

Biochemical and antibiotic properties of local Nigerian leech (*Aliolimnatis michaelsoni*) saliva extract

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ABSTRACT: Leeches have been used extensively in different medical fields for the treatment of various disorders. The local Nigerian leech (*Aliolimnatis michaelsoni*) contains an active biochemical compound in its saliva that can be used by traditional therapists, physicians and researchers as an effective remedy for disease management, especially antibiotic disorders. Biochemical screening of local Nigerian Leech Saliva Extract (LSE) showed positive reactions on Alkaloids, Balsams, Flavonoids, Saponins, reducing Sugar and Cardiac Glycosides, while Steroids, Anthraquinones, and Tannins were negative in chemical constituent screening. The antimicrobial activity of local Nigeria leeches saliva extract (LSE) tested against selected bacterial species using dilution method showed that it has a minimal inhibition Concentration (MIC) activity against *Streptococcus pyogenes* (360 µg/ml), *S. aureus* (360 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*, *Streptococcus pyogenes*, *P. aeruginosa* and *E. coli* are 0.36mg, 3.6mg, 0.36mg, 0.36mg, and 0.36mg respectively. This work has proved the bioactive compound and is likely to be the first work carried out on Biochemical and Antibiotic using local Nigeria Leech (*Aliolimnatis michaelsoni*) saliva extract.

Keywords: *Aliolimnatis michaelsoni*, antibiotic, biochemical, Leech, saliva, therapists.

INTRODUCTION

Leech is a freshwater annelid commonly having a black or brown coloured body of about 10 cm long. The organisms are segmented worms that belong to the phylum Annelida and comprise the subclass Hirudinea, which belongs to the genus *Hirudo* with more than 600 species. (Buchsbbaum *et al.*, 1987). Like other oligochaetes, such as earthworms, leeches share a clitellum and are hermaphrodite, but slightly differ from other oligochaetes with a lack of bristles and external segmentation of their bodies that 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, with two suckers, one at each end.

Traditionally, leeches have been long known and used in ancient times in countries like India and China by traditional healers, and also leeching has developed well in Arabic and European medicines (Wittke-Michalsen, 2007). In America and Europe, studies on the two most common medicinal leeches, *Hirudo medicinalis* and *Hirudo*

verbana, contain bioactive substances in their saliva that have attracted scientists as alternatives to synthetic drugs. Extracts of these active compounds in leech saliva have been used in health therapy (Elliott, 2017).

Antioxidants are substances at low concentration that are capable of preventing or delaying oxidative damage of lipids, proteins, and nucleic acids caused by reactive oxygen species include free radicals such as superoxide, hydroxyl, peroxy, alkoxy and non-radicals such as hydrogen peroxide, hypochlorous acid. These free radicals have been implicated as being responsible for several diseases, such as heart disease, cancer, stroke, diabetes, and ulcers. However, antioxidants are the molecules which inhibit the free radicals (Santos-Sánchez *et al.*, 2019). There are several reports that diseases are prevented by antioxidants, including ageing, allergies, asthma, arthritis, analgesia, atherosclerosis, autoimmune diseases, bronchopulmonary dyspepsia, and cancer. It also protects against cataracts, cerebral ischemia, eczema, diabetes,

inflammatory gastrointestinal diseases, and genetic disorders. These antioxidants are mainly classified into enzymes, proteins and small molecules (Moussa *et al.*, 2019). Particularly, it has been noted that natural protein extracts or purified proteins are used as antioxidants (Sarmadi and Ismail, 2010). The current global strategy is to find natural antioxidants that are both inexpensive and close to natural, hence this research. This study aims at exploring biochemical, antibiotic and antioxidant properties of local Nigerian leeches (*Aliolimnatis michaelsoni*) saliva extract collected in Minna, Niger State.

