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ANTICANCER EFFECT OF SALIVA EXTRACT OF THE NIGERIAN LEECH (*Aliolimnatis michaelseni*)

Mgbemena, C. C.[1], *Omalu, I. C. J.[2], Egwim, E. C.[3], Olayemi, I. K.[2], Ossai, P. C.[3] and Eke, S. S.[4]

[1] Dental Centre, General Hospital Minna, Niger State
[2] Department of Animal Biology, Federal University of Technology Minna, Niger State
[3] Department of Biochemistry, Federal University of Technology Minna, Niger State
4 Department of Biology, Admiralty University of Nigeria, Ibusa/Ogwashi – Uku, Delta State

***Corresponding author: omaluicj@futminna.edu.ng**

ABSTRACT
Leeches are haematophagous creatures widely distributed all over the world, and have found application in medicine for over three millennia. Leech saliva extract (LSE) contains proteins and peptides that have wide range of potential applications in life-threatening and other diseases, such as cancer metastasis. The aim of this study was to demonstrate the anticancer effects of the saliva extract of the Nigerian leech, *Aliolimnatis michaelseni*, against human breast cancer cells (MCF-7), *in vitro*. Leech samples were collected from freshwater bodies in Minna, Nigeria. MTT [3-4-5 dimthylthiazolyl-2]-2, 5-diphenyltetrazolium bromide] and Comet assays were deployed to study the cytotoxic and genotoxic potentials of these leech extracts. Results of the MTT assay showed that the saliva extract exacted a dose-dependent cytotoxic effect on the MCF-7 cells, with LC_{50} of 0.61% (v/v) or 1.55µg/ml. Comet assay results showed that the LSE had a significantly more potent genotoxic effect ($P<0.05$) than Cisplatin (standard drug for human breast cancer) 5%. LSE had a tail DNA of 9.74%±2.51 while 5% Cisplatin had a tail DNA of 2.7%±0.70. The study revealed that the protein contents of the saliva of *Aliolimnatis michaelseni* have a promising anticancer potential against human breast cancer cells.

***Keywords**: Leech saliva extract; Anticancer effects; Cytotoxicity; Genotoxic potentials*

INTRODUCTION
Leeches are haemotophagous creatures widely distributed all over the world, and have found application in medicine for over three thousand five hundred years. Egyptians according to records have used leech therapy for that long and have them as part of the hieroglyphics painting on walls (Munshi *et al*., 2008). The word "leech" was derived from the Anglo-Saxon "Loece", which means "toheal", the doctor at the time was called "a leech", and his medical book was called "leechdom (Hyson, 2005).

Secretions such as saliva, produced by haematophagous animals like leech have been known to contain compounds with biological activities, accounting for their therapeutic applications, since the beginning of civilization (Abdulkader *et al*., 2013). Recent studies of leech saliva have characterized the protein and peptides therein, and shown them to have antithrombin activity (hirudin, bufrudin), antiplatelet activity (calin, saratin), factor Xa inhibitors (lefaxin) and antibacterial activity (theromacin, theromyzin). For this reason, leech appears to be making a comeback as a new approach in the management of many chronic and life-threatening diseases, like cancer metastasis, cardiovascular problems, and infectious diseases (Abdulkader *et al*., 2013).

Cancer is front line cause of mortalities globally. It is said that 70 % of all cancer deaths occur in low and middle income countries such as Nigeria (Olakanmi *et al*., 2015). The heart of pathology of cancer is two-fold: first is the uncontrollable division of the affected cells which ultimately leads to tissue damage. Secondly, these cells are able to deposit at areas distant from the primary site through the lymphatic and/or vascular systems and form new cancer cells- a process known as metastasis.

Relying on data from the Nigerian cities of Abuja, Calabar and Ibadan cancer registries, Global Cancer Observatory stated that human breast cancer had the highest incident rate (26,310; 22.7%) among all cancers in both sexes and of all ages, in 2018 (IARC, 2019). The agency also recorded that among death related to cancer in 2018, those from breast cancer ranked highest at 16.4% (11,564). Crude saliva extract from the medical Malaysian leech showed promise in cytotoxicity against small cell lung cancer cell line (Merzouk *et al*., 2002). At the moment, there is no documented record of such work done with the crude saliva extract of *Aliolimnatis michaelseni* on any cancer cells.

