BIOCHEMICAL, ANTIBIOTIC AND ANTICOAGULANT PROPERTIES OF LOCAL NIGERIAN LEECH (*Hirudo medicinalis*) SALIVA EXTRACT

BY

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ABSTRACT

Leech ability to overcome blood clotting while sucking, keeping it in a liquid state for a long period of storage in their crops and displace antibacterial activities had attracted the attention of traditional therapists, physicians and researchers as an effective remedy for diseases management especially antibiotic and coagulation disorders. Biochemical screening of Leech saliva Extract (LSE) showed positive reactions on Alkaloids, Balsams, Flavonoids, Saponins, reducing Sugar and Cardiac Glycosides, while Steroids, Anthraquinones, and Tennins were negative in chemical constituent screening. The antimicrobial activity of LSE tested against several bacterial species using dilution method showed that it has a minimal inhibition Concentration (MIC) activity against *Strepy pyoge* (360 μg/ml), *S. aureus* (3600 μg/ml), *E .coli* (360 μg/ml), *P. aeruginosa* (360 μg/ml), and *B. subtilis* (360 μg/ml). Minimal bacteria concentration (MBC) against *B. subtilis*, *S. aureus*, *Strepy pyogen*, *P. aeruginosa* and *E. coli* are 0.36mg, 3.6mg, 0.36mg, 0.36mg, and 0.36mg respectively. The anticoagulant activity revealed that local Nigeria Leech saliva has anticoagulant properties with thrombin time that ranged from 7 – 53 minutes at different concentrations. This work is likely to be the first work carried out on Biochemical, Antibiotic and Anticoagulant using local Nigeria Leech saliva extract.

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CHAPTER ONE

1.0 INTRODUCTION

1.1 Background of the study

Leeches are segmented worms that belong to the phylum Annelida and comprise the subclass Hirudinea (Buchsbaum, Pearse, & Pearse, 1987). Like other oligochaetes such as earthworms, leeches share a clitellum and are hermaphrodites. Nevertheless, they differ from other oligochaetes in significant ways. For example, leeches do not have bristles and the external segmentation of their bodies does not correspond with the internal segmentation of their organs. Their bodies are much more solid as the spaces in their coelom are dense with connective tissues. They also have two suckers, one at each end.

The majority of leeches live in freshwater environments, while some species can be found in terrestrial. (Fogden & Proctor, 2009) and marine environments as well. Most leeches are hematophagous, as they are predominantly blood suckers that feed on blood from vertebrate and invertebrate animals (Sawyer, 2011). About 700 species of leeches are currently recognized, of which some 100 are marine, 90 terrestrial and the remainder freshwater taxa. (Boris & Peter, 2008).

Leeches, such as the *Hirudo medicinalis*, have been historically used in medicine to remove blood from patients (Payton, 2010). The practice of leeching can be traced to ancient India and Greece, and continued well into the 18th and 19th centuries in both Europe and North America. In modern times, the practice of leeching is much rarer and has been replaced by other contemporary uses of leeches, such as the reattachment of body parts and reconstructive and plastic surgeries (Adams & Zakrzewski, 2001) and, in

Germany, treating osteoarthritis (Teut & Warning, 2006; Michalsen, Moebus, Spahn., Esch, Langhorst, & Dobos., 2002).

The treatment of disease conditions with medicinal leeches is termed as Hirudotherapy (HT), a Latin term (Whitaker, Izadi, Oliver, Monteath, & Butler, 2004). In this non-invasive treatment methodology, medicinal leeches (*Hirudo medicinalis*) are used, hence the term "Hirudotherapy." The word 'leech' is supposed to be derived from an old English word for physician, laece (Davis, 2012; Irish, Gullane, Mulholland, & Neligan, 2000). Hirudotherapy (HT) takes the advantage of several biological properties of medicinal leeches. Among these, the earliest known fact was that leeches feed on the blood of their host (phlebotomy) and, during the course, release pain-killing (anesthetic) and blood-thinning substances (anticoagulants) along with their saliva. For centuries, leeches were the common tools of physicians, who were of the belief that diseases were the result of an imbalance of various humours and that the body can be stabilized by releasing blood (Bernard, 2009; Weinfeld, Yuksel, Boutros, Gura, Akyurek, & Friedman 2000). Later on with due course of time, physicians employed these spineless blood-sucking animals as a remedy for a large number of diseases and deformities, from congested limbs to the treatment of various eye diseases. Modern leech therapy differs from the ancient therapy; nowadays, only the leeches, which are grown in farms and which have undergone strict quarantine, are employed for the therapy. Wild leeches are not used anymore and a leech is used for a single treatment (Godfrey, 2011; Cole, 2013). Today, scientific studies concerning the active substances in the leeches have given us a better understanding of how these annelids work and have increased the field of applications of this ancient therapy.

Leech ability to overcome blood clotting while sucking and to keep it in a liquid state for a long period of storage in their crops had attracted the attention of traditional therapists, physicians and researchers as an effective remedy for diseases management especially coagulation disorders. Leeches have been the subject of many scientific researches to identify and characterize the blood-affecting constituents, especially peptides and proteins, in their salivary gland secretions (Wagner, 2004).

In the 20th century, leeches have been a model for many extensive studies to evaluate the usage of leech products, especially leech saliva, for therapeutic purposes (Corral-Rodríguez, Macedo-Ribeiro, Pereira, & Fuentes-Prior 2010; Koh & Kini, 2009). Consequently, a large number of peptides and proteins with tremendous clinical applications have been identified and characterized in leech extract, such as antithrombin agents (Electricwala, Sawyer, Jones, & Atkinson 2008), antiplatelet compounds (Deckmyn, Stassen, Vreys, Van Houtte, & Sawyer, 2006; Rhiouani, El-Hilaly, Israili, & Lyoussi 2008). Nowadays, leeches have been introduced as promising tools in microsurgery and reconstructive operations after many reports about better salvage of grafted tissues and amputated digits after leech application (Whitaker, Cheung, Chahal, Karoo, & Gulati, 2005).

With regards, to cancer and metastasis therapy, many researchers delineated the effective usage of leech saliva and leech salivary gland extract as an anti-metastatic agent. It has been outlined that salivary gland extract from *Haementeria ghilianii* and *Haementeria officinalis* inhibited the metastatic colonization of lung tumour cells which were injected intravenously into the experimental animals (Whitaker *et al*., 2005). Other research described a booming synthetic *hirudin* preparation as an efficacious metastasis inhibitor of a wide range of malignant tumour cells, such as pulmonary carcinoma,

osteocarcinoma, breast carcinoma, leukaemia (ATCC, 2007). Recently, intensive researches led to the isolation of a protein named *ghilanten* from the leech *H. ghilianii* salivary gland extract with factor Xa inhibitory and anti-metastatic activities (Promega, 2009).

1.2 Justification

Over the years, the extremely hazardous blood and infectious diseases have been of a big concern of many researchers looking for new efficacious alternative healing apart from synthetic drugs. Hirudotherapy is a treatment using medical leeches. This kind of therapy is known from the time of extreme antiquity and is still in practise. The fact that it is still being practiced shows its efficiency in healing various kinds of illnesses and diseases. Hirudotherapy have received much attention because of the extensive uses in many medical fields. Clinical usage of leech was outlined in Pharaohs' paintings, Roman manuscripts and Arabic Islamic writings (Whitaker *et al.,* 2004). Leech saliva contains active compounds which act as strong antibiotic and anticoagulants. But scanty scientific researchers were undertaken about medicinal Nigerian leeches. In Nigeria, leeches have been used in traditional therapy for various diseases treatment. With this concept in mind, the anticoagulant and antimicrobial activities of local Nigeria leech saliva extract has been the subject of this investigation**;** with respect of revival of Nature healing which will reducing the consumption of synthetic drugs.

1.3 Aims and Objectives

This study is aimed at determine the Biochemical, antibiotic and anticoagulant activities of local Nigeria Leech Saliva Extract (LSE), *Hirudo medicinalis*.

1.3.1 Objectives

The specific objectives of this study are:

1. To determine the Biochemical properties of Leech Saliva Extract (LSE).

2. To determine the antibiotic properties of Leech Saliva Extract (LSE).

3**.** To determine the anticoagulant properties of Leech Saliva Extract (LSE).

CHAPTER TWO

2.0 LITERATURE REVIEW

2.1 Biology of Leech

Leeches are blood sucking worms that have been used in medicine as far back as 2500 years ago. The word leech comes from an old English word laece‟ meaning physician‟. The spelling later became leech (Mory, Mindell, & Bloom 2001; Seigworth, 2009). In medieval England, leeches were linked with healing because of the etymology of the word. In old English the word “lacian” meant to heal and physicians were known as “leche” (Upshaw & O – Leary, 2000). Leeches are annelids or segmented worms, closely related to the earthworms and are anatomically and behaviourally more specialized. There is fresh water, terrestrial and marine leeches. Leeches breathe through the skin. The digestive system contains a crop or pouch, in which food can be stored for several months. One to four pairs of eyes are located at the anterior end (Encyclopaedia Britannica, 2003). All leeches have 34 body segments. The first 5 or 6 form a small anterior sucker and last 7 form a powerful posterior sucker (Encyclopaedia Britannica, 2010).

