 2000). It is commonly called medicinal leech or fresh water leech while locally, across some major ethnicity and tribe in Nigeria it has been severally described. It is referred to as "etu" in Igbo, "mujemuje" in Yoruba, "mosasaku" in Hausa, "kulun kulun" in Gwari and "tuturu" in Nupe. The worm is black or brown in colouration with two yellow marginal stripes at its sides. It can measure up to 12cm long (when they stretch), but generally it is smaller weighing one to one and the half a gram (1 to1.5g) before feeding. The adult form possesses eight pairs of eyes and two suckers (anterior and posterior). The head is located at the narrow tapered end (anterior) which comprises of the anterior suckers which is also the mouth part that consists of three jaws. These jaws have about sixty to hundred teeth which are used to bite deeply and causes prolong bleeding after detachment. The posterior sucker is used for attachment and it aids crawling. It is grouped as sanguivorous leeches because they are ectoparasites that feed on the blood of vertebrates; they are also a hematophagous feeder that feeds on the blood of humans and cattle. It sucks about two to twenty millilitres of blood within ten to thirty minutes, and then drops off immediately after being satisfied with no further intention of feeding (Knoblock, 2010).

The skin of *H. medicinalis* is used for breathing and they are considered as hermaphrodite because a matured worm possesses both male and female sex organs; but still they do not undergo self-fertilization.

Table 2.1: **Classification of *Hirudo medicinalis***

Taxonomic group	**Animal**
Dormain	Eukarya
Kingdom	Animalia
Phylum	Annelida
Class	Clitellata
Sub-Class	Hirudinea
Order	Arhynchobdella
Family	Hirudinidae
Genus	*Hirudo*
Species	*medicinalis*

Source: Whitman (1886)

2.3 Local Medicinal Uses of *Hirudo medicinalis*

Hirudo medicinalis in ancient times were locally used for various processes ranging from wound healing to bloodletting, and their use was attributed to their nature and desire to suck blood from vertebrates including humans. This was then referred to as "leeching". Recently surgeons did invested their attention towards the use of leeches for surgical practices such as plastic and reconstructive surgery with the leech aiding these surgeons in stimulating blood flow at post surgical sites (Abdualkader *et al*., 2013; Rehman, 2021).

With the advent of micro surgery, leeches have become beneficially important in plastic or traumatological surgery to resolve many problems due to insufficient venous drainage. Since the survival of re-implanted tissue hinges on an efficient venous return, and establishing a venous return is difficult to attain due to the fact that artery are more easily stitched to veins, surgeons have turned to the idea of the application of leech to locally resolve this challenge. By their suction, leeches helps in draining tissues in danger of necrosis and also restores capillaries match between the face of a wound where surgical suture is technically impossible (Michalsen *et al*., 2003; Abdualkader *et al*., 2013).

2.4 Effect of Some Environmental Factors on Leeches

For the purpose of medicinal treatments and their aid in reconstructive surgery, the overuse and environmental restraint of leeches has posed a major threat on their availability and diversity. Especially in closed basins such as marsh lands and lakes that receive heavy pollutants, pressure of these polluting compounds are lethal on the species of leeches that inhabits such environments (Saglam, 2018).

2.4.1 Agricultural factor

Wetlands such as “Fadama” areas have been for sometimes, a good land for agriculture and agricultural purposes especially rice farming. Areas as such inhabit a good number of leeches constituting to their high bioavailability. Due to the nature of land being wet and the water available for irrigation, wetlands thus poses a major threat in the survival of leech’s species that inhabits such environment. Some agricultural methods practiced in this region have led to the increased level of both nutrients and pollutants (i.e animal dung’s, pesticides and fertilizers). Remediation and regulation of wetlands and rivers with agricultural purpose in mind have led to the change in the duration and extent of water flow affecting critical life stages of water dependent species including leeches. Cultivation of

these wetlands also plays an important role of altering the ecological system function with the use of heavy machineries on it (AGDE, 2016; Saglam, 2018).

The life of leeches in wetlands is shortened as a result of the agricultural activities previously mentioned. Some leeches could lose their spawning and breeding environment and eventually dies as a result of pesticides and fertilisation application which spreads into wetlands faster through the air (in cases of air application)and also through surface flow during rainy seasons.

2.4.2 Endocrine-disrupting compounds on leeches

Compounds that have the ability to affect the growth, reproduction, general embryonic and morphology development of aquatic organisms are referred to as endocrine-disrupting compounds (EDC's) (Saglam, 2018). These compounds are found in various sources such as common household chemicals and wastes, industrial chemicals and wastes, municipal effluents containing natural hormones and synthetic steroids, alkyl phenolics, agricultural pesticides and run off (Wang and Zhou, 2013). These compounds exhibit a high level geno-toxicity which can disrupt genetic diversity and structure. Adverse effects of EDC's on the biomass and biodiversity in the wetlands can cause anomalies in leech offspring, leech embryos and disruption of leech reproductive system. These compounds can therefore be responsible for endangering of leech species leading to extinction.

2.4.3 Urbanisation factor

Rapid development in wetland areas daily has in a way added and piled up pressure causing a disproportionate effect on wetlands. Construction of roads and increase in the number of houses being built makes possible the direct transfer of sediments and other materials to streams which may kill leeches and disrupt the cocoon breeding fields in wetlands (Saglam, 2018).

2.5 Activity of Leech Salivary Extract

Leech salivary extract have been reported to have antibacterial activity against some bacteria. In a study revealed by Ojo *et al*. (2018), LSE was used in an *in vitro* investigation against *Mycobacterium tubercolosis* and the result revealed that the extract possessed anti-tubercular activity against *M. tubercolosis* at a concentration of 50 µL. Also, in other studies of "the anti-oxidative and antimicrobial activities of leech salivary extract" against *Staphylococcus aureus* and *Escherichia coli*, the LSE inhibited the growth of *Staphylococcus aureus* with zones of inhibition produced alongside (Malik *et al*., 2019). In a research conducted by Abdualkadeer *et al*. (2011) on the biological activities of leeches, the leech salivary extract also showcased activity on some bacteria pathogens such as *E. coli, P. aeruginosa*, *Bacillus cereus* and *S. aureus*. All these reveal the antibacterial potential of LSE to be used in this study.

2.6 Nanotechnology and Nanoparticles

Nanotechnology is a renaissance in science and technology that predates as far as the ninth century, when nanoparticles such as gold and silver were used to produce a sparkling effect on pots and other utensils by the artisans of the Mesopotamia age (Patra and Baek, 2014). Nanotechnology is defined as the use of various engineering, physical, chemical and biological methods in the exploitation of metallic substance at the atomic level to produce the metallic nanoparticles of its equivalence. Nanoparticles as described by the International Union of Pure and Applied Chemistry (IUPAC) are particles that cannot be seen with the naked eyes and range in size from 1 to 100 nanometer (nm) in dimension (Agbebati-Maleki *et al*., 2020). They are mostly synthesized from transition metals in the periodic table, and undergo reduction to the valence of zero (Hussain *et al*., 2016).

2.6.1 Methods of nanoparticle synthesis

2.6.1.1 Biological methods

The biological method of synthesizing various metallic nanoparticles is regarded as biogenic reduction or green synthesis. It is an alternative method, and solution to the demerits and toxic effects caused by both physical and chemical methods of synthesizing nanoparticle. Unlike the physical and chemical methods, biological method is far less expensive to carry out, it involves no toxic chemicals and it is eco-friendly (Parasharu, 2009; Hussain *et al*., 2016). Biological method makes use of natural products for example primary and secondary metabolites such as plant extracts, proteins, enzymes, carbohydrates, fat, nucleic acid, phenolic compounds, amines, alkaloids and pigments obtained from microorganisms, plants and animals that can function as capping and stabilizing agents which helps the biogenic reduction of metallic ion to a valence of zero and precipitate nanoparticles either intracellularly or extracellularly (Korbekandi *et al*., 2009; Iravani, 2011). These natural products of plant, animals and microorganisms used as both capping and stabilizing agents in the biogenic reduction of metal ions also helps in reducing the risk of toxicity of the process to the environment and control undesired agglomeration of the product formed. Some microorganisms for example yeast and algae are good detoxifier of heavy metals and thereby reducing it to nanoparticles of good morphology and size (Singhal *et al*., 2011). These nanoparticles are used across various fields of medicine science and engineering (Aghebati-Maleki *et al*., 2019).

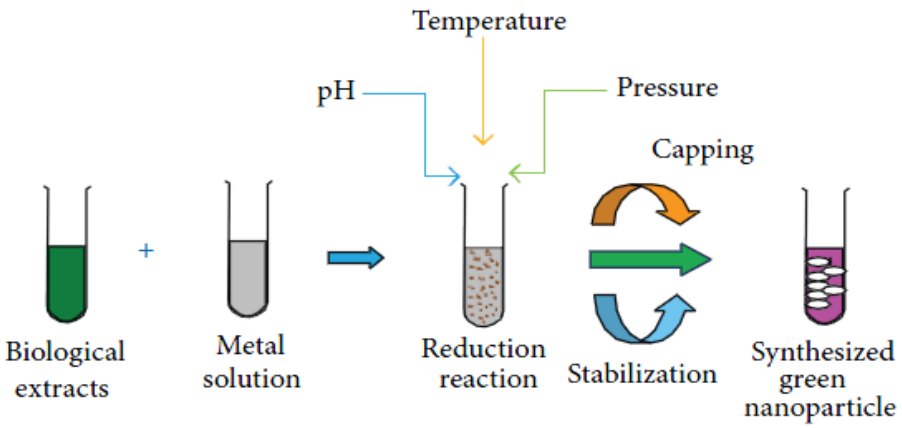

Figure 2.1: Process of Green Synthesis of Nanoparticles

(Source: Patra and Baek, 2014)

2.6.1.2 Chemical methods

Chemical method is the most common method of nanoparticle synthesis. Here, organic and inorganic chemicals are employed as capping and stabilizing agents in reducing metal ions to nanoparticles of its equivalence. These chemical agents although reduces metal ions, constitute a high degree of toxicity to the environment and even to human system if used as a drug delivery agent (Oliveira *et al*., 2005; Merga *et al*., 2007; Barkat *et al*., 2018).

2.6.1.3 Physical methods

This method holds a greater advantage over chemical methods because it does not require chemicals. This method makes use of very high heat in the synthesis of various metallic nanoparticles (silver, gold, lead). Method such as evaporation-condensation, laser ablation has been employed in synthesizing nanoparticles. Synthesis of nanoparticles using ceramic heaters with a heat source, and cooling down the evaporated vapor in a fast rate synthesizes nanoparticles by evaporation-condensation method. Likewise, nanoparticle can be synthesized from metallic bulk materials in solution by laser ablation method. The effectiveness of this method (laser ablation), and the characteristics of the nanoparticles synthesized depends on factors such as the wavelength of the laser impinging the metallic target, the duration of the laser pulses (in the femto, pico and nanosecond regime), the laser fluence, the ablation time duration and the effective liquid medium, with or without the presence of surfactants (Kim *et al*., 2005; Tarasenko *et al*., 2006; Barkat *et al*., 2018).