MATERIALS AND METHODS
Leech sampling and extraction of Saliva
Leech collection was done by the use of locally constructed trapping device and scooping net, as described by Pennuto and Butler (1993). The trapping device was funnel-shaped and made of plastic material, 17.5 cm in height and 14.5 cm in diameter. The top was covered with a net which funneled into the chamber, opening through an apex of 1 cm. The scoping net was 15 cm in diameter with a handle of 1.5 cm long. Baits of frozen fish parts were secured in the funnel traps. These were placed at intervals in littoral areas of the water, at depth of 0.5 – 1.0 m. the traps were deployed at dusk and retrieved at dawn. A total of 1156 Leeches (*Aliolimnatis michaelseni*) were collected over a period of 8 months (June 2014-January 2015) from shallow ponds and streams located within the vicinities of the Gidan Kwano and Bosso Campuses of the Federal University of Technology, Minna, Niger State, Nigeria. They were kept in well aerated 400litres plastic aquaria containing non-chlorinated water from the University borehole. They were kept at room temperature, with water pH of 6.5 and shielded from strong light. The water was changed every three days.

Samples of the collected leeches were sent to the American Museum of Natural History, New York, USA, for identification to the species level.

Prior to commencement of extraction, the leeches were fed with blood of cows from the local abattoir. Feeding was accomplished by placing the leeches in batches in plastic aquaria with enough cow blood to cover the bottom surfaces. Any leech fully fed will appear engorged and would initiate a climb out of the blood medium. Fully feed leeches were subsequently starved for two weeks. Saliva extraction was done according to the method described by Rigbi *et al*. (1987).

The leeches were transferred to glass containers (test-tubes), and placed in plastic containers filled with ice cubes for 10 minutes. The drop in temperature induced vomiting in the leeches. The vomited saliva, devoid of blood, was aspirated using syringe and needle and placed in sealed specimen bottles. To complete saliva extraction, the leeches were squeezed smoothly from the posterior towards the anterior sucker and this, without scarification on the leeches. This modified method of extraction was described by Abdulkader *et al*. (2011).

Anticancer effect of saliva extract of *Aliolimnatis michaelseni*

The study of the anticancer effect of the saliva extract from *Aliolimnatis michaelseni* was done in collaboration with the Nigeria Institute of Medical Research (NIMR), Lagos.

Two assays were deployed to determine the cytotoxic and genotoxic potentials of this extract, known respectively as MTT [3-4-5-dimethylthiazoly 1-2] -2,5-diphenyltetrazolium bromide] assay, and the comet assay.

MTT assay to study the cytotoxic potential of saliva extract of *Aliolimnatis michaelseni*

The method described by Mosmanu (1983), and modified by Southon *et al*. (2004) was used.

Media Preparation

A little (1L) of distilled water was measured out, sterilized and stored at room temperature, 9.8g of Minimum Essential Medium (MEM), 5.96g of HEPES buffer and 2.25g of Na_2CO_3 were introduced into the distilled water and carefully mixed. To this was added 20ml of 10% fetal bovine serum (FBS). This solution was then transferred to a T75 flask by filtration using a filter sterilizer. The flask was sealed with paraffin and placed in a vertical position inside a 5% CO_2 incubator for ease of sterility monitoring (Southon *et al*., 2004).

Cell culturing and maintenance of human breast cancer adenocarcinoma

Human breast adenocarcinoma (MCF-7) cell line was sourced from Sigma Aldrich, USA. It was preserved in MEM medium and 10% FBS (v/v) and 1% penicillin streptomycin was added as supplement, and kept in 5% CO_2 incubator at a temperature of 37^0C. The cells were regularly inspected for their physiologic and growth features. The indicators for these were the maintenance of even distribution, normal morphology and cell density. Contamination would make the medium cloudy, with a change in color from reddish pink to pale pink, in which case the affected cells were discarded.