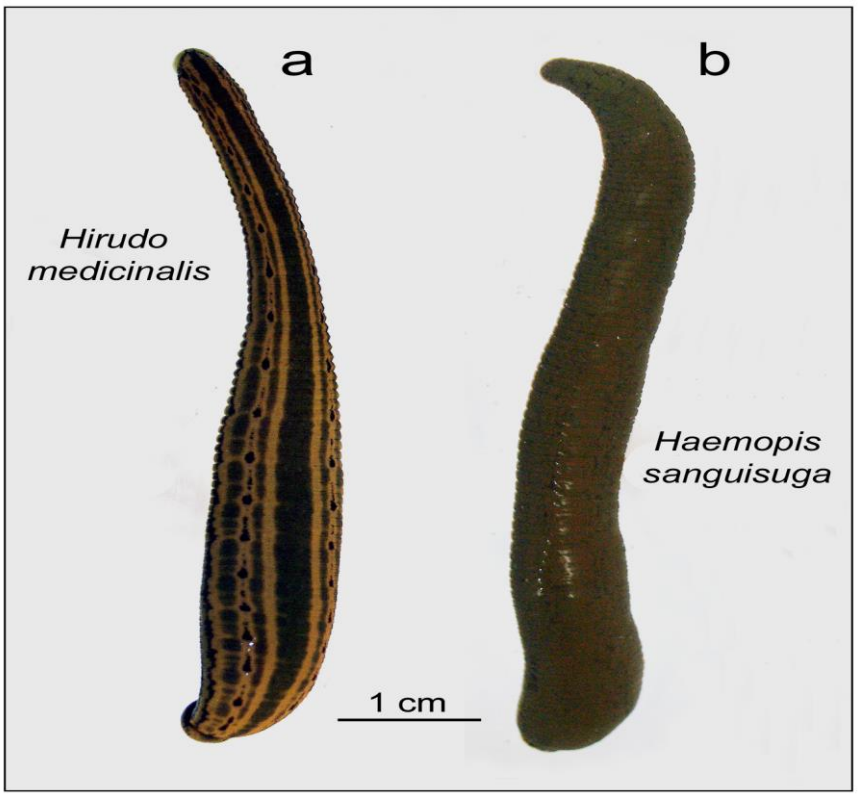
The medicinal leech (*Hirudo medicinalis*) is a segmented annelid belonging to Phylum: Annelids, Class: Clitellata and Subclass: Hirudinea (Lamark, 1818) (Davis & Appel, 1979; Mory, *et al.*, 2000). Leeches have two suckers, one on either extremity. The posterior suction cup helps it to move on dry surfaces and in attaching to its host; the anterior suction cup harbours a mouth and three sharp jaws. The bite looks like a Mercedes-Benz symbol. Each of the three jaws has 100 teeth, for a total of 300 teeth (Fort, 2001).

Medicinal leeches inhabit clean, fresh waters. Leeches swim around freely in water by undulating movements. Leeches are poikilotherms and can survive in the temperature

range of 0ºC to 30ºC; however, rapid temperature changes may stress these animals to death. They breathe in water with dissolved or atmospheric oxygen through their general body surface. Oxygen requirements are minimal and do not suffocate even in nearly completely closed containers. Harmful substances, like chlorine in water, even in low doses cause death of leeches. Leeches secrete a mucous layer over their body surface under unfavourable and stress conditions; it can thus act as a stress indicator of leeches. Leeches periodically shed their skin. Leeches are "protandrous" – first males then females lay eggs in cocoons 1-9 months post-copulation – about 4 cocoons each containing about 15 eggs and about 60 offspring per year. Young leeches feed on the blood of small water animals, like frogs and fish (Eroglu, Hokelek, Guneren, Ese., Pekba, & Uysal 2001).

Leeches usually remain attached to the host for 30 minutes to 6 hours for feeding, and during the course they get engorged with blood. It can suck about 5 to 15 ml of blood, but the bite continues to ooze for 4 to 24 hours (Fort, 2001). Leech saliva contains several bioactive substances, including anticoagulants, vasodilators and anaesthetics. Its saliva is rich in a potent anticoagulant - Hirudin. The benefits of leech therapy are mainly because of its salivary anticoagulants, vasodilators and anaesthetics as well as by its blood feeding (phlebotomy).

2.1.1 Scientific classification of Leech (*Hirudo medicinalis)*



Kingdom: Animalia

Subkingdom: Eumetazoa

Class: Hirudinea

Order: Gnathobdellia

Genus: *Hirudo*

Species: *medicinalis* (Encyclopaedia Britannica, 2003).

2.1.2 Zoological description of the leech

There are about 300 species of leeches, of the class Hirudinea, generally found in fresh water or humid environments. They are elliptical in shape, with 34 segments each and a sucker at both ends of the body (the large sucker on the posterior end). They can reach up to 16 inches (40cm) in length. The smallest leeches are 1cm in length, and most

species are 2 to 5 cm long, but the giant of the class is the Amazonian *Haementeria ghiliani*.

2.1.3 Taxonomy and Systematic

Leeches are presumed to have evolved from certain Oligochaeta, most of which feed on detritus. However, some species in the Lumbriculidae are predatory and have similar adaptations as found in leeches. As a consequence, the systematics and taxonomy of leeches is in need of review. While leeches form a clade, the remaining oligochaetes are not their sister taxon, but in a diverse paraphyletic group containing some lineages that are closely related to leeches, and others that are far more distant (Fort, 2001).

There is some dispute as to whether Hirudinea should be a class itself, or a subclass of the Clitellata. The resolution mainly depends on the eventual fate of the oligochaetes, which as noted above do not form a natural group as traditionally circumscribed. Another possibility would be to include the leeches in the taxon Oligochaeta, which would then be ranked as a class and contain most of the clitellates. The Branchiobdellida are leechlike clitellates that were formerly included in the Hirudinea, but are just really close relatives (Mory, *et al.*, 2000).

The more primitive Acanthobdellidea are often included with the leeches, but some authors treat them as a separate clitellate group. True leeches of the infraclass Euhirudinea have both anterior and posterior suckers. They are divided into two groups: Arhynchobdellida and Rhynchobdellida.

Rhynchobdellida are "jawless" leeches, armed with a muscular, straw-like proboscis puncturing organ in a retractable sheath. The Rhynchobdellae consist of two families:

Glossiphoniidae are flattened leeches with a poorly defined anterior sucker.

Piscicolida have cylindrical bodies and a usually well-marked, bell-shaped, anterior sucker. The Glossiphoniidae live in fresh-water habitats; the Pisciolidae are found in seawater habitats.

Arhynchobdellida lack a proboscis and may or may not have jaws armed with teeth. Arhynchobellids are divided into two orders:

Gnathobdela: In this order of "jawed" leeches, armed with teeth, is found the quintessential leech: the European medical leech, *Hirudo medicinalis*. It has a tripartite jaw filled with hundreds of tiny sharp teeth. The incision mark left on the skin by the European medical leech is an inverted Y inside a circle. Its North American counterpart is *Macrobdela decora*, a much less efficient medical leech (Payton, 2010). Within this order, the family Hirudidae is characterized by aquatic leeches and the family Haemadipsidae by terrestrial leeches. In the latter are *Haemadipsa sylvestris*, the Indian leech and *Haemadipsa zeylanica* (*yamabiru*), the Japanese mountain or land leech.

Pharyngobdella: These so-called worm-leeches consist of freshwater or amphibious leeche that have lost the ability to penetrate a host's tissue and suck blood. They are carnivorous and equipped with a relatively large, toothless, mouth to ingest worms or insect larvae, which are swallowed whole.
The Pharyngobdella have six to eight pairs of eyes, as compared with five pair in Gnathobdelliform leeches, and include three related families. The Erpobdellidae are some species from freshwater habitats.

2.1.4 Habitat, Habits and Reproduction

The preferred habitat is muddy, fresh water pools and ditches with plentiful weed growth in temperate climates. Leeches are distributed all over the world, except the polar zones, deserts and altitudes exceeding 3700 meter. Leeches may be aquatic, amphibious or terrestrial. European leeches live in fresh water and damp places (Anonymous, 2008).

Leeches are hermaphrodite but they are not self-fertilizing, the sperm of one individual fertilizes only the eggs of other individual. Development and growth are directed, without a larval stage (Anonymous, 2008).

The testes are four to ten pairs, arranged by segments, beginning with segment 12 to 13. The testes on each side of the body are connected with vas deference, a duct that leads indirectly to the male pore. The female reproductive system consists of one pair of ovisacs containing two ovaries, which although located in front of the testes, may extend same length posteriorly, depending on the animal. The ovaries connect to form an oviduct that forms either a female pore or in those species that copulate, a vagina (Anonymous, 2008). In Gnathobdelliae, sperm are transferred by the penis of one animal into the vagina of another. In two other families- Rhynchobdellae and Erpobdellidae, sperm transferred by sperm capsule, or spermatophore, on to the body of leech, after which the sperm leave the spermatophore and enter the ovary through the female pore to unite with the eggs. Leech eggs, numbering from one to more than 100, are usually deposited in cocoons, which may be oval or elongated in shape and are generally attached to rocks, vegetation or any submerged body or lodged in bank side burros (Anonymous, 2008). Little is known about the life span of leeches. One species of Erpodella requires a year to reach sexual maturity after which it lays cocoons once

and dies. Another species breeds once a year for two years and dies during the third. It has been said that annelids are the most highly organized animals with the power of complete regeneration. The powers of regeneration are greater in the polychaetes and lower oligochaetes than the higher oligochaetes that's why leeches lack the ability of regeneration (Anonymous, 2008).