MATERIAL AND METHODS

Study area

Minna is the capital of Niger State. Minna experiences two distinct seasons (Dry and Wet seasons). The annual rainfall varies from about 1600 mm in the south to 1200 mm in the north. The duration of the rainy season ranges from 150 to 210 days from north to south. The minimum temperature of Minna fluctuates between March and June, while the maximum temperature of 33 to 36°C is usually recorded between December and January.

Collection of leeches, maintainers and feeding

One hundred and twenty (120) leeches were collected in a local rice farm located at Fadikpe in Chanchaga Local Government Area, using the least mesh of a biological sieve net. The leeches were maintained in a well-aerated plastic aquarium filled with non-chlorinated water and kept in the laboratory at room temperature. Water was regularly changed every three days. The leeches were fed on sugar solution and animal blood collected from a slaughterhouse in Minna, Niger State.

Leech's saliva extraction

Leech saliva was extracted by using a method described by Abdulkader *et al.* (2013), without scarifying the animal. Leeches were starved for two weeks before the commencement of saliva extraction and prior to another saliva extraction; Leeches were fed a phagostimulatory solution made of a mixture of 0.001 M arginine and 0.15 M sodium chloride solution, which was heated at 37°C. This solution was placed in an inverted funnel coated with a parafilm sheet. Leeches were allowed to suck the solution through the parafilm until they were satiated and detached themselves from the parafilm. Saliva extract was obtained as described by Anwar and Mustafa. (2018) adding three leeches in a 10m test tube surrounded with an ice block for 15-20 min. This technique forces the leeches to regurgitate saliva through the mouth. To complete the saliva

extraction, leeches were squeezed smoothly from the posterior towards the anterior (mouth) sucker. All fluids that had been regurgitated were collected (bloody fluids were discarded) in another clean test tube and centrifuged at 4°C for 10 minutes at 9000 rpm. The supernatant was discarded, and the settlement fluid was named leech crude saliva extract and was stored for further use.

Preliminary biochemical analysis

Preliminary biochemical analysis of the local Nigerian leeches (*Aliolimnatis michaelsoni*) saliva for the presence of Alkaloides, Anthraquinones, Balsams, Cardiac Glycosides, Flavonoids, Saponines, Steroides, Tannines and Reducing Sugar, was performed according to the methods described by Shahidi and Zhong (2010).

Screening of test organisms

The test organisms used are *Staphylococcus aureus*, *Pseudomonas aeruginosa*, *Escherichia coli*, *Streptococcus pyogenes*, and *Bacillus subtilis* and were collected from the Microbiology Laboratory, Federal University of Technology, Minna. The isolated bacteria were cultured on nutrient agar and incubated at 37°C for 24 hours. The organisms were repeatedly subcultured to obtain pure isolates. Gram staining was carried out for proper identification. The organisms were finally inoculated into agar slants and stored at 4°C until further use (Junren *et al.*, 2021).

Standardisation of test organisms

A loopful of tested organisms was inoculated into 5.0 ml of nutrient broth and incubated 37°C for 24 hours. A 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 standardise the culture to 10⁶cfu/ml (Junren *et al.*, 2021).

Antimicrobial susceptibility screening

The screening antimicrobial activity was done by reconstitution of the local leeches' saliva extract and control (Ampiclox). About 1.8 g of crude leech saliva extract and 250 mg of control (Ampiclox) were added to a separate EDTA bottle that contains 5ml of sterile distilled water. A prepared nutrient agar was poured into a Petri dish and allowed to gel. A sterile cork borer (7 mm) was used to make two ditches in each Petri dish, one for crude leech saliva extract and the other for control. A stick swap was used to inoculate the test organism into the nutrient agar, and each plate was properly labelled. About 1 ml of

reconstituted leech saliva extract and control was dispensed into separate dishes. The plates were allowed for diffusion of leeches saliva and control before incubation at 37°C for a period of 24 hours. The zone of clearance produced around the ditches after incubation was observed, measured and recorded (Omalu *et al.*, 2015)

Determination of minimum inhibitory concentration (MIC)