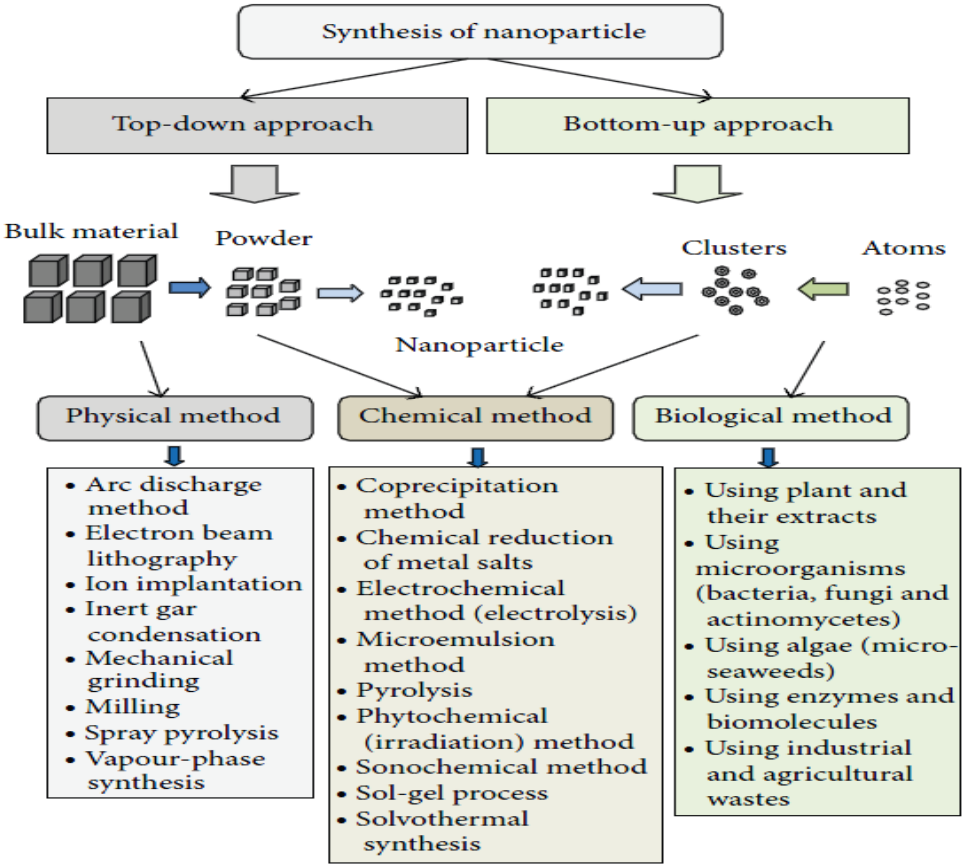

Figure 2.2: Methods Involved in the Synthesis of Nanoparticles

(Source: Patra and Baek, 2014)

2.7 Silver Nanoparticles

Silver is a basic, ductile and malleable natural occurring element that makes up the earth crust. Silver occurs in four different oxidation states: Ag^0, Ag^+, Ag^{2+} and Ag^{3+}. The most predominantly abundant in nature are Ag^0 and Ag^+ while Ag^{2+} and Ag^{3+} are unstable in aquatic environment. Metallic silver is insoluble in water, but salts of metallic silver such as $AgNO_3$ and AgCl are very much soluble in water (Ramya and Subapriya, 2012). Silver nanoparticles can be synthesized through three known and established methods which are

the physical, chemical and biological methods. Biological method has shown and proven to be the safest and economical method of synthesis (Pantidos and Horsefall, 2014).

2.7.1 Synthesis of silver nanoparticles

Synthesis of silver nanoparticles is best carried out by the biological method of silver reduction. Biogenic reduction through the use of organisms and natural product either secreted or extracted can be wholesomely classified into two phases: The use of microorganisms (bacteria, fungi and algae) and the use of natural products of plant and animal metabolites (Panigrahi, 2013).

Some organisms have the ability to form resistance against metal ion concentration (such as transition metals used in nanoparticle synthesis) and thereby are able to thrive in this condition by mechanisms such as secretion of enzymes, proteinous and organic substance that aids oxidation and reduction of metals bioaccumulation, efflux and transport of metals leading to intracellular and/or extracellular synthesis of nanoparticles (Husseiny *et al*., 2007). The first bacterium used in the synthesis of AgNp was *Pseudomonas stutzeri* AG259 strain obtained from silver mining sites. The in vitro synthesis of AgNp using the bacterium was observed to involve the reduced form of nicotinamide adenine dinucleotide phosphate (NADPH) dependent nitrate reductase (Vaidyanathan *et al*., 2010).

Fungi also are good synthesizer of silver nanoparticles. They synthesize larger amount when compared with bacteria, this is so because fungi secretes larger amount of proteins needed for the biogenic reduction of silver metals giving rise to a higher yield of silver nanoparticles. Fungal cells traps silver ions and anthraquinones, naphthoquinones present in the fugal system helps in the reduction of the silver ions 'Ag^{+}' to silver atom 'Ag^{0}' (Mukherjee *et al*., 2001; Mohanpuria *et al*., 2008). *Fusarium oxysporum* has been reported to possess (NADPH) dependent nitrate reductase and a ferry of quinine enzymes that are

responsible for the formation of nanoparticle (Ahmad *et al*., 2003). The nitrate reductase enzyme is a widely and conventionally agreed and acceptable mechanism in microorganisms for AgNp synthesis although the process is slower when compared with the use of plant and animal metabolites (Panigrahi, 2013).

Natural products from plant and animal have a broad variety of metabolites (primary and secondary) and are used in the synthesis of AgNp and many other metallic nanoparticles. These metabolites are easy to obtain and are regarded as nontoxic and safe. Plant and animals also possess the ability for instant reduction of silver ions to AgNp. Natural products used for the reduction process includes phytochemicals such as terpenoids, flavones, ketones, aldehyde, amides, carboxylic acids and animal proteins and saliva. The use of natural products from plant and animals in the synthesis of nanoparticles have been proven to be more time saving compared with using natural organisms (Parasharu *et al*., 2009)

2.7.2 Action and toxicity of silver nanoparticles

The specific mechanism of action of AgNp on microorganisms is not clearly ascertained, but various mechanism of action has been proposed. Silver nanoparticles have the ability to attach to cell surfaces and infiltrate the cell wall of microbes causing a structural alteration of the cell membrane leading to pit formation and permeability of the membrane and eventual death of the cell (Sondi and Salopek-Sondi, 2004; Chwalibog *et al*., 2010). Silver nanoparticles are believed to release silver ions which may react with thiol group of some enzymes leading to death or inhibition of some respiratory enzymes which may result in the release of some reactive oxygen species which attacks the cell (Kim *et al*., 2007; He *et al*., 2011). These particles can also act as a soft base which may react with sulphur and phosphorous components of the cell wall and genetic makeup of an organism (DNA)

disrupting its replication and thus terminating the microbes (Morones *et al*., 2005; Tripathi *et al*., 2017).

2.7.3 Characterization and application of silver nanoparticles

Characterization of nanoparticles is very cogent in knowing and comprehending nanoparticle synthesis and control. Parameters used in the characterization of NPs include: energy dispersive x-ray spectrophotometer (EDS) which is used alongside an emission scanning electron microscope, x-ray photoelectron spectroscopy (XPS), x-ray diffractometry (XRD), fourier transformation infrared spectroscopy (FTIR) and UV-vis spectroscopy which is used to confirm the formation of AgNps. X-ray diffractometry is used in the determination of the crystallinity of the NP synthesized. Fourier transformation infrared spectroscopy is used to evaluate the functional group(s) of organic compounds responsible for the biogenic reduction of the Ag^{+} to Ag^{0}. Scanning electron microscopy and transmission electron microscopy used in determining the shape, size, and structure of the NP and how relatively dispersed or uniform they are. Silver nanoparticles have been phenomenal in its application across wide areas of medicine, science and the industrial world. Nanoparticles are widely used in medical line due to their size and ability to deliver the drugs to the target site and their antimicrobial potential against pathogens (Barkat *et al*., 2018).

2.8 Medical Importance of Test Isolates

2.8.1 *Klebsiella* species

Klebsiella sp is from the kingdom; ‘Bacteria’, phylum; ‘Proteobacteria’, class; ‘Gamma proteobacteria’, order; ‘Enterobacteriales’, family; ‘Enterobacteriaceae’, and genus; *Klebsiella*. *Klebsiella* is a genus of non-motile, Gram-negative, oxidase-negative, rod shaped bacteria with a prominent polysaccharide-based capsule (Arulmozhi *et al*., 2014;

Paterson *et al*., 2014). This genus was named after Edwin Klebs (1834 to 1913), a German microbiologist. *Klebsiella* species are found everywhere in nature (ubiquitous) and this is thought to be due to differentiated sub lineages developing specific niche adaptations with associated biochemical adaptations which makes them better suited to a particular environment. They can be found in water, soil, animals, and human beings (Bagley, 1985; Vimont *et al*., 2014; Barati *et al*., 2016). According to Ristuccia and Cunha 1984, *Klebsiella* tend to be rounder and thicker than other members of their 'Enterobacteriaceae' family. They typically occur as straight rods with round or slightly pointed end. They can also be found singly, in pairs or in short chains. Diplocobacillary forms are found in vivo. *Klebsiella* grows best at a temperature between 35 °C and 37 °C and at pH of 7.2. The species of this genus are facultative anaerobes, most strains surviving with citrate and glucose as their sole carbon source and ammonia as the sole nitrogen source. *Klebsiella* species are routinely found in the human nose, mouth and gastrointestinal tract as normal flora however, they can also behave as opportunistic human pathogens leading to a wide range of disease states such as, pneumonia, urinary tract infections, septicemia, meningitis, diarrhea and soft tissue infection (Ristuccia and Cunha, 1984; Canda and Aksoz, 2014; Al-Bshabshe *et al*., 2020).

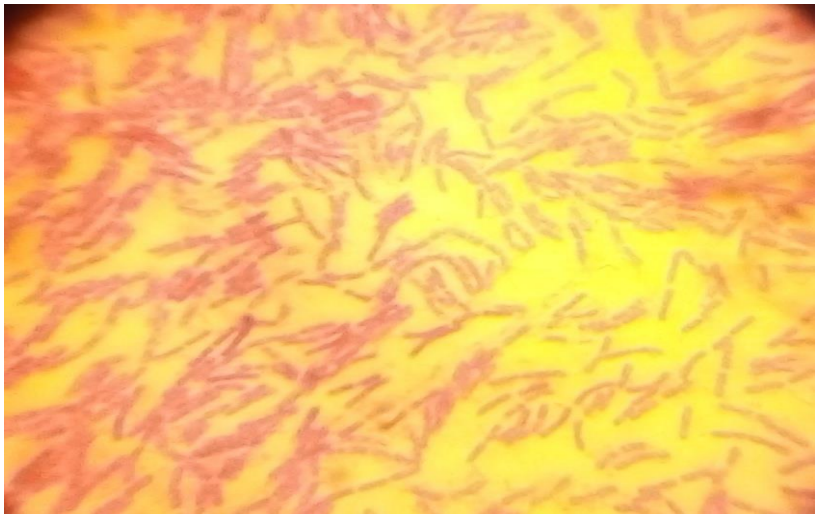

Plate I: Photo micrograph of *Klebsiella* spp under a x100 objective lens

2.8.2 *Pseudomonas aeruginosa*

Pseudomonas aeruginosa is taxonomically from the kingdom 'Monera', phylum 'Proteobacteria', class 'Gamma proteobacteria' sub-division, order 'Pseudomonadaceae', genus *Pseudomonas*, and species *aeruginosa*. The word 'Pseudomonas' is derived from two Greek words: *Pseudo* meaning 'false' and *monas* meaning 'single unit', *aeruginosa* 'greenish-blue' is from the latin *aerūgō* meaning 'rusted copper'. *Pseudomonas aeruginosa* is a heterotrophic, motile, Gram-negative rod shaped bacterium. It is a facultative aerobe that grows both aerobically and anaerobically with nitrate as its final electron acceptor. It is also a phototroph (not fastidious) and can make use of over a hundred (100) organic molecules as a source of carbon and/or energy (Diggle and Whiteley, 2020). It is an opportunistic organism in nature and it's medical importance is obviously seen in the infection of humans with weakened immune defences and it is the antecedent of very austere pulmonary diseases. Apart from this, *P. aeruginosa* is responsible for many diseases such as implant and burn, corneal and urinary tract

infections, cystic fibrosis, chronic otitis and many other conditions (Alhazmi, 2015; Soheili *et al*., 2019). The virulence of *P. aeruginosa* is highlighted by the organism's capabilities of producing several virulence factors and enzymes including transcriptional regulators, proteases, toxins and lipopolysaccharides (Soheili *et al*., 2019) thereby making it possible to evade and interfere with the host defences, facilitate adhesion, modulate or upset host cell pathways and target extracellular matrix (Alhazmi, 2015). Inspite of the discovery of pharmaceutical drug for several decades, the organism remains one of the most recalcitrant and difficult to treat due to its ability to form biofilms protecting it from drug (antibiotics) and the host immune system (Alhazmi, 2015; VanDrisse *et al*., 2021).