2.1.5 Leech Life Cycle

2.1.5.1 Egg Stage

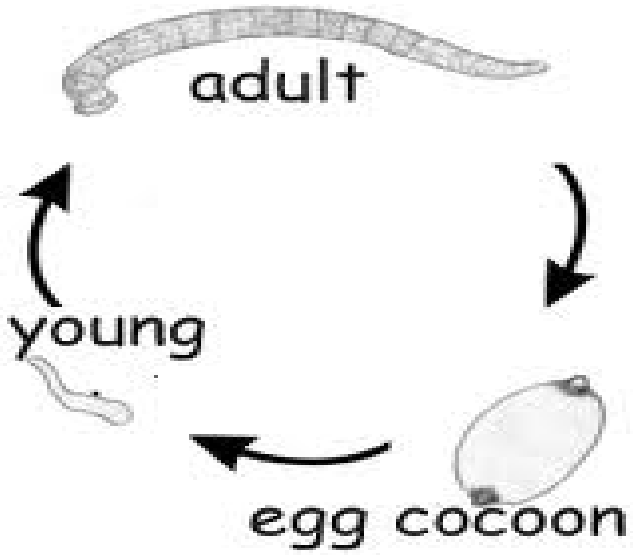
Once the eggs have been fertilized, the fertilized leech will build a cocoon to lay the eggs. This is the first stage of the leech's life cycle. A leech bearing fertilized eggs will secrete a cocoon from its glands that fully encases the body of the leech. Once the cocoon has been secreted, it will deposit the eggs and extricate itself from the cocoon. The eggs are then protected by the egg laying parent until they are ready to hatch. Incubation of the eggs, as well as type and location of the cocoon, varies by leech species.

2.1.5.2 Adolescent Stage

When the eggs hatch, the adolescent leeches break free from their cocoon and attach themselves to the egg-laying leech's body. During this stage of metamorphosis, the adolescent leech appears to be a smaller version of the parent leech. Unlike earthworms, the adolescent leeches do not develop additional segments, and grow by increasing the size of their segments. While the leech is in its adolescent stage it is unable to feed or defend itself, relying exclusively on the parent leech to care for it and protect it.

2.1.5.3 Adulthood

Adulthood is reached when the adolescent leech has grown close to the size of its parent and it is able to exist without being attached to it. When an adolescent leech can no longer be supported or protected by its parent, it releases from its body and quickly attaches to another host to rely on for food and protection. Another characteristic of this stage of leech metamorphosis is that they reach sexual maturity and are able to reproduce with other leeches, beginning the life cycle over again.



(Fernadez & Matte 2010)

Figure 2. Leech life Cycle

2.1.6 Development

Leeches are hermaphrodites, meaning each has both female and male reproductive organs (ovaries and testes, respectively). Leeches reproduce by reciprocal fertilization, and sperm transfer occurs during copulation. Similar to the earthworms, leeches also use a clitellum to hold their eggs and secrete the cocoon. During reproduction; leeches use hyperdermic injection of their sperm. They use a spermatophore, which is a structure containing the sperm. Once next to each other, leeches will line up with one's

anterior side opposite the other's posterior. The leech then shoots the spermatophore into the clitellur region of the opposing leech, where its sperm will make its way to the female reproductive parts.

The embryonic development of the leech occurs as a series of stages. During stage 1, the first cleavage occurs, which gives rise to an AB and a CD blastomere, and is in the interphase of this cell division when a yolk-free cytoplasm called teloplasm is formed (Fernadez, Olea, Tellez, & Matte 2010). The teloplasm is known to be a determinant for the specification of the D cell fate (Weisblat & Shankland, 2011). In stage 3, during the second cleavage, an unequal division occurs in the CD blastomere. As a consequence, it creates a large D cell on the left and a smaller C cell to the right. This unequal division process is dependent on actinomycin (Lyons & Weisblat, 2009), and by the end of stage 3 the AB cell divides. On stage 4 of development, the micromeres and teloblast stem cells are formed and subsequently, the D quadrant divides to form the DM and the DNOPQ teloblast precursor cells. By the end stage 6, the zygote contains a set of 25 micromeres, 3 macromeres (A, B and C) and 10 teloblasts derived from the D quadrant (Sandig & Dohle, 2008).

The teloblasts are pairs of five different types (M, N, O, P, and Q) of embryonic stem cells that form segmented columns of cells (germinal band) in the surface of the embryo (Berezovskii & shankland, 2013). The M-derived cells make mesoderm and some small set of neurons, N results in neural tissues and some ventral ectoderm, Q contributes to the dorsal ectoderm and O and P in the leech are equipotent cells (same developmental potential) that produce lateral ectoderm; however the difference between the two of them is that P creates bigger batches of dorsolateral epidermis than O (Welsblat & Shankland, 2011). The sludgeworm *Tubifex*, unlike the leech, specifies the O and P

lineages early in development and therefore, these two cells are not equipotent (Arai, Nakamoto & Shimizu, 2001). Each segment of the body of the leech is generated from one M, O, P cell types and two N and two Q cells types (Welsblat & Shankland, 2011).

The ectoderm and mesoderm of the body trunk are exclusively derived from the teloblast cells in a region called the posterior progress zone (Nardelli-Haefliger & Shankl, 2012; Shankland & bruce, 2010). The head of the leech that comes from an unsegmented region, is formed by the first set of micromeres derived from A, B, C and D cells, keeping the bilateral symmetry between the AD and BC cells (Shankl & bruce, 2010).

2.1.7 Anatomy

The anatomy of leeches is remarkably uniform. The body is typically dorsoventrally flattened and frequently tapered at the anterior. The segments at both extremities have been modified to form suckers. The anterior sucker is usually smaller than the posterior one, and frequently surrounds the mouth. The posterior sucker is disc-shaped and turned ventrally. There are no setae present. A clitellum is present and is always formed by segments IX, X and XI. The body of a leech can be divided in to five regions.

1. Head or Cephalic Region: it is composed of six segments and prostomium. Dorsally, the head bear a numbers of eyes and ventrally it bears the anterior sucker surrounding the mouth.

2. Preclitellar Region: it is composed of four segments.

3. Clitellar Region: it is composed of three segments. It bears single male gonopore and a single female gonopore.

4. Middle Region: it is composed of 15 segments. Comprises the greater part of the trunk.

5. Terminal or Posterior Region: it is eight fused segments. These segments are modified to form the ventral posterior sucker. The anus opens dorsally in front of the sucker.

The nervous system of leeches reflects their specializations of body structure. The cell bodies of ganglia are grouped into distinct masses, or follicles. Each ganglion is composed of six such follicles, arranged in two transfers 'traits. In the 5th and 6th segments, a large ganglionic nerve ring surrounds the pharynx or proboscis, represents the brain, the circum-pharyngeal connectives, and the sub-pharyngeal ganglia of other annelids, and the ganglion of the first three or four segments that have migrated posterior, although each pair of segmental ganglia are fused. The leeches can also contain various type of dispersed sensory cells joined together to form sensory papillae (Barnes, 2005). *Hirudo* possess no true blood-vascular system. The entire circulatory system is considered to represent a very much reduced coelom. It consists of a well developed system of vessels containing a red coelomic fluid (Brown, 2008). A true circulatory system persists only in the Acanthobdellida and the Rhynchobdellida. It is closed and usually lack haemoglobin 21. Special respiratory organs found only in some piscicolidae, as evaginations of the body wall connected with coelomic channels. Glossiphoniidae obtain their oxygen supply through the hypodermal coelomic channels. Other has a densenet of coelomic capillary channels between epidermal cells (Kaestner, 2007).

Leeches contain from ten to seventeen pairs of nephridia, located in the middle third of the body. The nephridial tube in leeches is especially peculiar. It is in part or completely composed of a cord of cells through the interior of which runs a non-ciliated, intracellular canal (Barnes, 2005).

2.1.8 Food and feeding

Leeches are carnivorous or blood sucking annelid worms. The medicinal leech *Hirudo* feeds principally on mammalian blood, but it also sucks blood from snakes, tortoises, frogs and fish. Most of them are permanent or temporary external parasites, attaching themselves to the host and sucking blood. The anterior sucker, jaws and muscular pharynx serve as an efficient apparatus for the abstraction of blood. Sucked blood is stored in lateral diverticulae of the crop and as the blood passes down the pharynx, it is mixed with a glandular secretion which prevents coagulation. A leech ingests at a single meal several times its own weight of blood which may suffice for several months (Anonymous, 2008).

2.2 Medicinal uses of Leeches

In medieval and early modern medicine, the medicinal leech- *Hirudo medicinalis* and its congeners was used to remove blood from a patient as part of a process to "balance" the "humours" that, according to Hippocrates, must be kept in balance in order for the human body to function properly and these four humours are blood, phlegm, black bile and yellow bile (Wikipedia, 2008).