The broth dilution method was used, where 9 ml of broth was dispensed into the 50 test tubes. The test tubes were arranged in 5×5, 1ml of reconstituted leech saliva was dispensed into 25 test tubes and 1ml of reconstituted control was poured into the remaining 25 test tubes. Serial dilution was done in all the test tubes. About 1ml of the tested organisms was inoculated in all the test tubes using a string and incubated at 37°C for 24 hours. The lowest dilution after incubation that shows no visible turbidity is regarded as the minimum inhibitory concentration (Green *et al.*, 2020)

Determination of minimum bactericidal concentration (MBC)

The test tubes that showed no visible turbidity after incubation of the batch of test tubes were sub-cultured on nutrient agar plates and incubated at 37°C for 24 hours. The concentration that shows no visible growth after incubation was considered the minimum bactericidal concentration (Green *et al.*, 2020)

Antioxidative activity

Antioxidative activity of leech saliva extract was performed according to the method of Blois (1985) using DPPH, although in this antioxidative test, 1,1-diphenyl-2-picrylhydrazyl. The DPPH was used as a free radical. Antioxidative activity of crude leech saliva extract was measured based on its ability to catch hydrogen released from DPPH. Crude leeches' saliva extract was diluted in Methyl alcohol solvent, and the solution was referred to as 3 times the crude leeches' saliva extract concentration. Three two-fold serial dilutions of Methyl alcohol crude leech saliva extract were prepared first by dissolving 100 µL crude leech saliva extract in 100 µL methanol. About 100 µL of this Methyl alcohol crude leech saliva extract solution was then dissolved in another 100 µL of methanol. The same step was repeated once again until Methyl alcohol crude leech saliva extract solutions with concentrations of 1:2, 1:4, and 1:8 were obtained. Then, into each tube, methanol solution was added until a final volume of 300 µl was reached. Simultaneously, DPPH was diluted in methanol to make a 0.002 M DPPH Methyl alcohol solution. 15 µl DPPH Methyl alcohol solution was

added to each tube, and after 15 minutes, an A516 absorbance reading was done. Phagostimulatory solution was used as a negative control. As a control reading, 15 µl DPPH was diluted in 300 µl methanol, and its A516 absorbance reading was done. The same procedures were used to prepare three two-fold serial dilutions of Methyl alcohol L-ascorbic acid (50 µg/ml) as a positive control (Ghawi *et al.*, 2012).

Statistical analysis

Data were subjected to an analysis of variance and a Duncan test.

RESULTS

Biochemical screening Biochemical screening showed positive (+) reaction where Alkaloids, Balsams, Cardiac Glycosides, Flavonoids, Saponins and Reducing Sugar, some negative (-) reactions which include: Anthraquinones, Steroid, Tannins (Table 1).

Antimicrobial susceptibility screening of local Nigerian leeches (*Aliolimnatis michaelsoni*) crude saliva extract

The saliva extract of the local Nigerian leech (*Aliolimnatis michaelsoni*) exhibited antibacterial activity against all the tested organisms when compared with the standard control antibiotic, Ampiclox. The zones of inhibition produced by the leech saliva extract were 20 mm for *Staphylococcus aureus*, 26 mm for *Pseudomonas aeruginosa*, 28 mm for *Escherichia coli*, 21 mm for *Streptococcus pyogenes* as shown in Plate 1 and 11 mm for *Bacillus subtilis* (Table 2). In contrast, Ampiclox produced larger zones of inhibition: 45 mm against *Staphylococcus aureus*, 48 mm against *Pseudomonas aeruginosa*, 46 mm against *Escherichia coli*, 38 mm against *Streptococcus pyogenes*, and 28 mm against *Bacillus subtilis*. Among the organisms tested, *Escherichia coli* showed the highest susceptibility to the leech saliva extract (28 mm), while *Bacillus subtilis* showed the lowest susceptibility (11 mm) (Table 2).