2.8.3 *Escherichia coli*

Escherichia coli are members of the family 'enterobacteriaceae' that colonizes the gastro-intestinal tract (GIT) of humans, infants and animals. It is a facultative anaerobe, capsulated, Gram negative, motile bacillus that is harmlessly confined to the gut of humans and animals (organisms that inhabit this region are also referred to as coliforms). It is part of the normal flora of human GIT and as part of it remains harmless. Although in imuno-compromised host, non-pathogenic strains of *E. coli* can cause infections especially the gastro intestinal barriers are violated (Nataro and Kaper, 1998; Cho *et al*., 2021). They are differentiated from other coliforms (organisms that constitute the normal flora of the intestinal tract) by its ability to breakdown tryptophan to produce indole which is indicated by a deep red ring colouration on addition of 2 to 3 drops of Kovac's reagent to a 24 hours old culture.

Majorly, pathogenic *E. coli* strains have undoubtedly been reported to cause infections such as diarrhea, abdominal pain, fever, nausea and food poisoning that range from non-

presenting to severe in humans and neonates (with neonates case usually life threatening). For example *E. coli* O157:H7 is a reputable strain renowned as the causative agent of traveller's diarrhea, it produces a powerful toxin known as 'shiga toxin'. Also pathogenic *E. coli* strains maybe localized to the mucosa surface of the gut or systemically disseminated throughout the body. This and can result in clinical syndromes which include urinary tract infection, septic shock, sepsis/meningitis and enteric diarrhea disease in both human and neonates with neonatal cases usually life threatening (Sapountzis *et al*, 2020).

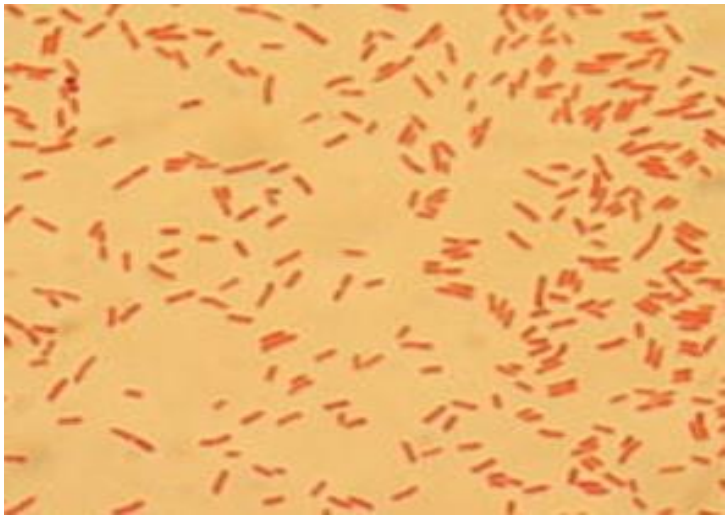

Plate II: Photo micrograph of *Escherichia coli* under a x100 objective lens

2.8.4 *Staphylococcus aureus*

Staphylococcus aureus is a Gram positive coccal bacterium which was discovered by Alexander Ogston. In 1880, he observed pus from 88 human abscesses under his microscope from which he pointed at Gram positive spherical. He named the organism "Staphylococcus" (from the greek word meaning grape shape). *Staphylococcus aureus* is spread by having direct contact with infected person and also through droplets of cough

and sneezes leading to skin infection, pneumonia and blood stream infection (septicemia). The treatment of this organism were limited to topical application of carboxylic acid as introduced by Joseph Lister and promoted by Alexander Ogston until the introduction of the first miracle drug (penicillin) in 1940. Strains of *S. aureus* produces penicillase an enzyme that specialises in interacting and /or interferring with the effect of penicillin (Myles and Datta, 2012; Islam *et al*., 2021).

Furthermore, strains of *S. aureus* secretes siderophores which is able to overcome the host attempt to starve it of iron from heme and transferrin in the blood stream. Finally, *S. aureus* is capable of producing toxins known as hemolysin (alpha, beta, gamma and delta) which is responsible for the lysing of red blood cells and it also causes toxic shock syndrome and food poisoning, making them virulent and dangerous (Myles and Datta, 2012; Perry *et al*., 2019).

CHAPTER THREE

3.0 MATERIALS AND METHODS

3.1 Study Area

Leeches were collected from Panda Development Area, Karu Local Government Area, Nassarawa State. Nassarawa is a North Central State of Nigeria with a population of 1,868,377 and land mass of 10,470 sq mi, with most of it populace fishermen and traders by occupation. It is situated on the on latitude 8° 32' N and longitude 8° 18' E.

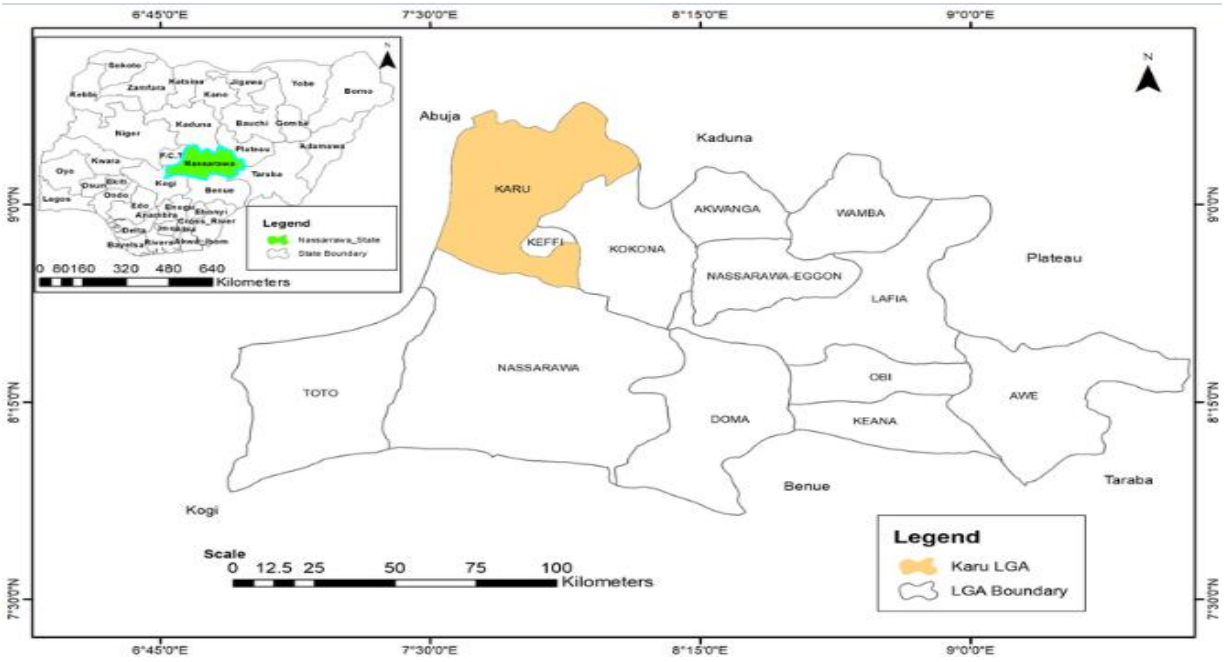

Fig 3.1: Study Area of Leech Collected in Panda Development Area Nassarawa State (Source: GIS, 2021)

3.2 Collection and Identification of Leeches

Leeches were collected with the aid of a scooping net at the shorelines of fresh water Dam, in Panda Development Area, Nassarawa State, Nigeria in the month of June, 2019. The worms

were transported to Minna with the aid of perforated plastic bottles identified in the Department of Animal Biology, Federal University of Technology, Minna, Niger State as *Hirudo medicinalis* (Plate III).

Plate III: *Hirudo medicinalis*

3.3 Laboratory Maintenance of Leeches

The leeches were maintained in well-aerated plastic containers filled with non-chlorinated water (borehole water), at room temperature (25 ± 2 °C) throughout the period of study. The water was frequently changed at 2 days interval and feeding of the leeches with cow blood was carried out 3 weeks interval after the extraction of their saliva (Abdualkader *et al*., 2014).

3.4 Extraction of Leech Saliva

The extraction of leech saliva was carried out by Ice-shocking method as described by Abdualkader *et al*. (2014) and Ojo *et al*. (2018). Two to three pieces of leeches were placed in a test tube and the test tube was positioned in a bowl packed with ice blocks for 15 to 20 minutes. Leeches were completely paralyzed and forced to regurgitate their intestinal content into a sterile test tube. The saliva was aspirated using a sterile hypodermic needle and syringe, and then transferred into sterile screw capped containers and preserved at −4 °C. (Plate IV).

Plate IV: Ice Shock Method of Leech Saliva Extraction Process and Leech Salivary Extract

3.5 Source and Identification of Test Organisms

The test organisms used in this study were isolated from clinical samples from patients admitted at Ibrahim Babangida Specialist Hospital Minna, Niger State. The clinical isolates were identified using the method described by Cheesbrough (2010). Morphological and biochemical tests (such as Gram's staining, catalase, oxidase, coagulase, citrate, urease, starch

hydrolysis, methyl red, indole, and sugar fermentation) were used in the isolates' identification. The organisms were maintained in slants until required.

3.5.1 Biochemical identification of test isolates

3.5.1.1 Gram staining

A smear of the bacterial isolates was made on a clean grease free slide with the aid of a droplet of sterile water. The slides were passed through flame twice to heat fix them and then allowed to air dry. The slides were placed on a staining rack and flooded with crystal violet (primary stain) and allowed to stand for 60 seconds. Afterwards, the slides were rinsed with water and drained, then flooded with Grams iodine and allowed to stand for 30 seconds. The slides were again rinsed with water after which 95 % alcohol was used to decolourise the primary stain. The alcohol was rinsed with water and the slides were flooded with safranin (a counter stain) and allowed to stand for 60 seconds before rinsing with water. The slides were viewed under a light microscope using oil immersion objective lens.