Hirudotherapy was introduced by Ibne Sina in the Canon of Medicine (1020s). He considered the application of leech to be more useful than cupping in "letting of the blood from deeper parts of the body". Leech therapy became a popular method in medieval Europe due to the influence of his Canon. A more modern use for medicinal leech was introduced by Abdul-ul-Latif-al Baghdadi in the 12th century, who wrote that leech, could be used for cleaning the tissue after surgical operations. Contemporary leech therapy is pioneered by surgeons (Deuraseh, 2004). The use of leeches in

medieval times was of greater benefit than cupping because the amount of blood removed would be more “predictable” and of a greater amount. The reporting of the practice of using leeches reached its climax between 1820 and 1845. Francois Broussais (1772-1832) proposed that all diseases resulted from excess of blood and that bloodletting was the only cure (Thorwald, 2009). Leeches seem to have made both a clinical and laboratory resurgence over the past several years. On the clinical front, plastic and microvascular surgeons in both Western Europe and North America have been utilizing the ability of this medicinal worm to inject anticoagulants and remove blood. The use of leeches has been reported as an adjacent for grafted skin flaps (Batchelor, Davidson, & Sully, 2012; Henderson, Matti, Laing, Morelli, & Sully, 2012) and breast reconstruction (Dicckson, Boothman, & Hare, 2010). Reports also indicate their utility in the reattachment of severed digits. Local removal of accumulated blood seems to be a task for which the leech is well suited. The leech has been applied to the suture lines of re-implanted digits in an attempt to reduce venous congestion, as following surgery, venous return may be impaired. The anti-coagulant in leech saliva may provide additional therapeutic benefits by causing the bites to bleed for many hours after the leech detaches. The leech has also been recently used in the evacuation of periorbital haematomas (Bunker, 2008). As treatment common to ancient man; leeches has also been reportedly applied to conditions such as hemorrhoids and varicose vein (Bunker, 2008).

Systemic utilization of the products of the leech salivary gland appears to be growing as the chemicals produced in their salivary glands are further analyzed. Salivary extracts are available from pharmaceutical firms, and their clinical effects are being studied (Wood head, 2009). Hirudin has been reported to prevent some pathological changes of

diffuse intravascular coagulation (DIC) such as consumption of clotting factors and development of multiple micro thrombi. It also reportedly inhibits localized hemorrhagic necrosis induced by endotoxin injection (Wood head, 2009). Secretion from Haementria salivary cells have been reported to interfere with the metastatic growth of some lung tumours (Iwakawn, Gasic, Viner, & Gasie. 2006) and the efficacy of a fibrinase on atherosclerotic plaques is reportedly under investigation (Sawyer, 2011).

2.2.1 Mode of action of Hirudotherapy

Leech therapy involves an initial bite, which is usually painless (leech saliva contains a mild anaesthetic), and an attachment period lasting 20 to 45 minutes, during which the leech sucks between 5 and 15 ml of blood. Its main therapeutic benefits are not derived from the blood removed during the biting (although this may provide dramatic relief at first), but from the anticoagulant and vasodilator contained in the leech saliva. These properties permit the wound to ooze up to 50 ml of blood for up to 4 hours. Leech bites usually bleed for an average of six hours (Ikizceli, Avsarogullari, Sözüer, Yürümez, & Akdur 2005; Kowalczyk, 2002).

Salivary glands of a medical leech contain more than 100 bioactive substances and the salivary gland secretion has anti-edematous, bacteriostatic, and analgesic effects; it possesses resolving activity, eliminates microcirculation disorders, restores the damaged vascular permeability of tissues and organs, eliminates hypoxia (oxygen starvation), reduces blood pressure, increases immune system activity, detoxifies the organism by antioxidant pathways, relieves it from the threatening complications, such as infarct and strokes, and improves the bioenergetic status of the organism (Glyova, 2005). The molecules existing in leech saliva and the most studied to date include:

Hirudin: An active principle in the salivary gland secretion of leeches, which acts as a potent anticoagulant (blood thinner). It inhibits blood coagulation by binding to thrombin (Eldor, Orevi, & Rigbi2010; Chang, 2012; Bichler & Fritz. 2013; Seemuller, Dodt, Fink & Fritz, 2008).

Hyaluronidase (spreading factor): Facilitates the penetration and diffusion of pharmacologically active substances into the tissues, especially in joint pain and has antibiotic properties (Adams, 2005).

Calin: Inhibits blood coagulation by blocking the binding of the Von Willebrand factor to collagen. It inhibits collagen-mediated platelet aggregation (Eldo, 2010; Glyova, 2005; Munro Jones, & Sawyer, 2007).

Destabilase: Dissolves fibrin and has thrombolytic effects (Glyova, 2005; Zavalova, Baskova, Lukyanov, Sass, 2000).

Hirustasin: Inhibits kallikrein, trypsin, chymotrypsin, and neutropholic cathepsin G (Sollner, Mentele, Eckerskorn, Fritz, & Sommerhoff, 2004; Sawyer, 2011).

Bdellins: Anti-inflammatory effect and inhibits trypsin, plasmin and acrocin (Eldor, 2010; Seemüller, Dodt, Fink, & Fritz, 2008).

Chloromycetyn: Potent antibiotic (Sawyer, 2011).

Tryptase inhibitor: Inhibits proteolytic enzymes of host mast cells (Eldor, 2010).

Eglins: Anti-inflammatory. They inhibit the activity of alpha-chymotrypsin, chymase, substilisin, elastase, and cathepsin G (Eldor 2010; Glyova, 2005).

Factor Xa inhibitor: Inhibits the activity of coagulation factor Xa (very important role during the treatment of Osteo-arthritis and Rheumatoid arthritis) (Hofmann, Nutt, & Dunwiddie, 2002; Rigbi, Jackson, & Latallo, 2008).

Anesthetic-like substances: Reduce pain during biting by a leech (Godfrey, 2011; Rigbi, Levy, Eldor, Iraqi, Teitelbaum, & Orevi, 2007).

Histamine-like substances: A vasodilator increases the inflow of blood at the bite site (Glyova, 2005; Kumar & Prakash, 2011).

Complement inhibitors: Replace natural complement inhibitors if they are deficient.

Carboxypeptidase - A inhibitors: Increase the inflow of blood (Reverter, Vendrell, Canals, Horstmann, Avilés, & Fritz, 2008; Reverter, Fernández-Catalán, Baumgartner, Pfänder, Huber, & Bode, 2000).

Acetylcholine: Vasodilator (Zaidi, Jameel, Zaman, Jilani, Sultana, & Khan, 2011).

Collagenase: Reduces collagen (Glyova, 2005; Rigbi *et al*., 2008).

2.2.2 Applications of Hirudotherapy in Human Surgery

Modern leech therapy utilizing *Hirudo medicinalis* is based on sound scientific principles and has resulted in important patient care enhancements. Leech therapy is most often used in the settings of localized venous congestion associated with flap reconstructions and surgical replantations. Hirudotherapy has also been used to treat

soft tissue swelling and hematomas in trauma (Munro *et al*., 2007; Knobloch, Gohritz, Busch, Spies, & Vogt, 2007).

2.2.3 Arthritis

The leech's saliva assists in the treatment of arthritis (Gileva, 2010). There are a number of substances and compounds in its saliva that help to reduce inflammation in a joint: some of these compounds are bdelins and eglins, acting as anti-inflammatories (Glyova, 2005). Apart from anti-inflammatory components, its saliva also has an anesthetic component that alleviates the pain felt in the joint and also contains a histamine-like substance that acts as a vasodilator (Zaidi *et al*., 2011). Acetylcholine, another component of the leech's saliva, is also a vasodilator (Zaidi *et al*., 2011). This is important for the treatment of arthritis because as the vessels dilate, it increases the flow of blood, thus removing the compounds from the site, thereby relieving pain and inflammation.

2.2.4 Skin flap

Leeches are being employed in skin flap transplantations (Knobloch *et al*., 2007). As soon as the leeches attach themselves to the skin flap site, they begin to suck blood. During this, they also release a component called hirudin from their saliva (Eldor *et al*., 2010; Glyova, 2005). This component is very vital for the inhibition of platelet aggregation and coagulation cascade. If these two detrimental complications continue to supervene in a skin flap, there will be marked venous congestion, which slows down the healing process of the skin graft. When venous outflow of the skin flaps is inadequate the flap becomes cyanotic and congested. The venous congestion further compromises the arterial circulation unless it is alleviated. Because of the presence of hirudin and the

Factor Xa inhibitor in the leech's saliva, these processes are inhibited. Presence of the vasodilator component in their saliva further reduces venous congestion, promoting good blood flow into the skin flap. After continuous medicinal leech therapy, the skin flap soon turns warm and pinkish, indicating an adequate blood supply to the flap.

2.2.4 Venous congestion

Leech therapy has been proven to help patients suffering from venous diseases (Weinfeld *et al*., 2000; Conforti, Connor, Heisey, & Hartig 2002). It can help reduce the pain and the swelling, due to varicose veins, and can help dissolve blood clots. However, leech therapy is not effective for diseases caused by insufficient valves and inadequate vessel dilation. The saliva of leeches is known to contain beneficial enzymes, which prevent blood from coagulating. Apart from this, there is also an enzyme that breaks up thrombi. These two properties function to make the blood thin, so that it flows freely in the veins. To further aid in this function, another enzyme acts as a vasodilator to allow better blood flow. A leech's saliva also has antibacterial properties (Eroglu *et al*., 2001), which helps individuals who have open sores complicated by venous disease. Leech therapy is best used in conjunction with compression stockings, weight management, diet, and exercise.