Cell passaging and harvesting of human breast cancer adenocarcinoma

The cells in the culture flask were inspected under the microscope to confirm cell growth of up to 70-90%. The growth medium was thereafter, discarded into a waste jar containing 10% bleach solution. In order to terminate the action of the FBS, freshly made phosphate buffer saline and 2ml of trypsin were added to the flask. This also helped to detach the cells of the MCF-7 from the walls of the flask, where they attached for support. The culture flask was thereafter, returned to the 5% CO_2 incubator for 4 minutes. Once the cells were completely detached, FBS was added for the flask to terminate the action of the trypsin. The cell suspension was transferred by means of pipettes, into the bleu cap falcon tubes and centrifuging was done for five minutes at 5,000rpm while the pellets of cell were left at the bottom of the tubes (Southon *et al*., 2004).

Fresh medium was put into the falcon tubes and mixed with the cells using stripette and pipet gun. 9ml of the fresh suspension medium were transferred to the T75 flask which was then sealed with paraffin and placed for preservation in the incubator. After 24 hours, the cells were inspected under the microscope to monitor their condition (Southon *et al*., 2004).

Trypan blue count

For cell counting, 10µl of cell suspension and 0.4%, trypan blue solution of the same volume were mixed and transferred into the chambers of a clean haemocytometer. Live and dead cells were counted, while percentage of viable cells and seeding concentration were determined using the formula:

$$\%\ viability = seeding\ concentration = x\ df\ x\ 10^{d}\ cells/ml$$

(dilution factor = df :1:1)

Cell plating

Centrifugation of cell suspensions was done for 5 minutes at 5000 rpm, at room temperature. The supernatant was discharged. Cell pellets were thereafter, re-suspended in growth medium. 1.5 x 105 cells were seeded into each well of a flat-bottomed 96-well microtitre plate and incubated at 37^OC in 5% CO2 incubator for 24 hours (Southon *et al*., 2004).

Dosing of cells

At this stage, the cells were treated with the Leech saliva extract. The starting concentration was 10ml. From the highest concentration; serial dilution was done to obtain different concentration of the leech saliva extract. Incubation of the treated cells was done for 24 hours. MTT assay was done after this incubation period (Southon *et al*., 2004).

MTT assay

To each of the wells in the flat-bottomed 96-well plate was added 10µl of MTT solution using the multi-channel pipette. Incubation of the Plate was done for 3 hours and 100µl of fresh Dimethyl Sulfoxide (DMSO) was introduced to each of the wells to solubilize the crystals of formazin formed by enzymes action on the MTT. Finally, the absorbance of the dissolved formazan crystals was measured at 492nm and 630nm for each well, on the Bio-Rad colorimetric multi-well plate reader (Bio-Rad Laboratories India Pvt Ltd). This test was done in triplicates according to (Southon *et al*., 2004).

Comet Assay to study the Genotoxicity Potential of Saliva Extract from *Aliolimnatis michaelseni*

This method was described by Singh *et al*. (1988) and modified by Hartman and Speit (1995) and further by da Silva *et al*. (2000). The following reagents were prepared:

1. Phosphate Buffer Saline (PBS) was prepared using8.0g (137mM) of sodium chloride, 0.25g (1.4mM) of potassium dihydrogen phosphate, 0.2g (2.7mM) of potassium chloride, and 1.44g (4.3mM) of disodium hydrogen phosphate dissolved in 800ml of double distilled water. The pH was adjusted to 7.4 and the volume made up 1000ml. This solution was autoclaved and stored at room temperature (Silva *et al*., 2000).