3.5.1.2 Catalase test

A drop of 3 % hydrogen peroxide was made on clean grease free slides, then, a smear of the 24 h culture of bacterial isolates were made on the slides with the aid of a sterile wire loop and it was observed for bubble formation which indicated the reaction was positive.

3.5.1.3 Citrate utilization test

Sterile Simon citrate medium were kept in slanted position and allowed to solidify. A straight, sterile wire was used to streak the slopes and stabbed the bottom of the slopes with the bacterial isolates and then incubated for 48 h at 37 ºC. Bright blue colouration indicated a positive citrate reaction while no change in colour indicated a negative citrate reaction.

3.5.1.4 Coagulase test

A drop of human plasma was placed on clean grease free slides, with the aid of a sterile wire loop, a colony of the bacterial isolates were picked and smeared on the slides. The formation of a clump by the bacterial isolates indicated a positive result for coagulase.

3.5.1.5 Starch hydrolysis

Nutrient agar (4 g) was added to 0.3 g of soluble starch in a conical flask. One hundred milliliter (100 mL) of distilled water was added to the mixture. It was pre-heated and then sterilized by autoclaving at 121 ^{o}C for 15 min. The medium was allowed to cool to 40 ^{o}C and aseptically poured into sterile Petri dishes and allowed to solidify. Each test bacterial isolate was inoculated by streaking, while a plate was left un-inoculated (to serve as control) and incubated at 37 ^{o}C for 24 h. After incubation, the plates were flooded with Gram's iodine and observed for colour change. A clear zone shown around the colonies of the test organism confirmed a positive result, while blue-black colouration with Gram's iodine indicated a negative result.

3.5.1.6 Methyl red test

Sterile peptone broth was dispensed into sterile test tubes and allowed to cool. The test tubes containing the broth medium were inoculated with bacterial isolates and incubated at 37 ^{o}C for 48 h. At the end of the incubation period, four drops of methyl red indicator were added and gently mixed. Positive test was indicated by bright or brick red colour while those that were negative were indicated by yellow colour.

3.5.1.7 Indole test

Sterile peptone broth was dispensed into sterile test tubes and allowed to cool. The test tubes containing the broth medium were inoculated with bacterial isolates and incubated at 37 ^{o}C for 48 h. At the end of the incubation period, four drops of Kovac's reagent were added and

gently mixed. Positive test was indicated by a brown ring while those that were negative were indicated by absence of the brown ring.

3.5.1.8 Sugar fermentation

Seven point five grams (7.5 g) of Peptone water was prepared and 0.1 g of phenol red was added. The medium was divided into 5 beakers and 1 g of each sugar (glucose, lactose, maltose, sucrose, and fructose) was added to the respective beakers containing the medium. This was then dispensed into test tubes and Durham tubes were introduced in an inverted position into each test tube, they were sealed and autoclaved at 121 °C for 15 min. After autoclaving, the media were allowed to cool and bacterial isolates were inoculated into each sugar medium in the test tube and then incubated afterwards at 37 °C for 48 h. At the end of incubation, the tubes were observed for gas production (via the Durham tubes) and change in colour from red to yellow indicated positive test while a change from red to any colour different from that of the control indicated a negative test.

3.5.1.9 Oxidase test

A drop of oxidase reagent was added on a filter paper, colonies from 24-h cultures were picked and smeared on the spot where the reagent was dropped on the filter paper and allowed to stand for 10 seconds. Oxidase positive isolates developed a bluish-purple colouration while oxidase negative isolates remained colourless.

3.5.1.10 Urease test

One point four grams (1.4 g) of urea agar base was dispensed into a 250 mL conical flask and then autoclaved at 121 °C for 15 min and allowed to cool, 40 % of urease salt (4 g in 10 ml of autoclaved water) was also prepared and added to the cooled urea agar base. The resulting mixture was swirled together gently and then dispensed into sterile test tubes and kept in a slanted position until it solidified. Bacterial isolates were inoculated on to the slant at 37 °C

for 24 h and then observed. Positive result showed a colour change from yellow to light red or pink while a negative result showed no colour change.

3.6 Antimicrobial Assay

3.6.1 Standardization of test organisms

Following the method of NIPRD, (2006) and Babayi *et al*. (2018[a]) 0.2 mL of an overnight culture of each test organism was dispensed into 20 mL of freshly prepared sterile nutrient broth and incubated for 5 h to standardize the culture to 10^6 Cfu/mL. The absorbance of the standardized culture was obtained using a spectrophotometer at 625 nm.

3.6.2 Screening for antibacterial activities of crude leech salivary extract

The neat crude leech salivary extract at one hundred micro litres (100 µL) and two hundred micro litres (200 µL) respectively, were assayed for antibacterial activity on the test isolates using agar well diffusion technique described by Malik *et al*. (2019). Mueller Hinton agar (MHA) with a thickness of 4 mm was prepared. Standardised isolates (a loopful) were seeded on the agar plate with the aid of a sterile wire loop and swab sticks. Four holes were bored on the plate using a sterile cork borer (5 mm in diameter). Two concentrations of 100 µL and 200 µL of neat crude Leech salivary extract were dispensed into the first two holes (labeled 'A and B') while the other two holes were filled with a standard broad spectrum antibiotic (Augmentin (8 µg/ml) labeled '+') and sterile distilled water (labeled '-'). Afterwards, the plates were made in triplicates and incubated at 37 °C for 24 h and diameter of zones of inhibition were observed, measured and recorded at the end of the incubation period.

3.6.3 Synthesis of Leech salivary extract silver nanoparticles

The method of Pirtarighat *et al*. (2019) and Gavamukulya *et al*. (2019) were used for the synthesis of silver nanoparticles from LSE with slight modifications. A solution of silver nitrate (2 mM) was prepared by dissolving 0.034 g of the salt in 100 mL of sterile distilled

water. A measured quantity of 10 mL of the prepared 2 mM silver nitrate solution was dispensed into a conical flask and 1 mL of the leech salivary extract (LSE) was added to it, to make up a ratio of 1:10. The resulting mixture was placed under the sun for 30 min. A dark brown colour indicated a changed in colouration in the reaction mixture and a suspected formation of silver nanoparticles. The wavelength of the particles was determined with the aid of a UV-Vis spectrophotometer (Shidmadzu, UV-1800 series) at 625 nm and the size was determined by a nanosizer (Zetasizer version 7.01 by Malvern instrument limited)

3.6.4 Screening for antibacterial activities of leech salivary extract silver nanoparticles

The leech salivary extract silver nanoparticles (LSE-AgNp) at 100 µL and 200 µL were assayed for antibacterial activities on the test isolates using agar well diffusion technique described by Malik *et al*. (2019). Mueller Hinton agar (Oxoid) with a thickness of 4 mm was prepared. Standardised isolates (a loopful) were seeded on the agar plate with the aid of a sterile wire loop and swab sticks. Four holes were bored on the plate using a sterile cork borer (5 mm in diameter). Two concentrations of 100 µL and 200 µL of crude Leech salivary extract were dispensed into the first two holes (labeled 'A and B') while the other two holes were filled with a standard broad spectrum antibiotic (Augmentin (8 µg/mL) labeled '+') and sterile distilled water (labeled '-'). Afterwards, the plates were made in triplicates and incubated at 37 °C for 24 h and diameter zones of inhibition were observed, measured and recorded at the end of the incubation period.

3.6.5 Determination of minimum inhibitory concentration of leech salivary extract silver nanoparticles

The minimum inhibitory concentration (MIC) of the leech salivary extract silver nanoparticles was determined using the micro broth dilution method by Ojo *et al*. (2018). Two hundred

micro litres (200 μL) of sterile Mueller Hinton's broth was dispensed into a micro titre plate (from well 2 to 12) with the aid of a multiple channel pipette. Two hundred micro litres of neat LSE-AgNp was then dispensed into well 1 and well 2. Two hundred micro litres of the resulting mixture of broth and LSE-AgNp in well 2 was pipetted and dispensed into well 3 (making a double fold dilution). This was carried out for well 4 through well 10. Two hundred micro litres of standardized culture of *P. aeruginosa* was dispensed into well 1 through well 9 and well 11 respectively. The plate was incubated for 37 °C for 24 h. At the end of the incubation period 50 μL of tetrazolium dye was added to the wells and further incubated for 20 min to check for colour change from yellow to pink. The same procedure was repeated for the MIC of the LSE-AgNp for *Klebsiella* spp. (Ojo *et al*., 2018). Well 1, 10, 11 and 12 served as the LSE-AgNp sterility control, organism viability control and broth sterility control respectively.

3.6.6 Determination of minimum bactericidal concentration of leech salivary extract silver nanoparticles

The MBC of the LSE-AgNp was determined using the micro broth dilution method as described by Ojo *et al*. (2018). Two hundred micro litres (200 μL) of sterile Mueller Hinton's broth was dispensed into a micro titre plate (from well 2 to 12) with the aid of a multiple channel pipette. Two hundred micro litres of neat LSE-AgNp was then dispensed into well 1 and well 2. Two hundred micro litres of the resulting mixture of broth and LSE-AgNp in well 2 was pipetted and dispensed into well 3 (making a double fold dilution). This was repeated for well 4 through well 10. Two hundred micro litres of standardized culture of *P. aeruginosa* was dispensed into well 1 through well 9 and well 11 respectively. The plate was incubated for 37 °C for 24 h. At the end of the incubation period 50 μL of tetrazolium dye was added to the wells and further incubated for 20 min to check for colour change from yellow to pink. Two

wells before and after the MIC well was sub-cultured on Mueller Hinton agar at 37 °C for 24 h, the least concentration that killed the organisms on sub-culturing was taken as the MBC.

3.7 Animal Studies

3.7.1 Laboratory animals

Twenty-seven Wister rats (*Rattus novergicus*) with an average weight of 147 g were used for the acute and sub-acute toxicity studies. The rats were obtained from Jos, Plateau State, Nigeria and then allowed to adapt for 2 weeks. In compliance with Good Laboratory Practice (GLP), the rats were housed in plastic cages bedded with clean dried wood shavings (saw dust) well aerated and were kept in well-spaced and ventilated house. The rats were fed with standard animal feeds and tap water *ad libitum*. Sanitation and changing of the soiled wood shaving was observed on a daily basis (Babayi *et al*., 2018[b]). The weight, feed and water intake were measured and recorded appropriately.

3.7.2 Toxicological studies of leech salivary extract silver nanoparticles

The acute and sub-chronic toxicity studies of LSE-AgNp were conducted as described by Shittu *et al*. (2015). The studies included the determination of LD_{50}, evaluation and prolonged effect of low concentration of the Nanoparticles on haematological parameters and serum biochemical indices in rats.