2.2.5 Vascular diseases

Vascular disorders and diseases are now being cured by leech therapy (Ahmad, Anwar, 2009; Niqar & Alam, 2011; Pospelova & Barnaulov, 2010; Porshinsky, Saha, Grossman, Beery, Ii PR & Stawicki, 2011). Their saliva has over 100 bioactive substances that are very beneficial. One such component is hirudin, which acts as an anticoagulation agent (Glyova, 2005; Bichler *et al*., 2013). Calin is another component

that also inhibits blood coagulation (Munro *et al,* 2007; Riede, Koenen, Goerdt, Ehmke, & Faulhaber 2010). A component that dissolves fibrin clots as well as inhibits the formation of thrombus is the destabilase (Zavalova *et al*., 2000). Leech saliva also contains a Factor Xa inhibitor, and this compound restrains the coagulating effect of the coagulation Factor Xa (Hofmann *et al*., 2002; Rigbi *et al*., 2008). It also has hyaluronidase that enhances the viscosity of the interstitial fluid (Adams, 2005). For a vasodilating effect, it has acetylcholine and histamine-like substances as well as carboxypeptidase - A inhibitors (Reverter *et al*., 2000; Zaidi *et al*., 2011). These three can increase blood flow by dilating constricted vessels.

These are just some of the very useful components in leech saliva, which work in milieu to decrease the viscosity of the blood, so as to promote better flow. Blood that has a thick consistency makes it prone to clot formation as well as increases the blood pressure of an individual. These clots can travel to different parts of the body and can block a vessel, which could then cause a stroke or heart attack. Thick blood poses a risk that the distal extremities, especially the tips of the fingers and toes, will not receive adequate oxygenated blood and the nutrients they need. Therefore, the anticoagulation component in a leech's saliva is vital, as it naturally reduces viscosity of blood and works to inhibit platelet coagulation. As stated above, the saliva's vasodilating components dilate or widen the blood vessels, thereby promoting a better blood flow. With all these components working together, there will be a remarkable improvement in the vascular status of the patient.

CHAPTER THREE

3.0 MATERIALS AND METHODS

3.1 Study Area

Minna is the capital of Niger state. Minna experiences two distinct seasons (Dry and Wet season). The annual rainfall varies from about 1600mm in the south to 1200mm in the north. The duration of the rainy season ranges from 150 – 210 from north to south. The minimum temperature of minna fluctuates between March and June, while the maximum 33℃ to 30.6C is usually recorded between December and January. Most part of the state comes under the influence of the tropical continental air mass which blows from the north. Niger State lies between latitude 3.20º' East and 9º.25'N of the equator and between longitude 11.30º North and 9º 37' E of the equator. According to National Population Commission (2006) the population of Niger state during the 2006 National Population Census was 3,950,249.

3.2 Leech collection

One hundred and twenty (120) Leeches were collected in local rice farm located at Fadikpe in Chanchaga Local Government Area, using lest mesh of biological sieve net.

3.2.1 Maintenance

The leeches were maintained in well-aerated plastic aquarium filled with non chlorinated water and kept in a separate room at room temperature. Water was regularly changed every three days.

3.2.2 Leech Feeding

Leeches were fed on sugar solution and animal blood

3.2.3 Leeches Saliva Extraction

Leeches were kept in aquarium containing water at 23°C for the period of experiment. Water was changed every three days. Leeches were starved for two weeks before the commencement of saliva collection and prior to another leeches saliva extraction; the leeches were served with sugar solution and animal blood. Saliva was obtained by a small variation as described by Rigbi (2008). Three to four leeches were then put in a test tube surrounded by ice block for 15-20 min. This technique forces the leeches to vomit saliva through the mouth. To complete the saliva collection, leeches were squeezed smoothly from the posterior towards the anterior (mouth) sucker. All fluids that have been vomited were collected (bloody fluids were discarded) in clean test tubes and centrifuged at 4°C for 10 minutes at 9000 rpm, the supernatant were discarded and settlement fluid was named leech crude saliva.

3.3 Preliminary biochemical analysis

Preliminary biochemical analysis of the local Nigerian leech saliva for the presence of Alkaloides, Anthraquinones, Balsams, Cardiac Glycosides, Flavonoids, Saponines, Steroides, Tannines and Reducing Sugar, performed according to the methods described by Culei (1982) & Sofowora (1984).

3.3.1 Test for Alkaloids

Two grammes (2g) of leech saliva was treated with 10ml of I % Hydrochloric acid in a water bath for 10 minutes. The filtered solution was then treated with a few drops of Dragendorfs reagents: Development of turbidity or precipitate in the fitterate indicated the presence of alkaloids (Culei, 1982; Sofowora, 1984).

3.3.2 Test for Anthraquinones

Five grammes of leech saliva were added to 10ml of Benzene. This was filtered and 5ml of 10% ammonia solution was added and shaken vigorously, the presence of a pink red or violet colour in the ammonia phase (bottom of the test tube) indicated the presence of anthraquinones (Culei, 1982; Sof'owora, 1984).

3.3.3 Test for Balsams

Two (2) drops of alcoholic ferric chloride solution was added to 5ml of local leech saliva collection. A dark green colour indicates the presence of balsam.

3.3.4 Test for Cardiac Glycosides

A small quantity of the leech saliva was dissolved in pyridine and a few drops of 2% sodium nitroprusside was added followed by the addition of a few drops of 20% sodium hydroxide. The appearance of deep red color which faded to a brownish - yellowish indicated the presence of cardiac glycosides (Culei, 1982; Sofowora, 1984).

3.3.5 Test for Reducing sugars

Zero point two gram (0.2g) of leech saliva collection was hydrolyzed by boiling 5cm of diluted Hydrochloride acid (HCL) and resulting solution was neutralized with Sodium hydroxide (NaOH aq). A few drop of Fehling's solution A and B were added after which the mixture was heated on water bath for two (2) minis. Formation of reddish precipitate (Cuprous Oxide) indicates the presence of combined Sugar (Trease *et al* 1983).

3.3.6 Test for Flavonoids

Iron (III) chloride test

The saliva leech extract was boiled, two (2) ml of the boiled leech saliva was added with few drops of 10% Iron (III) chloride solution were added. Blue – violet coloration was an indication of the presence of the phenolic hydroxyl group (Trease *et al* 1983).

3.3.7 Test for Saponins

Zero point five grams (0.5g) of the local Nigeria leech saliva collection was mixed with water and observation of foam which persists on warming confirms the presence of saponin (Sofowora, 1982).

3.3.8 Test for Tannins

A small quantity leech saliva was mixed with water and heated in water bath. To this solution, iron (III) chloride (FeCl) solution was added. A blue- black or green or blue-green precipitate indicates the presence of tannins.

3.3.9 Test for Steroids

Five drops (5) of concentrated H_2SO4 were added to 2ml of local Nigeria leech Saliva extract. A reddish brown color indicates the presence of steroids.

3.4 Screening of test organisms

The test organism used (*Staphylococcus aureus, Pseudomonas aeruginosa, Escherichia Coli, Strepy pyogen, Bacillus subtili*) were collected in microbiology laboratory Federal University of Technology Minna. The isolated bacteria were culture on nutrient agar

and incubated at 37ºC for 24 hours. The organisms were repeatedly sub- cultured in other to obtain pure isolates. Gram staining was carried out for proper identification. The organism were finally inoculated into agar slants and stored at 4ºC until further use.

3.5 Standardization of test organisms

A loopful of tested organisms was inoculated in to 5.0 ml of nutrient broth and incubated 37ºC for 24 hours. 0.2 ml from the 24 hours culture of the organism was dispensed into 20 ml sterile nutrient broth and incubated for 3 - 5 hours to standardize the culture to 10^6cfu/ml (Babayi *et al*, 2004).

3.6 Antimicrobial susceptibility screening of local Nigeria Leech crude saliva extract

Screening antimicrobial activity started by reconstitution of the local leech saliva collection and control (Ampiclox). This was achieved by dissolving 1.8g of leech saliva and control in separate EDTA bottle respectively in 5ml of sterile distilled water.

The agar diffusion method was used. Sterile nutrient agar plates were prepared. The nutrient agar was pure into a Petridis and allow to gel. A sterile cork borer (7 mm) was used to make two (2) ditches in each plat, one for leech saliva and the other one for control. A stick swap was used to inoculate the test organism into 19 ml of nutrient agar and each plate was properly labelled. 1ml each out of reconstituted local leech saliva collection and control was pure into a ditched. The plates were left to allow diffusion of leech saliva and control before incubation at 37ºC for a period of 24 hours. The zone of clearance produce around the ditches after incubation were observed, measure and recorded.

3.7 Determination of Minimum Inhibitory Concentration (MIC)

Broth dilution method was used. 50 test tubes were collected. 9ml of broth were dispensed into the 50 test tubes, the test tube were arranged in 5×5, 1 ml of reconstituted leech saliva was dispensed into 25 test tubes and 1 ml of reconstituted control was purred into remaining 25 test tubes. Serial dilution was done in all the test tubes. 1 ml of tested organisms was inoculated in all the test tubes using siring; this was incubated at 37ºC for 24 hours. The lowest dilution after incubation that shows no visible turbidity is regarded as minimum inhibitory concentration (Rotimi, 1987).

3.8 Determination of Minimum Bactericidal Concentration (MBC)

The test tubes that show no visible turbidity after incubation of the batch of the test tubes were sub- cultured on nutrient agar plates and incubated at 37ºC for 24 hours. The concentration that shows no visible growth after incubation was considered the minimum bactericidal concentration.

3.9 Anticoagulant activities of local Nigeria Leech saliva extract

These were done using thrombin time (TT) method. Ten (10) test tubes were arranged in test tubes racker. 2ml of blood were purred into a test tube using siring; the time of coagulation was recorded. 2ml of blood were dispensed into test tube and introduce Ethylenediaminetetra acetic acid (EDTA), which is already known anticoagulant; the blood was observed and recorded. Another 2ml of blood were purred into test tube and 10µg/ml of leech saliva was dispensed, the time of coagulation was recorded, this was repeated with 20µg/ml - 80µg/ml and the times of coagulation was recorded respectively.