2. Normal Melting Point Agarose (NMPA) 0.75% v/v was prepared by adding 188mg of NMPA to 25ml of PBS in a 100ml beaker and sealed with Aluminum foil. The NMPA was melted in a microwave oven at low power for 2 minutes.
3. Low Melting Point Agarose (LMPA) 0.5% v/v was prepared in a 100ml conical flask by adding 125mg of LMPA to 25ml of PBS. The mouth of the conical flask was sealed with aluminum foil, and the LMPA melted in the microwave oven at low power for 2 minutes.
4. Lysis Stock solution with pH set at 10 and the final volume made up to 890ml with double distilled water was gotten from a mixture of 146.1g (2.5m) of sodium chloride, 37.2g (100mM) of disodium ethylene diamine tetra acetic acid (EDTA) and 1.2g of tris to 700ml of double-distilled water, stirred until dissolved and 12g of sodium hydroxyl pellets were added and stirred to dissolution. Thereafter, 10g of sodium 1auryl sarcosinate was added and stirred to dissolution. The solution was fattened and stored at room temperature. A Working solution was prepared prior to use, by adding 1.6ml of Triton X-100 to 108ml of stock lysis solution. This mixture was cooled in a refrigerator for 60 minutes.
5. Electrophoresis buffer was prepared using a mixture of 30ml Stock solution I (200g of sodium hydroxide dissolved in 500ml double-distilled water) and 5ml Stock solution II (14.89g of disodium EDTA (200mm) dissolved in 200ml of double distilled water and the pH adjusted to 10, using sodium hydroxide). The volume was made up to 1000ml with cold double distilled water and adjusted to fill the electrophoretic tank.
6. Neutralization buffer was made with 48.5g of tris (0.4m) dissolved in 800ml of double distilled water with pH adjusted to 7.5 using concentrated hydrochloric acid. The final volume was made up to 1000ml.
7. Reagents for fluorescent staining Stock solution: 10mg of ethidium bromide was dissolved in 50ml of double-distilled water and a Working solution was gotten by adding 1ml of stock solution to 9ml of double-distilled water.

Sample Preparation

Using the same procedure as was done in the MTT assay, a new batch of cells of Human breast adenocarcinoma (MCF-7) sourced from Sigma Aldrich, USA, was grown on Minimum Essential Medium. Cell samples were handled under dimmed light to avoid DNA damage from ultraviolet light. The buffers used were cooled to 4^{o}C to prevent endogenous damage happening during sample preparation. Centrifugation of cell suspensions was done, the supernatant discharged and cell pellets re-suspended in growth medium. Thereafter 1.5 x 105 cells were seeded into 24 wells of a flat-bottomed 96-well microtitre plate and incubated at 37^{o}C in 5% CO_2 incubator for 24 hours. The Wells were divided into four groups of six wells each and the cells in Groups 1 to 4 treated with 0.75% v/v concentration of the extract; 5% v/v concentration of the extract, 5% v/v concentration of Cisplatin, and 2ml of the growth medium as negative control respectively. Groups 3 and 4 served as controls. Incubation of the treated cells was done for 24 hours.

Slides preparation

Six roughened slides were used for this experiment, two slides for each test. The slides were immersed briefly; into 1.5% hot normal melting agarose (NMA) in phosphate-buffered saline (PBS). The slides were dried and stored in a refrigerator of 4^{o}C, coating of each of the slides with boiled 300µl of 10% NMA in PBS was done. A coverslip was placed over each of the slides to flatten the layer of molten agarose, and stored in a humidified box at 4°C until ready for use 24 hours after. This was to enable the agarose to gel.

A 0.75% low melting agarose was added to the treated cells and placed on six of the prepared slides at the rate of 1000 cell density per ml, two slides each, for each test group. Rightly-sized coverslips were used to spread this on the slides and placed in a moist box to gel at 4°C. After the gelling of the agarose, the slides were transferred into a cold freshly-prepared lysing solution, for one hour. Alkaline unwinding of leech saliva extract was done by taking out **t**he slides from the lysing solution and putting them in the electrophoresis chamber already filled with freshly-prepared alkaline buffer. This exposure was done for 30 minutes at 300mA and 25V and is useful for the facilitation of the unwinding of the DNA and to express the alkali labile sites. After electrophoresis, the slides were placed horizontally and washed with 0.4MTris buffer, three times, at 5 minutes per wash. This was done to neutralize the excess alkali.