3.7.3 Determination of acute toxicity studies of leech salivary extract silver nanoparticles

In order to establish a safe range of doses to be administered to rats, the lethal dose 50 (LD_{50}) of the LSE-AgNp was determined using Lorke's method of 1983 with slight modifications (Babayi *et al*., 2018[b]). Twelve (12) rats were fasted for 12 hours before the administration of the conjugate. The study was conducted in two phases. In phase one, nine (9) rats were grouped into three (3) groups of three (3) rats each and were administered with 10 mg/kgbw,

100 mg/kgbw and 1000 mg/kgbw of the Nanoparticles respectively with the aid of an oral canular. Phase two was setup as phase one except that the numbers of rats per group was one and the range of doses were 1600 mg/kgbw, 2900 mg/kgbw and 5000 mg/kgbw respectively (Babayi *et al*., 2018$_b$; Olabayotan and Akin-Osanaiye, 2019). The rats in both phases and in each group were observed for 48 hours for physical signs of chemical intoxication, reaction and mortality. The volume of the LSE-AgNp to be administered was calculated in relation to the weight of the rats using equation 3.1

$$\text{Volume (mL)} = \frac{\text{Weight of animal (g) X Dose to administer}}{\text{Concentration (mg/mL)}} \text{ X } \frac{1000\text{g}}{1} \quad (3.1)$$

3.7.4 Determination of sub-acute toxicity of leech salivary extract silver nanoparticles

The method of Shittu *et al*. (2015) was employed for the determination of the sub-chronic toxicity of the LSE-AgNp. Fifteen (15) rats were selected and divided into five groups of three rats each. Group one through three were administered with the main treatment of LSE-AgNp at concentration of 25 mg/kgbw, 50 mg/kgbw and 100 mg/kgbw respectively. Group four were administered with 0.2 mL of normal saline which served as the negative control while group five (5) were administered with 0.2 ml of 2 mM silver nitrate ($AgNO_3$) solution as the positive control for a period of twenty one (21) days. The body weight, the feed and fluid intake of the rats were taken daily for the 21 days. The rats were starved overnight before being sacrificed under chloroform anesthesia at the end of the twenty one days treatment. The blood was collected in heparin bottles for haematology test and plain bottles for serum biochemistry (Shittu *et al*., 2015). Body organs such as the liver, heart, kidneys, lungs and spleen were removed from each rat and weighed.

The relative organ weight (ROW) of the rats was calculated in relation to the weight of the rats using equation 3.2.

$$\text{ROW} = \frac{\text{Organ weight of animal r}}{\text{Body weight of animal on sacrifice day (g)}} \text{X } \frac{100}{1} \quad (3.2)$$

3.8 Statistical Analysis and Data Evaluation

Data generated in this study were expressed as the mean value ± Standard Error of Mean (S.E.M). Comparisons between different groups were carried out by Analysis of Variance (ANOVA). Significant differences between the control and experimental groups were determined by Duncan Multiple Range Test (DMRT) using the Statistical Package for Social Sciences (SPSS) version 21.

CHAPTER FOUR

4.0 RESULTS AND DISCUSION

4.1 Results

4.1.1 Identification of test isolates

Table 4.1 shows the microscopic, morphological and biochemical characteristics of the test isolates. The organisms isolated included: *Pseudomonas aeruginosa*, *Escherichia coli*, *Staphylococcus aureus* and *Klebsiella* spp.

4.1.2 Antibacterial activities of crude Leech salivary extract on test isolates

Table 4.2 revealed the antibacterial activities of crude leech salivary extract on the test isolates. The neat crude leech salivary extract was unable to inhibit the growth of all the test isolates at 100 µL and 200 µL. The standard antibiotics (Augmentin) used as the positive control inhibited the growth of the test isolates at 20 µg/ml with zones of inhibition of 20.00 ± 0.33 mm, 25.00 ± 0.57 mm, 27.00 ± 0.88 mm and 31.00 ± 1.20 mm against *S. aureus*, *Klebsiella* species, *E. coli* and *P. aeruginosa*. Distilled water which served as the negative control showed no inhibition on the growth of the test isolates.

Table 4.1: Microscopic, Morphological and Biochemical Characteristics of Test Isolates

GR	SHAPE	MO	CA	CO	CI	SH	IN	MR	H_2S	OX	UR	Sugar Fermentation					Suspected Organisms
												G	F	L	M	S	
G-	Rod	+	+	-	+	-	-	-	-	+	-	-	-	-	-	-	*P. aeruginosa*
G-	Rod	-	+	-	+	+	-	-	-	-	+	+	-	+	+	+	*Klebsiella* sp
G-	Rod	+	+	-	-	+	+	+	-	-	+	+	-	+	-	+	*Escherichia coli*
G+	Cocci	-	+	+	+	+	-	+	+	-	+	+	+	+	+	+	*Staphylococcus aureus*

GR: Gram's reaction, G+: Gram positive, G-: Gram negative, MO: Motility test, CO: Coagulase, CA: Catalase, CI: Citrate utilization, SH: Starch hydrolysis, IN: Indole, MR: Methyl red, H_2S: Hydrogen sulphide production, OX: oxidase, UR: Urease, G: glucose, F: Fructose, L: Lactose, M: Maltose, S: Sucrose.

Table 4.2: Antibacterial Activities of Crude Leech Salivary Extract on Test Isolates

Test isolates	Diameter Zones of inhibition (mm)			
	LSE (100 µL)	LSE (200 µL)	Dw (100 µL)	Aug (8 µg/mL)
P. aeruginosa	0.00 ± 0.00^{a}	0.00 ± 0.00^{a}	0.00 ± 0.00^{a}	31.00 ± 1.20^{b}
Klebsiella sp	0.00 ± 0.00^{a}	0.00 ± 0.00^{a}	0.00 ± 0.00^{a}	25.00 ± 0.57^{b}
Escherichia coli	0.00 ± 0.00^{a}	0.00 ± 0.00^{a}	0.00 ± 0.00^{a}	27.00 ± 0.88^{b}
Staphylococcus aureus	0.00 ± 0.00^{a}	0.00 ± 0.00^{a}	0.00 ± 0.00^{a}	20.00 ± 0.33^{b}

Values are means ± standard deviation. Values with the same superscript in a row have no significant difference at ($P > 0.05$). LSE: Leech salivary extract, µL: Micro litre, Dw: Distilled water, Aug: Augmentin, µg/ml: Microgram per millilitre mm: millimetre.

4.1.3 Characteristics of Leech salivary extract silver nanoparticles

The LSE-AgNp formed was a dark brown precipitate. The wavelength of the precipitate was at 456 nm and the absorbance was 0.37 (Figure 4.1a). The size of the particle was 98 nm (Figure 4.1b).

(a)

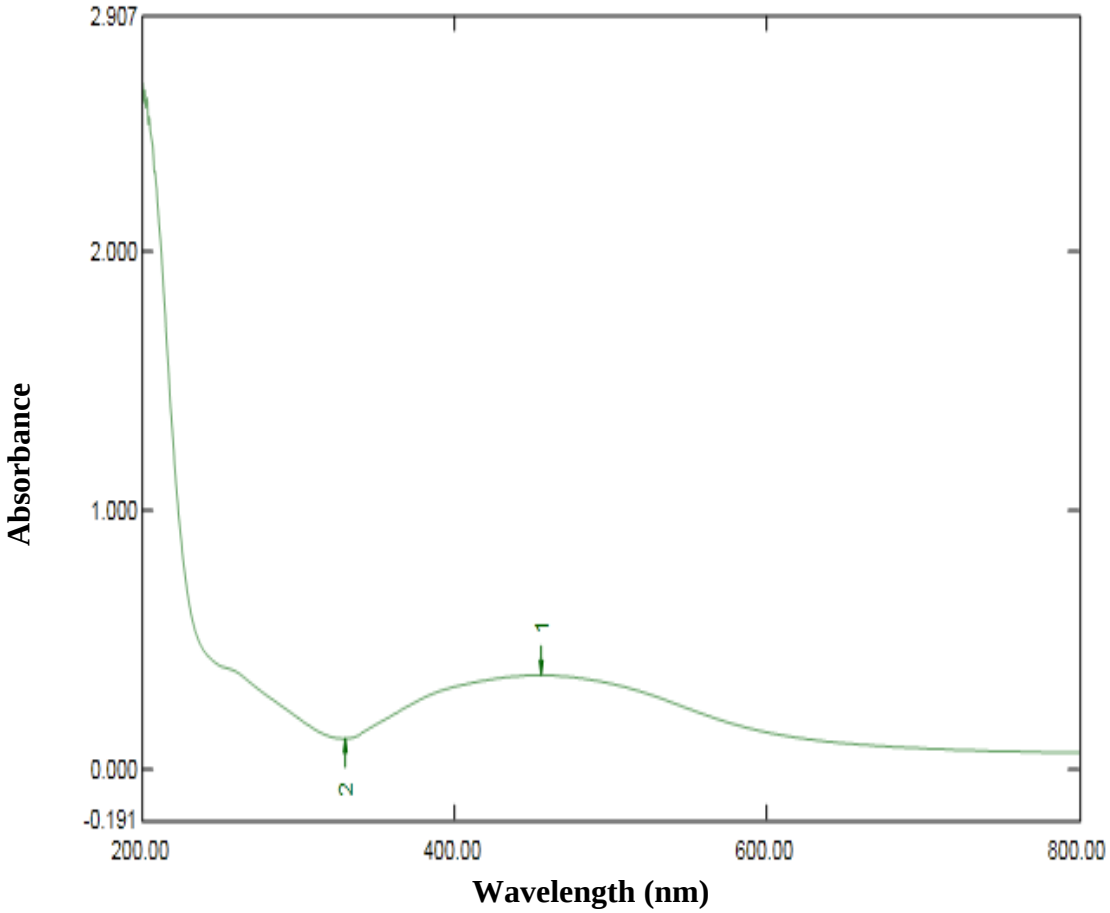

Figure 4.1a: Ultraviolet-Visible-Spectrum of Leech Salivary Extract Silver Nanoparticles

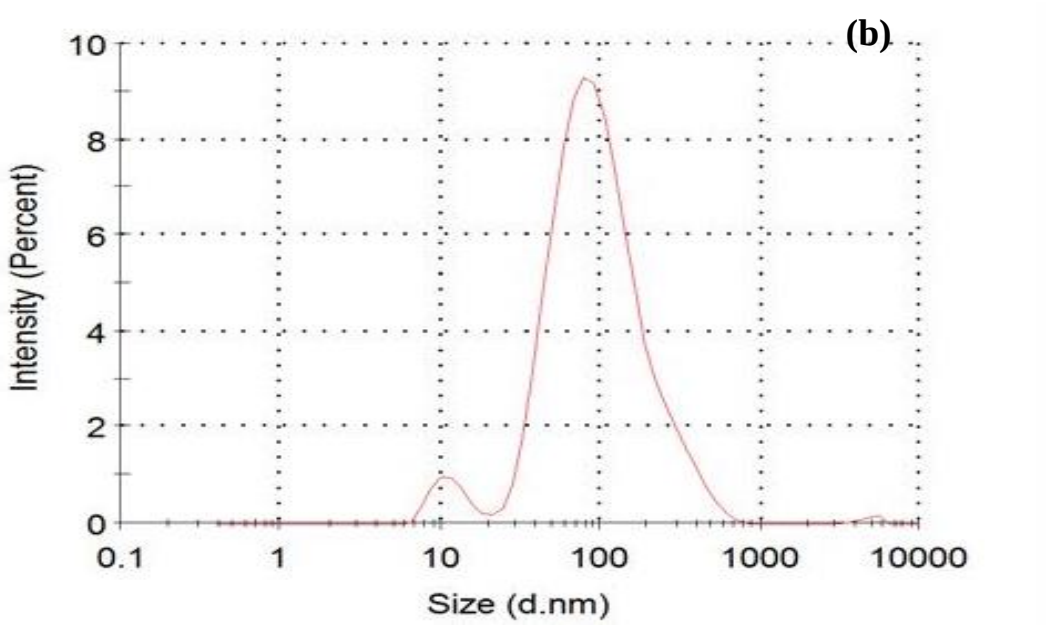

Figure 4.1b: Zeta Nano Size Distribution Pattern of Leech Salivary Extract Silver Nanoparticles

4.1.4 Antibacterial activities of Leech salivary extract silver nanoparticles on test isolates

The antibacterial activities of leech salivary extract silver nanoparticles against the test isolates at 100 µL and 200 µL respectively are shown in Table 4.3. The diameter zones of inhibition produced by LSE-AgNp are 8.33 ± 0.88 mm and 12.33 ± 0.33 mm for *P. aeruginosa* at 100 µL and 200 µL respectively. It produced 12 ± 0.57 mm and 12.33 ± 0.33 mm for *Klebsiella* spp. at 100 µL and 200 µL respectively while at the same concentration *E. coli* and *S. aureus* were resistant to it. The activities of the LSE-AgNp was compared with Augmentin which produced diameter zones of inhibition of 20.33 ± 0.33 mm, 25.00 ± 0.57 mm, 27.33 ± 0.88 mm and 31.33 ± 1.20 mm, for *S. aureus*, *Klebsiella* spp., *E. coli* and *P. aeruginosa*.