CHAPTER FOUR

4.0 RESULTS AND DISCUSSION

4.1 Biochemical screening

Biochemical screening showed positive (+) reaction where Alkaloids, Balsams, Cardiac Glycosides, Flavonoids, Saponins and Reducing Sugar, some negative (-) reactions which includes; Anthraquinones, Steroid, Tennins, the result is shown in (Table 4.1).

4.2 Antimicrobial susceptibility screening of Local Nigeria Leech crude saliva

Antibacterial activities of the local Nigerian leech saliva extract are shown in Table 4.2. The result revealed that leech saliva extract were active against the tested organisms when compared with control (Ampiclox). Activities of tested organism showed that *Staphylococcus aureus, Pseudomonas aeruginosa, Escherichia Coli, Strepy pyogen, Bacillus subtilis,* 20mm, 26mm, 28mm 21mm and 11mm, when compared to control (Ampiclox) that showed 30mm in three (*Staphylococcus aureus, Pseudomonas aeruginosa, Escherichia Coli*), *Strepy pyogen,* 28mm and *Bacillus subtilis,* 15mm respectively. The highest activities were *Escherichia Coli* with 28mm against control (Ampiclox) 30mm and lowest *Staphylococcus aureus* 20mm against control (Ampiclox) 30mm.

Table4:1 Biochemical screening of local Nigeria Leech saliva collection

Phytochemical Component	Leech Saliva Extract
Alkaloid	+
Anthraquinones	-
Balsams	+
Cardiac Glycosides	+
Flavonoids	+
Saponin	+
Steroid	-
Tennin	-
Reducing Sugar	+

KEY

+, present

- , absent

Table: 4. 2 Antimicrobial susceptibility screening of local Nigeria Leech crude saliva

Organisms	Leech Saliva Extract mm	Control (Ampiclox)mm
Staphylococcus aurous	20	30
Pseudomonas aeruginosa	26	30
Escherichia Coli	28	30
Strepy pyogen	21	28
Bacillus subtilis	11	15

4.3 Minimum Inhibitory Concentration (MIC) of Local Nigeria Leech crude saliva

The result of minimum inhibitory concentration (MIC) result is shown in Table 4.3 The result revealed that the minimum inhibitory concentration (MIC) of tested organism, *Bacillus subtilis Pseudomonas aeruginosa*, *Staphylococcus aureus, Strepy pyogen*, *Escherichia Coli*, 360µg/ml, 360µg/ml, 3600µg/ml, 360µg/ml and 360µg/ml. when compared to the Ampiclox (control) that showed 360µg/ml in two 3600µg/ml, 360µg/ml and 3600µg/ml respectively.

4.4 Determination of Minimum Bactericidal Concentration (MBC)

The result of minimum bactericidal concentration (MBC) result is shown in Table 4.4. The result revealed that the MBC of *Bacillus subtilis*, 0.36mg/ml, *Staphylococcus aurous*, 3.6mg/ml, *Strepy pyogen*, 0.36mg/ml, *Pseudomonas aeruginosa*, 0.36mg/ml and *Escherichia Coli* 0.36mg/ml, when compared with the Ampiclox (control) *Bacillus subtilis, Staphylococcus aurous, Strepy pyogen, Pseudomonas aeruginosa, Escherichia Coli;* 0.36mg/ml, 3.6mg/ml , 0.36mg/ml, 0.36mg/ml and 3.6mg/ml.

Table 4.3 Minimum inhibitory concentration (MIC) of local Nigeria Leech crude saliva

Organism	Leech Saliva Extract µg/ml	Control (Ampiclox)µg/ml
Bacillus subtilis	360	360
Pseudomonas aeruginosa	360	360
Staphylococcus aurous	3600	3600
Strepy pyogen	360	360
Escherichia Coli	360	3600

Table 4.4 Minimum bacteria concentration (MCB) of crude local Nigeria Leech saliva

Organism	Leech Saliva Extract Mg	Control (Ampiclox) mg
Bacillus subtilis	0.36	0.36
Staphylococcus aurous	3.6	3.6
Strepy pyogen	0.36	0.36
Pseudomonas aeruginosa	0.36	0.36
Escherichia Coli	0.36	3.6

4.5 Anticoagulant activities of local Nigeria Leech saliva

The result of anticoagulant activities is shown in table 4.5. The result revealed that anticoagulant activities of local Nigerian leech saliva extract vary with increase in volume. The lowest was recorded form 10µg/ml**,** 20µg/ml, 30µg/ml, 40µg/ml - 70µg/ml, 80µg/m lat 07:54TT, 15:20TT, 23:40TT, 28:40TT – 45:34TT, 52:18TT while the highest was recorded 80µg/ml in 52.18TT respectively.

Table 4.5 Anticoagulant activities of local Nigeria Leech saliva

SAMPLE	LSC (µg/ml)	Thrombin TIME (TT)
Control Blood	20	0:56
Control (EDTA)	100	Infinity
Crude Saliva Extract	10	0.7:54
	20	15:20
	30	23:40
	40	28:40
	50	31:12
	60	36:17
	70	45:34
	80	52:18

KEY

EDTA =Ethylenediaminetetra acetic acid.

LSE = Leech Saliva Collection.

4.6 Discussion

The wide biodiversity of leech species all over the world has led to set up many researches focusing on the antimicrobial and anticoagulant activity of their salivary gland secretion. This study established that local Nigeria leech saliva has antibiotic and anticoagulant properties. The preliminary Biochemical screening of the local Nigeria Leech saliva revealed the presence of alkaloids (a group of Nitrogen compound that are active as drugs), Balsams (An aromatic resinous substances), Cardiac Glycosides (compound found in plant used in treatment of heart failure), Flavonoids (An antibiotic), Saponins (Amphipathic glycoside). The presence of alkaloids and flavonoids revealed the efficacy of the saliva against pathogenic bacteria. This work agrees with the findings of Ebana *et al,* (1991) who worked on plants, reported that alkaloids and cardiac glycosides inhibit pathogenic bacteria. The presence of saponins agrees with Abdullah *et al,* (2003) who stated that leech are used traditionally in the treatment of venereal disease, and diabetes. The presence of Flavonoids confirms the assertion of Ingrid *et al,* (2006) who stated that leech is one of the highly potential antibiotic organisms. The Anthraquinones (phenol) found in the extracts of Z*zallthoxyloides* and *P. africaia* were also reported by Nguji, (1988). His investigation stated that phenols in plant tissues are usually oxidized to co-quinones which the serum subsequently form cross-Links with the serum protein of the skin to arrest bleeding and effect healing. This is because Leech saliva extract has properties that do not allow blood clotting, hence the Anthraquinones is negative (-) in Biochemical constituent of local Nigeria Leech extract.

The Nigeria leech saliva revealed a broad spectrum antibacterial activity against both Gram-positive and Gram-negative bacteria strains. The Minimal inhibitory concentration (MIC) is the lowest concentration of a drug that prevents growth of particular microorganisms (Presscort, Harley & Klein, 1990). Thus the MIC test gives some idea of the effectiveness of a chemotherapeutic agent against a microorganism. The results of the minimal inhibitory concentration (MIC) test indicated that different concentrations acted as the MIC volume for the respective organism. It is most probable that some of the organism" are more (sensitive) vulnerable to the action of the antimicrobial substance present in the extracts (which was manifested in their lower MIC volume). The results indicate that the organisms were inhibited by a minimal concentration of between 360µg/ml and 3600µg/ml of local Nigeria leech saliva. Higher MICs were recorded for *Streptococcus* sp than that of *Bacilllls* sp, *Pseudomonas aeruginosa, Strepy pyogen, Escherichia Coli.* The high MIC volume for *Streptococcus* sp indicate that these organism are more resistant to the leech saliva than *Bacilllls* sp, *Pseudomonas aeruginosa, Strepy pyogen, Escherichia Coli.* This is probably due to differences in genetic constitution of the organisms. However, this finding seems to show that *Staphylococcus* sp present difficulties in the therapy of bacterial infection by requiring more drugs at high concentrations. In addition this research work agree with work done by Bishop (2005), on Honey bees stated that Honey's antioxidants, amino acids and vitamins play a role in reducing inflammation. The antibacterial activity of honey rapidly kills the pathogens that cause typhoid fever, bacterial pneumonia, strep throat and bacterial dysentery. In fact, in 1998 honey was proven to be more effective than silver sulphadiazine, the antibacterial ointment most widely used on burns in hospital situations, in the treatment of burns.

Results revealed that the Local Nigeria leech Saliva extract exhibited an antithrombin activity since it prolonged TT in a linear dose dependent manner with an apparent inhibition constant (K), at 80mg/ml the TT shows 52: 18sec. Antithrombin components are a major component of the local Nigeria leech crude saliva, hence the organism can sock blood from animal for some minutes without coagulation. This result agrees with the work done in Malaysian by AbdualKader, (2011) that the saliva concentration which increases TT by 100% (IC_{100}) is 43.20µg/ml plasma. The Maleysian Leech was fed with phagostimulatory solution which may interfere with protein content.