Minimum inhibitory concentration (MIC) of local Nigerian leeches (*Aliolimnatis michaelsoni*) crude saliva extract

The result of the minimum inhibitory concentration (MIC) revealed that the minimum inhibitory concentration (MIC) of the tested organisms, *Bacillus subtilis*, *Pseudomonas aeruginosa*, *Staphylococcus aureus*, *Streptococcus pyogenes*, and *Escherichia coli*, were 360 µg/ml, 360 µg/ml, 360 µg/ml, 360 µg/ml and 360 µg/ml, respectively,

Table 1. Biochemical screening of local Nigerian leech saliva collection.

Phytochemical Component	Leeches saliva extract
Alkaloid	+
Antraquinones	-
Balsams	+
Cardiac Glycosides	+
Flavonoids	+
Saponin	+
Steroid	-
Tennin	-
Reducing Sugar	+

Key: + Present; - absent.

**Plate 1.** Inhibited plate of leech extract and control.**Table 2.** Antimicrobial susceptibility screening of local Nigerian leeches (*Aliolimnatis michaelsoni*) crude saliva.

Organisms	Leeches saliva extract (mm)	Control (Ampiclox) (mm)
<i>Staphylococcus aureus</i>	20	45
<i>Pseudomonas aeruginosa</i>	26	48
<i>Escherichia coli</i>	28	46
<i>Streptococcus pyogenes</i>	21	38
<i>Bacillus subtilis</i>	11	28

Table 3. Minimum inhibitory concentration (MIC) of local Nigerian leeches (*Aliolimnatis michaelsoni*) crude saliva.

Organism	Leeches' saliva extract µg/ml	Control (Ampiclox) µg/ml
<i>Bacillus subtilis</i>	360	3600
<i>Pseudomonas aeruginosa</i>	360	3600
<i>Staphylococcus aureus</i>	360	3600
<i>Streptococcus pyogenes</i>	360	3600
<i>Escherichia coli</i>	360	360

Table 4. Minimum bacteria concentration (MCB) of crude local Nigerian leeches (*Aliolimnatis michaelsoni*) saliva.

Organism	Leeches saliva (mg)	Extract control (Ampiclox) mg
<i>Bacillus subtilis</i>	0.36	0.36
<i>Staphylococcus aureus</i>	3.06	3.60
<i>Streptococcus pyogenes</i>	0.36	0.36
<i>Pseudomonas aeruginosa</i>	0.06	0.36
<i>Escherichia coli</i>	0.36	3.60

Table 5. Inhibition concentration 50 L - ascorbic acid and crude leech saliva extract.

Extract	Inhibition concentration 50 ($\mu\text{g/g}$)	\pm SEM
Leech saliva extract	108.048	\pm 0.465
L-ascorbic acid	3.312	

when compared to the Ampiclox (control) that showed 3600 $\mu\text{g/ml}$ in four and 360 $\mu\text{g/ml}$, respectively (Table 3).

Minimum bacteria concentration (MCB) of crude local Nigerian leeches (*Aliolimnatis michaelsoni*) saliva

Table 4 shows the minimum bacterial concentration (MBC) of crude saliva from local Nigerian leeches (*Aliolimnatis michaelsoni*) compared with Ampiclox. The leech saliva extract was active against all tested organisms, with the lowest MBC observed against *Pseudomonas aeruginosa* (0.06 mg). Similar MBC values were recorded for *Bacillus subtilis* and *Streptococcus pyogenes* (0.36 mg) for both treatments. The extract showed lower MBC values than Ampiclox against *Staphylococcus aureus* and *Escherichia coli*, indicating comparatively higher antibacterial activity.

Antioxidant (Inhibition concentration 50 L - ascorbic acid and crude leech saliva extract)

Antioxidative activities of crude leech saliva extract in this study is shown in Table 5. Inhibition concentration 50 IC₅₀, defined as the concentration of the tested substance causing the loss of DPPH activity by 50%, was used as a parameter. The loss of DPPH activity as a free radical occurs as the tested substance donates a hydrogen (H) atom to DPPH.