Table 4.3: Antibacterial Activities of Leech Salivary Extract Silver Nanoparticles on Test Isolates

Test isolates	**Diameter Zones of Inhibition (mm)**			
	LSE (100 µL)	**LSE (200 µL)**	**Dw (100 µL)**	**Aug (8 µg/mL)**
Pseudomonas aeruginosa	8.33 ± 0.88^{b}	12.33 ± 0.88^{c}	0.00 ± 0.00^{a}	31.33 ± 1.20^{d}
Klebsiella spp.	12.33 ± 0.33^{b}	12.00 ± 0.57^{b}	0.00 ± 0.00^{a}	25.00 ± 0.57^{c}
Staphylococcus aureus	0.00 ± 0.00^{a}	0.00 ± 0.00^{a}	0.00 ± 0.00^{a}	20.33 ± 0.33^{b}
Escherichia coli	0.00 ± 0.00^{a}	0.00 ± 0.00^{a}	0.00 ± 0.00^{a}	27.33 ± 0.88^{b}

Values are means ± standard error of mean. Values with the same superscript in a row have no significant difference at (P > 0.05). LSE: Leech salivary extract, µL: Micro litre, Dw: Distilled water, Aug: Augmentin, µg/mL: Microgram per millilitre mm: millimetre.

4.1.5 Minimum inhibitory and bactericidal concentrations of Leech salivary extract silver nanoparticles on test isolates

Table 4.4 revealed the minimum inhibitory and bactericidal concentration of leech salivary extract silver nanoparticles on test isolates. The MIC of the Nanoparticles against both *P. aeruginosa* and *Klebsiella* spp. was at 100 µL while the MBC was 200 µL respectively.

Table 4.4: Minimum Inhibitory and Bactericidal Concentration of Leech Salivary Extract Silver Nanoparticles on Test Isolates

Test Isolates	Concentrations of LSE-AgNp (µL)									
	200	**100**	**50**	**25**	**12.5**	**6.25**	**3.12**	**1.56**	**MIC**	**MBC**
P. aeruginosa	+	-	-	-	-	-	-	-	100	200
Klebsiella spp.	+	-	-	-	-	-	-	-	100	200

+: growth, -: no growth, LSE-AgNp: leech salivary extract silver nanoparticles,

MIC: minimum inhibitory concentration, MBC: minimum bactericidal concentration.

4.1.6 Acute toxic dose of Leech salivary extract silver nanoparticles in Wister rats

Table 4.5 shows the acute toxic dose (LD_{50}) of LSE-AgNp in Wister albino rats. The administration of doses ranging from 100 to 5000 mg/kgbw of the nanoparticles was not accompanied by physical and behavioural changes. The LD_{50} of the nanoparticles was greater than 5000 mg/kgbw.

Table 4.5: Acute Toxic Dose of Leech Salivary Extract Silver Nanoparticles in Wister Rats

Group	Dosage (mg/kgbw)	Mortality/No. of rats
1	100	0/3
2	500	0/3
3	1000	0/3
4	1600	0/3
5	2900	0/3
6	5000	0/3

mg/kgbw: milligram per kilogram body weight, No.: number of rats

4.1.7 Effect of Leech salivary extract silver nanoparticles on the body weight of Wister rats

The effect of LSE-AgNp on the body weight of Wister rats are shown in Table 4.6. The body weight of rats in all the groups (25 mg/kgbw, 50 mg/kgbw and 100 mg/kgbw) were not significantly ($P > 0.05$) different from the control groups (rats treated with $AgNO_3$ and normal saline).

Table 4.6: Effect of Leech Salivary Extract Silver Nanoparticles on Body Weight of Wister Rats

Treatment (mg/kgbw)	Body Weight of Rats Over Time (weeks)			
	0	**1**	**2**	**3**
25	148.77 ± 15.63[a]	159.62 ± 17.62[a]	161.89 ± 16.89[a]	177.91 ± 16.81[a]
50	157.02 ± 8.54[a]	148.82 ± 6.96 [a]	152.07 ± 5.47[a]	160.56 ± 6.14[a]
100	161.23 ± 13.094[a]	172.91 ± 20.48[a]	190.17 ± 42.21[a]	161.14 ± 9.44[a]
Ns	150.87 ± 13.99[a]	161.83 ± 18.21[a]	167.29 ± 17.77[a]	181.62 ± 12.39[a]
$AgNO_3$	132.20 ± 10.63 [a]	146.84 ± 6.03[a]	154.41 ± 4.05[a]	162.99 ± 7.95[a]

Values are means ± standard error of mean. Values with the same superscript in a column have no significant difference at ($P > 0.05$). $AgNO_3$: silver nitrate (positive control), mg/kgbw: milligram per kilogram body weight, Ns: normal saline (negative control).

4.1.8 Effect of Leech salivary extract silver nanoparticles on the relative organ weight of Wister rats

Table 4.7 shows the effect of LSE-AgNp on the relative organ weight of Wister rats. The results revealed that the weight of the heart, lungs, kidney and liver of the rats in all the treated groups (25 mg/kgbw, 50 mg/kgbw and 100 mg/kgbw) were not significantly ($P > 0.05$) different from the control groups (rats treated with $AgNO_3$ and normal saline). However, the spleen of the rats exposed to 100 mg/kgbw was significantly ($P < 0.05$) increased from the treated and control groups respectively.

Table 4.7: Effect of Leech Salivary Extract Silver Nanoparticles on Relative Organ Weight of Wister Rats

Treatment (mg/kgbw)	Relative Organ Weight (g)				
	Heart	**Kidney**	**Liver**	**Spleen**	**Lungs**
25	0.35 ± 0.00^{a}	0.53 ± 0.01^{a}	3.60 ± 0.51^{a}	0.16 ± 0.02^{a}	0.77 ± 0.01^{a}
50	0.27 ± 0.06^{a}	0.68 ± 0.06^{a}	3.77 ± 0.33^{a}	0.19 ± 0.03^{a}	0.93 ± 0.28^{a}
100	0.45 ± 0.11^{a}	0.81 ± 0.18^{a}	4.85 ± 0.62^{a}	0.36 ± 0.03^{b}	0.88 ± 0.12^{a}
Ns	0.32 ± 0.01^{a}	0.55 ± 0.02^{a}	3.71 ± 0.62^{a}	0.22 ± 0.01^{a}	0.76 ± 0.02^{a}
$AgNO_3$	0.41 ± 0.02^{a}	0.61 ± 0.03^{a}	4.01 ± 0.07^{a}	0.20 ± 0.02^{a}	0.73 ± 0.01^{a}

Values are means ± standard deviation. Values with the same superscript in a column have no significant difference at ($P > 0.05$). $AgNO_3$: silver nitrate (positive control), mg/kgbw: milligram per kilogram body weight, Ns: normal saline (negative control), g: grams.

4.1.9 Effect of Leech salivary extract silver nanoparticles on feed intake of Wister rats

Table 4.8 displays the effect of LSE-AgNp on the feed intake of Wister rats. At week 1, there was no significant difference at $P > 0.05$ in any of the treatment groups. At week 2, the feed intake in group 1, 3 and 5 were not statistically ($P > 0.05$) different from one another other. The animals given normal saline (negative control) had the highest feed intake followed by those given 100 mg/kgbw of the nanoparticles while animals given 50 mg/kgbw had the least feed intake. At week 3, there was no statistical difference at $P > 0.05$ in any of the treatment groups. Animals given 100 mg/kgbw of the nanoparticles had the least feed intake while animals given 50 mg/kgbw had the highest feed intake.

Table 4.8: Effect of Leech Salivary Extract Silver Nanoparticles on Feed Intake of Wister Rats

Treatment (mg/kgbw)	Feed Intake of Rats Over Time (weeks)		
	1	2	3
25	49.66 ± 2.84^{a}	44.66 ± 7.26^{ab}	45.89 ± 6.27^{a}
50	44.99 ± 6.82^{a}	40.29 ± 11.12^{a}	51.15 ± 8.03^{a}
100	41.24 ± 5.94^{a}	50.92 ± 6.91^{ab}	36.00 ± 5.74^{a}
Ns	49.77 ± 3.45^{a}	67.30 ± 8.45^{b}	50.05 ± 4.04^{a}
$AgNO_3$	44.96 ± 1.39^{a}	53.23 ± 5.39^{ab}	50.02 ± 5.83^{a}

Values are means ± standard error of mean. Values with the same superscript in a column have no significant difference at ($P > 0.05$). $AgNO_3$: silver nitrate (positive control), mg/kgbw: milligram per kilogram body weight, Ns: normal saline (negative control).

4.1.10 Effect of Leech salivary extract silver nanoparticles on the fluid intake of Wister rats

Table 4.9 shows the effect of LSE-AgNp on the fluid intake of Wister albino rats. At week 1, the rats administered silver nitrate had the least fluid intake when compared with other groups while groups administered with 25 mg/kgbw and 50 mg/kgbw of the nanoparticles had similar fluid intake. In week 2, groups administered 25 mg/kgbw, 50 mg/kgbw and normal saline had similar fluid intake while groups administered 100 mg/kgbw and $AgNO_3$ equally had similar fluid intake. In week 3, rats administered with 100 mg/kgbw had the least fluid intake when compared with other groups while groups administered with 50 mg/kgbw and normal saline had similar fluid intake. The group administered with 25 mg/kgbw had the highest fluid intake.

Table 4.9: Effect of Leech salivary extract silver nanoparticles on fluid intake of Wister rats

Treatment (mg/kgbw)	Fluid Intake of Rats Over Time (weeks)		
	1	2	3
25	285.71 ± 17.70^{c}	328.33 ± 18.15^{b}	322.85 ± 9.68^{d}
50	267.14 ± 15.54^{c}	300.00 ± 0.00^{b}	278.51 ± 12.03^{c}
100	180.00 ± 9.51^{ab}	188.33 ± 14.00^{a}	185.71 ± 13.42^{a}
Ns	218.57 ± 10.10^{b}	283.33 ± 23.47^{b}	275.71 ± 9.47^{c}
$AgNO_3$	165.71 ± 18.63^{a}	218.33 ± 10.13^{a}	245.71 ± 4.28^{b}

Values are means ± standard error of mean. Values with the same superscript in a column have no significant difference at ($P > 0.05$). $AgNO_3$: silver nitrate (positive control), mg/kgbw: milligram per kilogram body weight, Ns: normal saline (negative control).