CHAPTER FIVE

5.0 CONCLUSIONS AND RECOMMENDATIONS

5.1 Conclusion

It may at first glance hardly seem compatible with modern medicine, but local Nigerian leeches Saliva extract have indeed re-established themselves as an inherent part of contemporary healthcare.The results reported here has proved that the Nigerian leech saliva extract possesses a similar antithrombin activity to that of the Malaysian specie, raising the question of the actual structure of the active peptide, whether it has similarity with the other known antithrombin agents or not.

As well, Nigerian leech saliva extraction seems to contain broad spectrum antibacterial peptides which are supposed to have a novel structure that needs to be investigated. The MIC of local Nigerian leech saliva extraction was comparable to that of Ampiclox. If the extracts are purified and processed they can be used as antibacterial drugs since they inhibit the growth of bacterial isolates. As a result of the antimicrobial effect of this saliva, there may be a scientific basis for their utility in traditional medicine basis for the treatment.

5.2 Recommendation

States and Federal Government should establish Leech farming centers, more especially in Universities to enable more researches carryout more work and also where leech can be export to other counters.

Pharmaticule industry in Nigeria should prescribe Leech as an alternative drug for an Antibiotic drug for those patients who has allergic reaction for Ampiclox, hence this

work prove Antimicrobial activities, Hence world is enlighten the uses of Natural treatment.

Furthermore, work should be carryout on Antibiotic in other to quantify the percentage or milligram that can suppress action of bacterial activities in living organism.

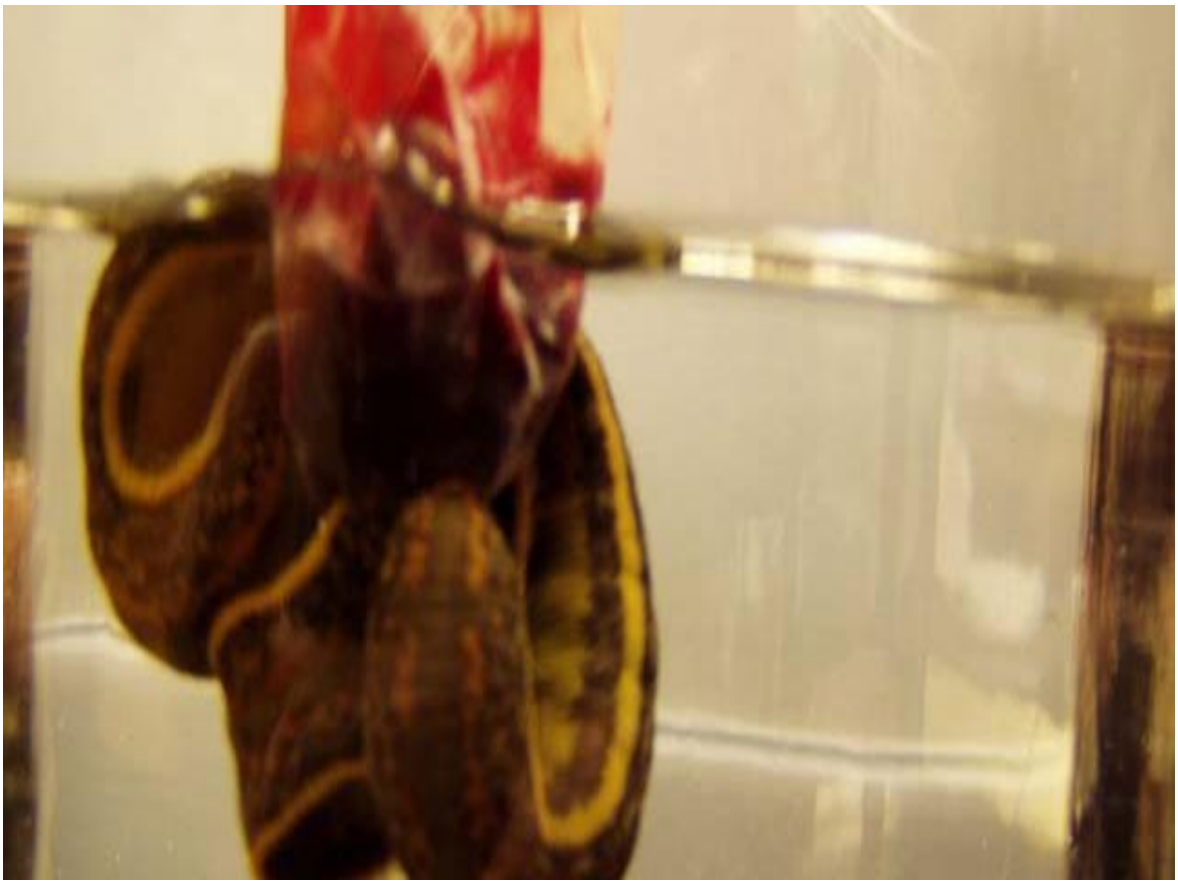


Figure 1. Leech feeding with blood

Plate .I: *Escherichia Coli* Inhibition

L = Leech saliva

C = Control (Ampiclox)

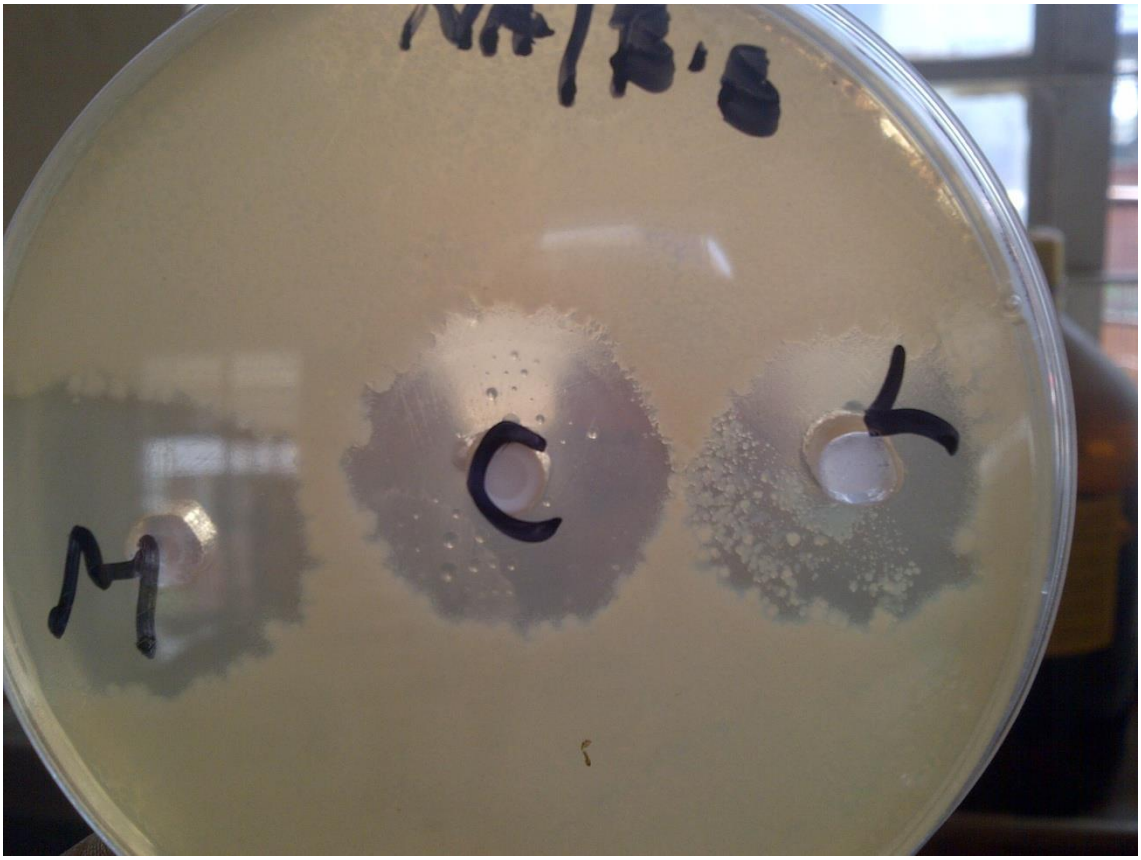


Plate. II: *Bacillus subtilis* inhibition

L = Leech saliva

C = Control (Ampiclox)