DISCUSSION

The wide biodiversity of leech species all over the world has led to the establishment of many research studies focusing on the antimicrobial and antioxidative activity of their salivary secretion. This study established that local Nigerian leeches (*Aliolimnatis michaelsoni*) saliva extract

has antibiotic and antioxidant properties in their saliva. The preliminary Biochemical screening of the local Nigerian leeches (*Aliolimnatis michaelsoni*) saliva revealed the presence of alkaloids (a group of Nitrogen compounds that are active as drugs), Balsams (aromatic resinous substances), Cardiac Glycosides (compounds found in plants used in the treatment of heart failure), Flavonoids (An antibiotic), and saponins (amphipathic glycosides). The presence of alkaloids and flavonoids revealed the efficacy of the saliva against pathogenic bacteria. This work agrees with the findings of Alagwa *et al.* (2019), who worked on plants, reported that alkaloids and cardiac glycosides inhibit pathogenic bacteria. The presence of saponins agrees with Abdulkade *et al.* (2013), who stated that leeches are used traditionally in the treatment of venereal disease and diabetes. The presence of Flavonoids confirms the assertion of Ingrid (Alagwa *et al.*, 2019), who stated that leeches are one of the potential antibiotic organisms.

Antioxidants are also known as radical scavengers, which inhibit the oxidation process and eliminate free radical intermediates by oxidising themselves even at very low concentrations. Oxidative processes produce oxygen reactive species, which are a potent precursor of systemic cell and tissue damage. In this research, the extract of therapeutic local Nigerian leech saliva revealed free radical scavenging activity. A previous report on saliva extract revealed high free radical scavenging activity (Omalu *et al.*, 2015).

The antimicrobial activity is owned by annelids in the form of peptides found in the body fluid of annelids. Peptides B are antimicrobial peptides found in the body of *H. medicinalis* and *Theromyzon tessulatum* (Tasiemski *et al.*, 2004). These antimicrobial peptides were found to have antibacterial activity on Gram-positive bacteria.

The minimal inhibitory concentration (MIC) is the lowest concentration of a drug that prevents the growth of particular microorganisms. 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 organisms 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 between 360 and 3600 µg/ml of local Nigerian leeches (*Aliolimnatis michaelsoni*) saliva. Higher MICs were recorded for *Streptococcus sp* than those of *Bacillus sp*, *Pseudomonas aeruginosa*, *Streptococcus pyogenes*, and *Escherichia coli*. The high MIC volume for *Streptococcus sp* indicates that these organisms are more resistant to the leech's saliva extract than *Bacillus sp*, *Pseudomonas aeruginosa*, *Streptococcus pyogenes*, and *Escherichia coli*. This is probably due to differences in the 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 agrees with work done by Bishop (2005), on Honey bees, stating that Honey's antibiotics, 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 (Bishop, 2005).

Vitamin C (L-ascorbic acid) was used as a positive control, as this acid is a very strong alternative antioxidant. The 50% inhibition concentration value of ascorbic acid was used as a comparison to the 50% inhibition concentration values obtained from crude leeches' salivary extracts to determine the strength of the crude leeches' salivary extracts' antioxidative properties. Results showed that the antioxidative power of crude leeches' salivary extraction in this study was very low when compared to that of ascorbic acid. Antioxidative activity of leech saliva extract was first reported by Malaysian researchers (Ghawi *et al.*, 2012). The crude leech's salivary extraction they got from Malaysian local leeches had an inhibition concentration 50 value of 7.282 µg/ml, and the inhibition concentration 50 value of the ascorbic acid they used was 5.803 µg/ml.

Conclusion

It may at first glance hardly seem compatible with modern medicine, but local Nigerian leeches' saliva extract has indeed re-established itself as an inherent part of contemporary healthcare. The results reported here have proved that the Nigerian leech's saliva extract possesses a similar antithrombin activity to that of the Malaysian

species, 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 its utility in traditional medicine for the treatment.

CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

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