4.1.11 Effect of Leech salivary extract silver nanoparticles on the haematological parameters of Wister rats

Table 4.10 reveals the effect of LSE-AgNp on the haematological parameters of Wister rats. The haematological parameters (packed cell volume, haemoglobin, red blood cells, white blood cells, platelets, mean cell volume, mean corpuscular haemoglobin and mean corpuscular haemoglobin concentration) in all the treated groups (25 mg/kgbw, 50 mg/kgbw and 100 mg/kgbw) were not significantly ($P > 0.05$) different from the control groups (rats treated with $AgNO_3$ and normal saline).

Table 4.10: Effect of Leech Salivary Extract Silver Nanoparticles on the Haematological Parameters of Wister Rats

Treatment (mg/kgbw)	Haematological parameters							
	Pcv (%)	**Hb (g/dL)**	**Wbc ($x10^9$)**	**Rbc ($x10^9$)**	**Platelet ($x10^6$)**	**Mcv (fL)**	**Mch (pg)**	**Mchc (g/dL)**
25	34.00 ± 4.00[a]	12.70 ± 0.00[a]	5.70 ± 0.60 [a]	5.70 ± 0.40[a]	208.00 ± 2.00[a]	66.00 ± 6.00[a]	22.00 ± 2.00 [a]	37.80 ± 4.40[a]
50	42.50 ± 2.50[a]	14.15 ± 0.80[a]	4.30 ± 0.20 [a]	4.65 ± 0.35[a]	255.00 ± 65.00[a]	92.00 ± 12.00[a]	31.00 ± 4.00 [a]	31.80 ± 1.50[a]
100	43.00 ± 5.00 [a]	14.50 ± 1.50[a]	4.85 ± 0.80 [a]	4.85 ± 0.80[a]	230.50 ± 19.50[a]	91.00 ± 7.00[a]	30.50 ± 2.40[a]	33.30 ± 0.00[a]
Ns	41.00 ± 7.00 [a]	13.65 ± 2.30 [a]	5.00 ± 1.00[a]	5.45 ± 0.70[a]	215.00 ± 5.00[a]	74.50 ± 2.50[a]	25.00 ± 1.00[a]	33.20 ± 0.10[a]
$AgNO_3$	45.50 ± 3.50[a]	15.15 ± 1.10[a]	4.80 ± 0.40[a]	4.75 ± 0.30 [a]	248.50 ± 61.50[a]	96.50 ± 14.50[a]	32.20 ± 4.80[a]	33.30 ± 0.00[a]

Values are means ± standard error of mean. Values with the same superscript in a column have no significant difference at ($P > 0.05$). $AgNO_3$: silver nitrate, Ns: normal saline, mg/kgbw: milligram per kilogram body weight, Pcv: packed cell volume, Hb: haemoglobin, Rbc: red blood cells, white blood cells, Mcv: mean cell volume, Mch: mean corpuscular haemoglobin, Mchc: mean corpuscular haemoglobin concentration.

4.1.12 Effect of Leech salivary extract silver nanoparticles on differential blood count of Wister rats

The effects of LSE-AgNp on the differential blood count on Wister rats are shown in Table 4.10. The differential blood count (neutrophils, lymphocytes, monocytes, eosinophils and basophils) in all the treated groups (25 mg/kgbw, 50 mg/kgbw and100 mg/kgbw) were not significantly ($P > 0.05$) different from the control groups (rats treated with $AgNO_3$ and normal saline).

Table 4.11: Effect of Leech Salivary Extract Silver Nanoparticles on Differential Blood Count of Wister Rats

Treatment (mg/kgbw)	Neutrophil	Lymphocyte	Monocyte	Eosinophil	Basophil
25	52.50 ± 2.50^a	41.00 ± 4.00^a	4.00 ± 1.00^a	2.50 ± 0.50^a	0.00 ± 0.00^a
50	46.50 ± 1.50^a	48.50 ± 1.50^a	3.50 ± 0.50^a	1.50 ± 0.50^a	0.00 ± 0.00^a
100	50.00 ± 10.00^a	40.50 ± 10.50^a	5.00 ± 1.00^a	3.50 ± 0.50^a	0.50 ± 0.50^a
Ns	51.00 ± 2.00^a	44.00 ± 4.00^a	3.00 ± 0.00^a	2.00 ± 2.00^a	0.00 ± 0.00^a
$AgNO_3$	34.50 ± 0.50^a	59.00 ± 1.00^a	4.50 ± 0.50^a	2.00 ± 1.00^a	0.00 ± 0.00^a

Values are means ± standard error of mean. Values with the same superscript in a column have no significant difference at ($P > 0.05$). $AgNO_3$: silver nitrate, Ns: normal saline, mg/kgbw: milligram per kilogram body weight.

4.1.13 Effect of Leech salivary extract silver nanoparticles on liver indices of Wister rats

Table 4.12 reveals the effects of LSE-AgNp on the liver of Wister rats. The levels of total protein, Albumin, Alkaline phosphatase, Aspartate amino transferase and Alanine amino transferase in all the treated groups (25 mg/kgbw, 50 mg/kgbw and 100 mg/kgbw) were not

significantly (P > 0.05) different from the control groups (rats treated with $AgNO_3$ and normal saline).

Table 4.12: Effect of Leech Salivary Extract Silver Nanoparticles on the Liver Indices of Wister Rats

Treatment (mg/kgbw)	Protein (g/dL)	Albumin (g/mL)	AST (u/L)	ALT (u/L)	ALP (u/L)
25	6.50 ± 0.10^a	3.40 ± 0.20^a	37.00 ± 5.00^a	18.60 ± 2.40^a	49.00 ± 7.00^a
50	5.70 ± 0.50^a	3.70 ± 0.10^a	41.00 ± 1.00^a	18.15 ± 2.85^a	53.00 ± 1.00^a
100	5.65 ± 1.05^a	2.40 ± 10.20^a	42.00 ± 6.00^a	14.65 ± 2.45^a	48.50 ± 9.50^a
Ns	4.90 ± 0.50^a	2.40 ± 1.40^a	38.00 ± 6.00^a	14.10 ± 2.10^a	44.50 ± 8.50^a
$AgNO_3$	6.40 ± 0.20^a	3.45 ± 0.10^a	47.20 ± 1.00^a	16.30 ± 0.10^a	55.00 ± 1.00^a

Values are means ± standard error of mean. Values with the same superscript in a column have no significant difference at (P > 0.05). $AgNO_3$: silver nitrate, Ns: normal saline, mg/kgbw: milligram per kilogram body weight, g/dL: grams per decilitre, mg/dL: milligrams per decilitre, u/L: micro litre, ALP: Alkaline phosphatase, AST: Aspartate amino transferase, ALT: Alanine amino transferase.

4.1.14 Effect of Leech salivary extract silver nanoparticles on kidney indices of Wister rats

Table 4.13 shows the effects of LSE-AgNp on the kidney indices of Wister rats. The concentrations of creatinine , urea, potassium and sodium in all the treated groups (25 mg/kgbw, 50 mg/kgbw and100 mg/kgbw) were not significantly (P > 0.05) different from the control groups (rats treated with $AgNO_3$ and normal saline).

Table 4.13: Effect of Leech Salivary Extract Silver Nanoparticles on the Kidney Indices of Wister Rats

Treatment (mg/kgbw)	Urea (mmol/L)	Sodium (mmol/L)	Potassium (mmol/L)	Creatinine (mg/dL)
25	3.90 ± 0.30^{a}	145.00 ± 3.00^{a}	4.90 ± 0.30^{a}	0.50 ± 0.00^{a}
50	3.60 ± 0.40^{a}	126.00 ± 0.00^{a}	4.55 ± 0.35^{a}	0.45 ± 0.05^{a}
100	3.30 ± 0.50^{a}	129.00 ± 17.00^{a}	3.75 ± 1.15^{a}	0.50 ± 0.30^{a}
Ns	3.00 ± 0.40^{a}	119.00 ± 9.00^{a}	3.30 ± 1.00^{a}	0.50 ± 0.10^{a}
$AgNO_3$	3.65 ± 0.15^{a}	145.00 ± 3.00^{a}	4.65 ± 0.05^{a}	0.70 ± 0.10^{a}

Values are means ± standard error of mean. Values with the same superscript in a column have no significant difference at ($P > 0.05$). $AgNO_3$: silver nitrate, Ns: normal saline, mg/kgbw: milligram per kilogram body weight, mmol/L: millimoles per litre, mg/dL: milligram per decilitre.

4.2 Discussion

In the present study, the test isolates were catalase positive (*P*seudomonas *aeruginosa*, *Escherichia coli*, *Staphylococcus aureus* and *Klebsiella* spp), oxidase negative (*E. coli*, *S. aureus* and *K*lebsiella spp), H_2S negative (*P. aeruginosa*, *Klebsiella* spp and *E. coli*), urease hydrolyser (*E. coli*, *S. aureus* and *Klebsiella* spp), glucose, lactose and sucrose fermenters (*E. coli*, *S. aureus* and *Klebsiella* spp), starch hydrolyser (*E. coli*, *S. aureus* and *Klebsiella* spp). This is similar with the findings of Alhubail *et al*. (2020) that identified similar organisms using biochemical tests. Gram staining of the test isolates revealed Gram negative rods (*P. aeruginosa, E. coli* and *Klebsiella* spp) and Gram positive coccus (*S. aureus*). This is similar to the report of Abdallah *et al*. (2016) who used Gram staining to classify the same organisms.

In the present study, the crude leech salivary extract (LSE) showed no inhibitory effect on the test isolates. This could be as a result of low concentrations of bioactive molecules in the extract. This result contradicts the report of Malik *et al*. (2019) who reported that, leech salivary extract showed inhibitory effect on some of the test isolates used in this study. The Leech salivary extract silver nanoparticles were observed to inhibit the growth of *P. aeruginosa* and *Klebsiella* spp while *S. aureus* and *E. coli* were resistant to it. Ojo *et al*. (2018) reported that bioactive compounds such as proteins and antimicrobial peptides (AMP), 4-bromobutyric acid, 6, 17-Octadiene-1-ol acetate and octahydro-1, 4, 9, 9-tetramethyl to be responsible for LSE's activity. The observed activity in the LSE-AgNp as against the crude LSE counterpart could be due to the submicron (minute) size of silver nanoparticles which enables it to act as a drug carrier by incorporating bioactive substances (hydrophilic and hydrophobic) into its matrix. The bioactive substances in the matrix of the nanoparticles may inhibit the growth of microorganisms by reacting with thiol group of some enzymes or respiratory enzymes leading to death or the release of reactive oxygen species which attacks microbial cells or accumulates itself in pits that form on the cell wall and punching holes in the membrane of microorganisms leading to denaturation and cell death (Shikha *et al*., 2020). This is result corroborates with the report of Malik *et al*. (2019) who attributed the activity of silver nanoparticles against some pathogenic organisms to it size.

In this study, formation of silver nanoparticles using Leech salivary extract was viewed by a colour change, from colourless to dark brown. Similarly, Saravana *et al*. (2018) reported that silver nanoparticles exhibited striking colour change from colourless to dark brown in the aqueous solution which is due to excitation of surface Plasmon resonance. By using UV-vis spectrum, the maximum absorbance peak was 456 nm. Similarly, Saravana *et al*.