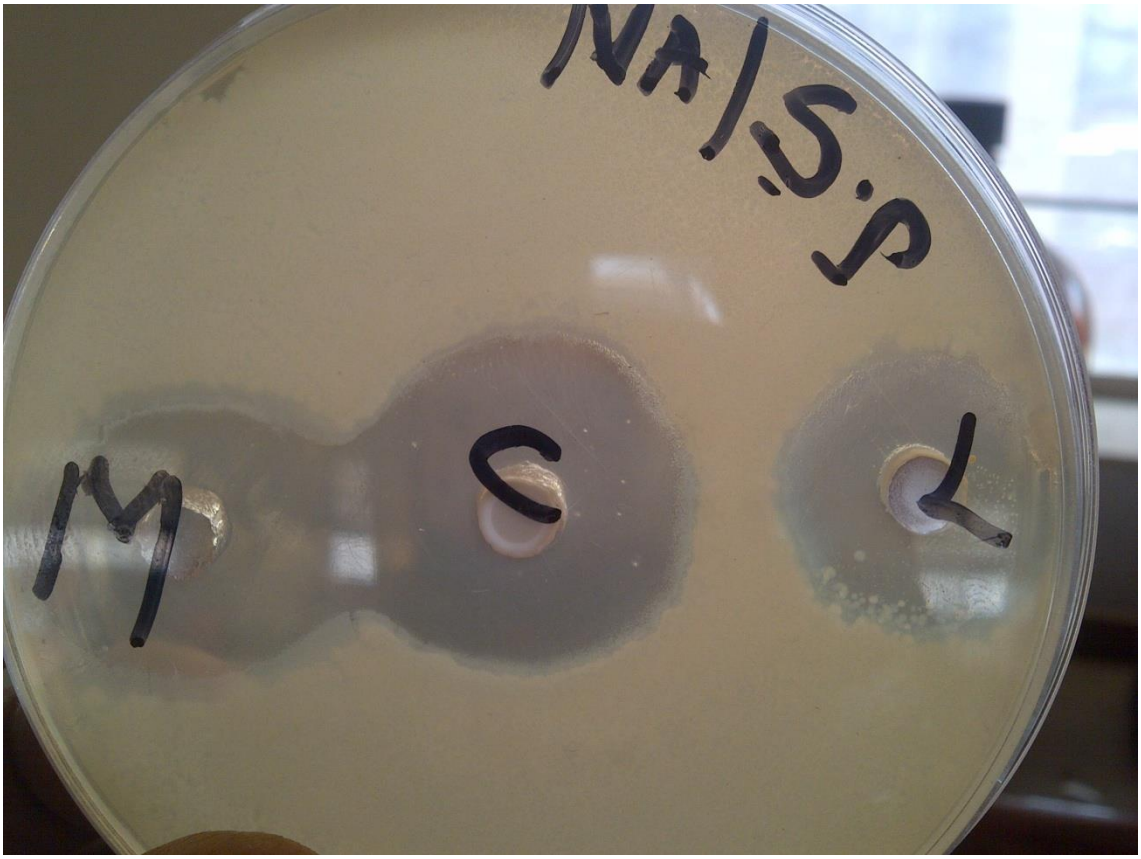


Plate. III. S*trepy pyogen* inhibition

L = Leech saliva

C = Control (Ampiclox)

Plate . IV. *Pseudomonas aeruginosa* inhibition.

L = Leech saliva

C = Control (Ampiclox)

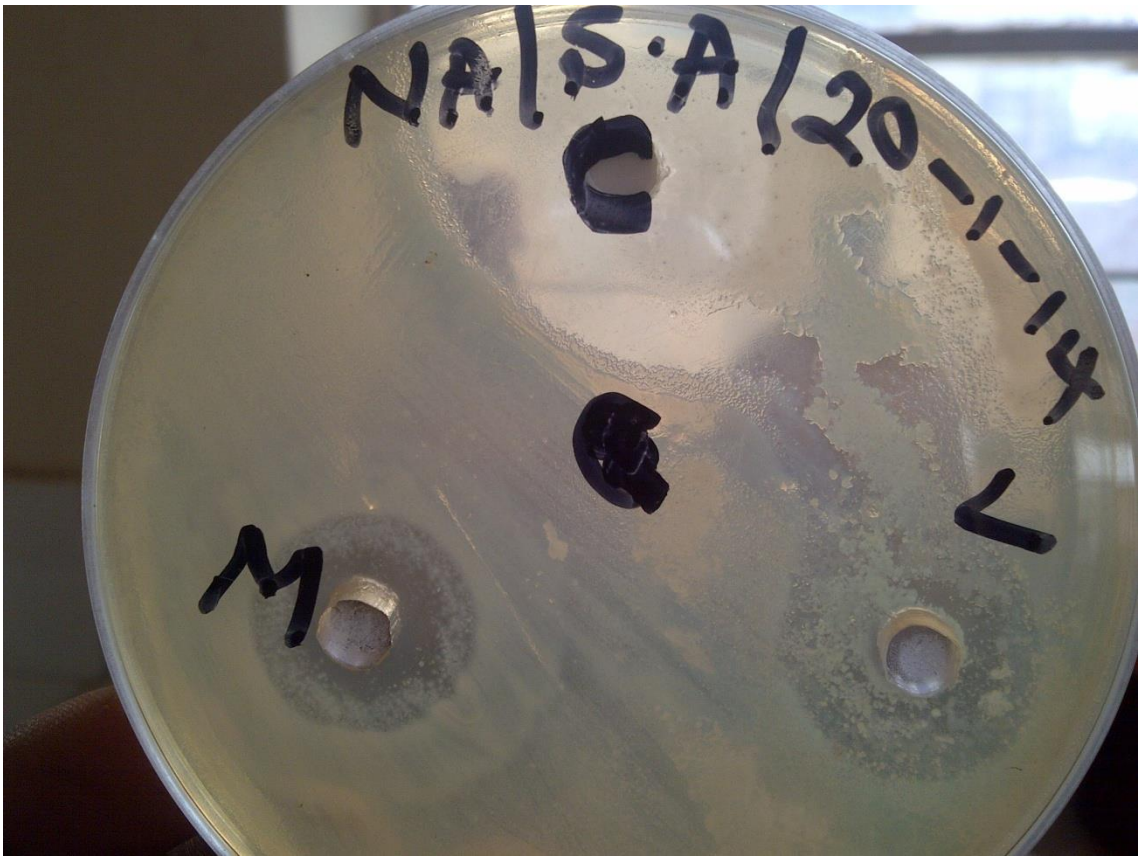


Plate. V. *Staphylococcus aurous* inhibition

L = Leech saliva

C = Control (Ampiclox)

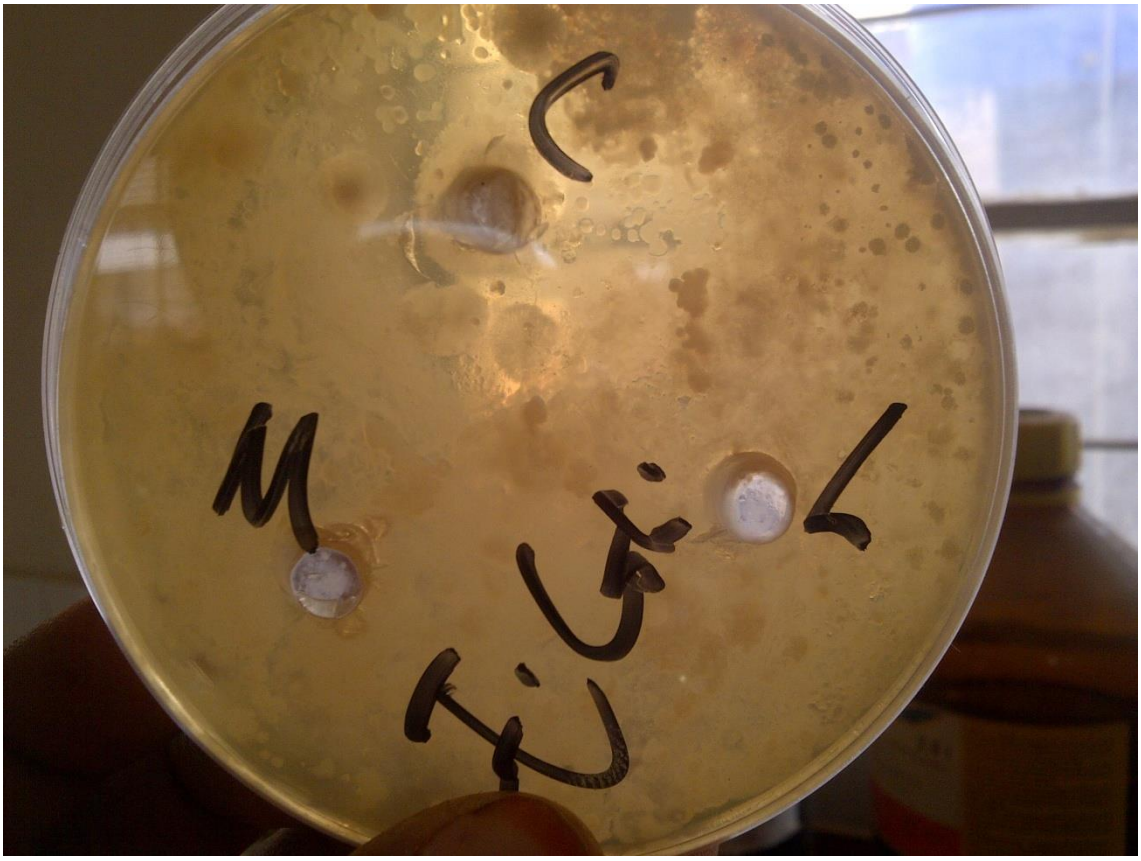


Plate. VI. Growth of *E. coli*. Without an antibiotic

L = Leech saliva

C = Control (Ampiclox)

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Appendix A

Convection form ‘gram (g)/ml’ to µg/ml.

1.8g/5ml

1.8/5 × 1000 = 1800g

$\frac{1800mg}{5ml} = 360mg/ml$

$\frac{360mg}{10} = 36mg/ml$ 36 × 1000 = 36,000µg

$\frac{36mg}{10} = 3.6mg/ml$ 3.6 × 1000 = 3600µg

$\frac{3.6mg}{10} = 0.36mg/ml$ 0.36 × 1000 = 360µg

$\frac{0.36mg}{10} = 0.036mg/ml$ 0.036 × 1000 = 36µg

$\frac{0.036mg}{10} = 0.0036mg/ml$ 0.0036 × 1000 = 3.6µg

1mg = 1000µg

0.1mg = 100µg

0.01mg = 10µg

0.001mg =1µg

Appendix B

Live leeches on plastic aquerum.