Staining of DNA and visualization of Comet

Finally, the DNA-specific dye, ethidium bromide, 70µl, was introduced to each slide, inside the staining box. The staining was sustained until a greyish colour appeared on the slides. The slides were then placed in a tray that contained the stopping solution for five minutes, until a yellow-brown colour appeared. Thereafter, the slides were washed with distilled water, placed in an inclined position and dried at room temperature.

Microscopy

The slides were viewed using a fluorescent microscope and 25 randomly selected images were viewed in each of the slides. The images were captured with a charge-coupled device camera and subsequently subjected to computer analysis using the Casplab 1.2, 3b2 exe computer software. This applied a metric based on the percentage of migrated DNA

Data Analysis

Data analysis was done using SPSS software 20.0. Two sample t-test was used to determine if the head and tail DNA of the 5% LSE and 5% Cisplatin differed significantly, at $P<0.05$.

RESULTS

MTT Cytotoxic Assay

Results showed that the saliva extract of *Aliolimnatis michaelseni* demonstrated a potent anti-proliferation activity against Human Breast Cancer cells (MCF-7) in a dose-dependent manner. The total protein concentration of the extract required to produce inhibition of the growth of 50% of the treated cells after 24 hours of incubation (LC_{50}) was 0.61% (v/v) or 1.55µg/ml. The lowest viability of the MCF-7 cells of 28.50% (v/v) was at 12.50% (v/v) of the extrac
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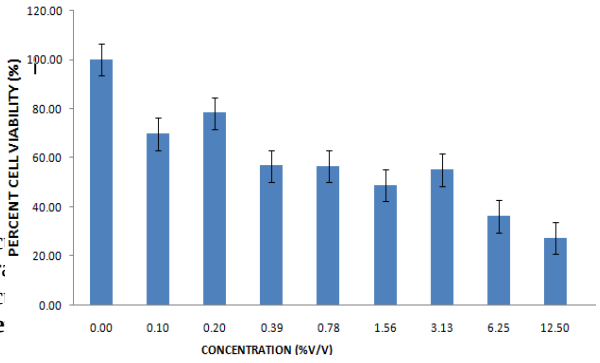

Figure 1: The effect of 24-hour exposure of Human Breast Cancer Cells (MCF-7) to varying concentrations of saliva extract of *Aliolimnatis michaelseni* (LC_{50} of 0.61 (v/v))

Investigation of the genotoxicity and ability of the saliva extract of *Aliolimnatis michaelseni* for Deoxyribo-nucleic Acid (DNA) damage showed a dose-related effect with tail moments of 1.75 ± 0.54 and 0.98 ± 0.25 by 5% and 0.75% V/V concentrations of the extract, respectively while 5% concentration of the standard anti-cancer drug which was used as positive control gave tail moment of 0.46 ± 0.13.

The medium, MEM, which was the negative control showed a tail moment of 0.15 ± 0.221. The tail DNA for 0.75% concentration of the extract was 4.88% ± 1.14, while the head DNA for the same concentration was 95.14% ± 1.94.

The head DNA for 5% concentration of the extract was 90.30% ± 2.31 while the tail DNA was 9.74 ± 2.51. The negative control yielded head DNA of 99.43% ± 0.57 and tail DNA of 0.57% ± 0.23.(Table 4.8). Plate 10 shows the DNA damage induced by exposure of MCF-7 cells to different concentrations, of leech saliva extract, Standard anti-cancer drug Cisplatin and the growth medium.