(2018) also reported absorption spectra of silver nanoparticles found in the reaction media to range from 250 to 600 nm. The size of the particle synthesized in this study, was revealed to be 98.04 nm. This result is in agreement with the report of Ganna *et al*. (2020) who described evaluated the characteristics of *Curcumin* loaded magnesium oxide nanoparticles and described the size of nanoparticles as a particle size ranging from 1 to 100 nm.

In this study, the minimum inhibitory concentrations (MIC) were at 100 µL for *Pseudomonas aeruginosa* and *Klebsiella* spp. The minimum bactericidal concentrations (MBC) of the LSE-AgNp were at 200 µL for *Pseudomonas aeruginosa* and *Klebsiella* spp. This result contradicts the findings of Loo *et al*. (2018) who reported the MIC for *Klebsiella* sp to be 3.9 µg/mL when evaluating the in-vitro activity of green synthesized silver nanoparticles against selected Gram negative food borne pathogens such as *Escherichia coli*, *Klebsiella* spp, *Salmonella* Typhimurum and *Salmonella enteritidis*.

The value of LD_{50} for a test substance is the dose required to slay half the number of a tested population after a period of time. The results obtained from the study revealed that the administration of LSE-AgNp in rats was not accompanied by mortality and other signs of toxicity at a dose limit of 5000 mg/kgbw. This result agrees with the report of Olobayotan and Akin-Osanaiye, (2019) that observed the biosynthesized silver nanoparticles from *Saccharomyces cerevisae* exhibited LD_{50} above 5000 mg/kgbw and was therefore non-toxic acutely. Ojo *et al*. (2018) also supported this claim when they reported that the LD_{50} of crude Leech salivary extract was above 5000 mg/kgbw.

Alterations in the body weight of rats serve as a sensitive indication of the general health status of the animal and also one of the critical signs of toxicity. Increase in body weight may be presumed as normal while decrease in body weight may be seen as a sign of ill

health. In this study, the body weight of the animals increased non-significantly ($P > 0.05$) at 25 mg/kgbw, 50 mg/kgbw and 100 mg/kgbw with the exception of 100 mg/kgbw in week 3 which non-significantly ($P > 0.05$) reduced. This might be an indication that the nanoparticles did not hinder the growth of the animal as reported by Luaibi and Qassim (2018) who observed that silver nanoparticles did not significantly ($P > 0.05$) affect the body weight of rats.

The organ weight is a very integral component in the assessment of chemical compounds for their potential harmful effects on the organs. It is a sensitive indicator of the effect exerted by an experimental substance. Results of this study showed that there was no significant difference at $P > 0.05$ in the relative weights of kidneys, liver, heart, and lungs with the exception of the spleen at 100 mg/kgbw. This implied that at 100 mg/kgbw and above, there is tendency for the LSE-AgNp to cause damage to the spleen of the rats. This result contradicts the findings of Babayi *et al*. (2018[b]) who reported that the administration of crude Leech salivary extract at 25 mg/kgbw 50 mg/kgbw and 100 mg/kgbw in rats showed no significant ($P > 0.05$) effect on the weight of the spleen of rats.

The feed intake in rats provides useful hint to the effectiveness of a treatment. In the present study, the results of the feed intake of rats decreased significantly ($P < 0.05$) in week 2 when compared with the control groups (rats treated with $AgNO_3$ and normal saline). The observed decrease in feed intake could be as a result of the nanoparticles interfering with the hypothalamus of the rats. This result contradicts the report of Luaibi and Qassim (2018) who noted that the administration of rats with silver nanoparticles synthesized chemically did not significantly ($P > 0.05$) affect their feed intake. The results of the study also contradicts the findings of Babayi *et al*. (2018[b]) who noted an insignificant

($P > 0.05$) increase in the feed intake of rats when fed with crude Leech salivary extract for 28 days.

In the present study, the fluid intake increased significantly ($P < 0.05$) in all the treatment groups when compared with the control groups. The observed increase may imply that the nanoparticles could stimulate dehydration in rats. This result contradicts the findings of Luaibi and Qassim (2018) who noted that the administration of rats with silver nanoparticles synthesized chemically did not significantly ($P > 0.05$) affect their fluid intake.

Assessment of the haematological parameters: red blood cells, white blood cells, and platelets, indicates the presence or absence of foreign compounds in blood constituent of a living system and the investigation of these parameters help in identifying several constituents that are of nutritional, pathological and physiological benefits to the status of a living system. Indices of the differential blood count: Basophils, Neutrophils, Lymphocytes, Eosinophils and Monocytes are haematological parameters which confer immunity to a living system defending the body against foreign bodies. The results of this study showed that there was no significant alteration in the differential blood counts of the tested animals compared with the control. This suggests that the consumption of the LSE-AgNp did not induce toxic effect that may trigger immune response or allergic reactions in rats. Similar observation was made in previous report by Babayi *et al*. (2018_b) in which prolonged administration of crude Leech salivary extract had no significant ($P > 0.05$) effect on the values of the haematological parameters of rats.

The values derived from the haematological parameters of the rats treated with the nanoparticles in this study were in tandem with the standard reference range in humans for WBC (4 to 10 x 10^9/L), RBC (4.5 to 6.5 x 10^{12}/L), Platelets (150 to 400 x 10^9/L), PCV (40

to 52 %), HB (13 to 17 g/dL) and MCHC (30 to 35 g/dL) except for the group of rats exposed to 25 mg/kgbw of the conjugate. MCH (27 to 32 pg) and MCV (80 to 100 fL) were also similar to human values except in groups treated with 25 mg/kgbw and normal saline (Farinde, 2019).

The values also derived from the parameters of the differential blood count of Wister rats treated with the nanoparticles were similar for Monocytes (2 to 8 %) and Eosinophils (1 to 4 %) while Neutrophils (55 to 70 %) and Basophils (1 to 4 %) were slightly below the reference range for humans. Values of Lymphocytes (20 to 40 %) were slighty above the human reference range (Choladda, 2019).

Liver is one of the organs of the body of humans and animals. It is tasked with the responsibility of regulating homeostasis and metabolism of toxic chemical substances or drugs when introduced into the body system, hence making it so vulnerable to impairment. The assessment of serum biochemical indices such as: Alanine amino transferase (ALT), Aspartate amino transferase (AST), Alkaline phosphatase (ALP), Albumin and Total proteins are thus germane in predicting the integrity of liver following the administration of drugs, plant extracts, animal metabolites and nanoparticles (synthesized from plant and animal metabolites). Alterations in the concentration of any of these indices are a conventional indication of the dysfunctionality of the liver. In the present study, the results showed no significant ($P > 0.05$) alterations in the serum concentration of ALT, AST, ALP, Albumin and Total proteins when compared with the control values. This could suggest that, the functional integrity of liver of the rats was not compromised during and after the treatment period. This result corroborates with the findings of Yusuf *et al*. (2018) who reported that the administration of *Zingiber officinale* in rats for 28 days caused no significant alterations in the levels of ALT, AST, ALP, Albumin and Total proteins.

The value obtained from the liver indices of Wister albino rats in this study were within the standard reference values in humans for ALT (5 to 30 u/L). It was slightly lower to human reference value for AST (5 to 30 u/L), Albumin (35 to 50 g/L), ALP (50 to 100 u/L) at 25 mg/kgbw, 50 mg/kgbw and normal saline treatment groups. The total protein (60 to 80 g/L) was also slightly lower in range at 50 mg/kgbw, 100 mg/kgbw and normal saline treatment groups (Farinde, 2019).

The kidneys of animals and humans play a very cogent role in regulating the excretion and re-absorption of substances such as urea, creatinine and electrolytes that can potentially cause harm to the body system when found in an increased concentration in the serum. The renal function test conventionally determines the functionality and dysfunctionality of the renal organ in performing their excretory duties of removing (by filtration) harmful substances in the body. In the present study, the results of treating rats with the nanoparticles caused no significant ($P > 0.05$) alterations to the serum concentration of creatinine, urea, sodium and potassium when compared with the control values. This could imply that the consumption of LSE-AgNp at concentration of 25, 50 and 100 mg/kgbw may not cause any glomerular impairment to the kidney. This finding contradicts the report of Adeyemi and Adewumi (2014) that, the administration of nanoparticles significantly ($P < 0.05$) elevated the levels of urea, creatinine and electrolytes in rats. This result further corroborates with the findings of Babayi *et al*. (2018[b]) that crude leech salivary extract administration for 28 days had no significant ($P > 0.05$) effect on the electrolyte levels as well as the creatinine and urea levels of the rats. The values obtained from the kidney indices of Wister rats treated with the nanoparticles in this study, were not above the standard reference range in humans for creatinine (≥ 4.0 mg/dL) and potassium (≤ 2.5 mmol/L or ≥ 6.5 mmol/L) and sodium (135 to 145 mmol/L). Urea (1.2 to 3.0 mmol/L) was

slightly above the human reference range except for the group treated with normal saline (Gary, 2019; Gates, 2019).

CHAPTER FIVE

5.0 CONCLUSION AND RECOMMENDATIONS

5.1 Conclusion

The present study revealed that crude leech salivary extract (LSE) exhibited no antimicrobial activity against *Escherichia coli*, *Staphylococcus aureus*, *Pseudomonas aeruginosa* and *Klebsiella* spp at 100 µL and 200 µL. However, the Leech salivary extract-silver nanoparticles (LSE-AgNp) showed antimicrobial activity against *P. aeruginosa* and *Klebsiella* spp at 100 µL and 200 µL respectively. The minimum inhibitory and bactericidal concentration of LSE-AgNp for *P. aeruginosa* and *Klebsiella* species was at 100 µL and 200 µL respectively. The LSE-AgNp was bactericidal on the test isolates.

The LSE-AgNp formed was at wavelength 456 nm and size 98.04 nm. Oral LD_{50} of the LSE-AgNp was above 5000 mg/kgbw. Oral administration of LSE-AgNp for 21days caused no alterations of haematological indices (Pcv, Mch, Hb, Mcv, Mchc and platelets), white blood cell and differential blood count (Basophils, Neutrophils, Eosinophils, Monocytes and Lymphocytes). The nanoparticles reduced the feed intake and increased the fluid intake of rats as treatment progressed. The LSE-AgNp produced no toxic effect on the relative weight of the lungs, liver, heart and kidneys of rats but significantly ($P < 0.05$) increased the weight of the spleen at 100 mg/kgbw.

The chronic administration of the LSE-AgNp had no toxic effects on the serum total protein, aspartate amino transferase, alanine amino transferase and the alkaline phosphate, sodium, potassium, creatinine and urea levels of the rats.

5.2 Recommendations

It is recommended that:

i. the ability of the LSE-Ag to exert bactericidal effect on *Pseudomonas aeruginosa* and *Klebsiella* species is a significant step in the hunt for novel drug for the treatment of their infections.

ii. the LSE-AgNp could be considered as a novel drug in the treatment of infections.

iii. the LD_{50} of the LSE-AgNp was above 5000 mg/kgbw indicating that it is acutely non toxic and therefore can be used on short term basis. This is beneficial in providing immediate therapy for local communities.

iv. the antimicrobial potential of LSE-AgNp revealed in this study supports further research to discover new chemical structures that can cure microbial infections.

v. the mode of action of the nanoparticles should be elucidated in further research.

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Appendix A

LSE and $AgNO_3$ Mixture Undergoing Reduction under Sunlight and Leech Salivary Extract Silver Nanoparticles

Appendix B

Diameter Zones of Inhibition for *Klebsiella* species and *Pseudomonas aeruginosa*