Table 1. Effect of 24-Hour exposure of Breast Cancer (MCF-7) Cells DNA to Saliva Extract of *Aliolimnatis michaelseni*

Agent concentration (%v/v)	Tail DNA %	Head DNA %	Tail moment
LE (0.75%)	4.88 ± 1.14	95.14 ± 1.04	0.98 ± 0.25
LE (5%)	9.74 ± 2.51	90.30 ± 2.31	1.75 ± 0.54
Cisplatin (5%)	2.7 ± 0.70	97.34 ± 1.07	0.46 ± 0.13
Negative Control (2ml MEM)	0.57 ± 0.77	99.43 ± 0.53	0.15 ± 0.221

Cisplatin: standard drug, control: MEM, LE: Leech Extract. t-test = 0.05

DISCUSSION

The findings of the MTT assay of the effect and viability of Human Breast Cancer Cells (MCF-7) exposed to the crude saliva extract of the leech, *Aliolimnatis michaelseni* for 24 hours showed a dose-dependent cytotoxic effect on the cancer cells of the human breast. The total protein concentration that inhibited 50% of cell growth during the period of exposure was 0.61% (v/v), equivalent to 1.55µg/ml. The highest concentration of the extract at 12.50% (v/v), equivalent to 31.84µg/ml yielded the lowest viability of 28.51%.

In a similar work by Merzouk *et al.* (2002) to study the anticancer effect of the Medical Malaysian leech saliva extract, a dose-dependent cytotoxicity against small cell lung cancer cell line (SW1271) was recorded. This time however, the total protein concentration needed to inhibit 50% of cell growth was 119.844µg/ml while the total protein concentration required to produce the lowest cell viability of 40% was 625.490µg/ml. Though these figures are much higher than the corresponding figures seen in this study, it may not be sufficient to make comparative statement on potencies because of the difference in the type and nature of cancer cells to which the extracts were exposed.

The findings of this study also demonstrated that the crude extract of the saliva of *Aliolimnatis michaelseni* has a dose-dependent genotoxic effect on the DNA of Human Breast Cancer cells (MCF-7), when exposed to same for 24 hours.

These crude saliva extract of *Aliolimnatis michaelseni* is significantly more genotoxic against Human Breast Cancer Cells, at $P<0.05$, than Cisplatin, a standard anticancer drug.

On the basis of published data, this work is the first to show the cytotoxic and genotoxic effects of the saliva extract of *Aliolimnatis michaelseni* against Human Breast Cancer Cells. Other authors showed the anti-metastatic capacity of the saliva extract from *Haemanteria ghilianii* and *Haemanteria officinalis* and ascribed this activity to the anticoagulant factors within the extracts (Merzouk *et al*., 2012; Gasic *et al*., 1983). Merzouk *et al*. (2002) demonstrated the cytotoxic effect of the saliva of the medicinal Malaysian leech, *Hirudo manillensis* against small cell lung cancer cell line SW 1271.

Other researchers also indicated that *Hirudino medicinalis* mediated its anti-metastasis through its antithrombin peptide, hirudin (Merzouk *et al*., 2012). There are equally, natural substances sourced from plants, animals, marine organisms and microorganisms that play important roles in cancer therapeutics (Gasic *et al*., 1983).

Some studies have indicated the possibility of a relationship between antimicrobial and anticancer activities. The first indication of such a relationship was in 1940 when the compound Actinomycin D, derived from *Actinomyces antibioticus* was found to exert both antimicrobial and anticancer effects (Nobili *et al*., 2009). Some proteins extracts from the annelid earthworm have also concomitantly shown antimicrobial and anticancer effects (Yanqin *et al*., 2007; Waksman *et al*., 1941). This body of evidence may suggest that the saliva extract of *Aliolimnatis michaelseni* may owe its antimetastatic activities to the presence of antimicrobial components. Isolation and purification of the crude extract would be required to identify specific active substance(s) responsible for these observed anticancer effects, with the attendant possibility of development of a chemotherapeutic agent against human breast cancer cells

CONCLUSION

This work has demonstrated the potency in vitro, of the crude saliva extract of *Aliolimnatis michaelseni* against human breast cancer cells (MCF-7).

CONSENT

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The anticancer effect of saliva extract of the Nigerian leech was conducted at the National Institute of Medical Research (NIMR), Lagos, Nigeria.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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Contribution of Authors

1. C, C. Mgbemena and I. C. J. Omalu designed the study
2. E. C. Egwim, I. K., Olayemi and P. C. Ossai carried out the Laboratory work
3. S. S. Eke performed the data analyses
4. All authors read and approved the manuscript