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A study on bioactive compounds from *Potamon ebonyicum* (Freshwater crab) in Ebonyi River Basin Nigeria

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Abstract
Bioactive compounds from freshwater crab (*Potamon ebonyicum*) was extracted and characterized in Ebonyi River Basin. Chitosan was extracted by standard methods which involved demineralization, deproteinization and deacetylation of chitin from male and female crabs. Bioactives from the chitosan (polysaccharide, peptide, alkaloid, and lipid) were quantified and analysed using one way analysis of variance (ANOVA). The result revealed sex based variation in bioavailability and accumulation of the bioactive compounds. The female crab recorded more chitosan yield, and significantly higher ($p < 0.05$) polysaccharide than the male crab. The male has higher concentration of peptide, alkaloid and lipid than the female crab. Subsequently and in terms of production of polysaccharide, the female *P. ebonyicum* should be recommended while the male could be used for the production of peptide, alkaloid and lipid. The findings might strategically support the development of local extraction and utilization of bioactive compounds for positive impacts on pharmaceutical industries and the economy.

Keywords: Bioactives, Crab, *Potamon ebonyicum*, Ebonyi river basin

Introduction

Freshwater crabs are an important component of aquatic biodiversity in Nigeria. They have been observed in river systems like the Cross River, Imo River, and the Niger Delta tributaries, suggesting a broad ecological adaptation across various freshwater habitats in southern Nigeria (Yushau *et al*., 2023) [18]. The Ebonyi River Basin in southeastern Nigeria is one of the key habitats for *Potamon ebonyicum*, a species endemic to the region (Akpaniteaku *et al*., 2019) [4]. These crabs are commonly found in muddy or rocky substrates along riverbanks and are often associated with burrows or submerged vegetation where they shelter and forage.

The distribution of freshwater crabs is often closely tied to water quality, substrate type, temperature, and availability of food. They tend to favor oxygen-rich environments with moderate to slow currents and prefer freshwater systems that are not heavily polluted or disturbed. Moreover, endemic species such as *Potamon ebonyicum*, exhibit restricted distribution and may be vulnerable to habitat degradation, pollution, and overharvesting.

Conservation of these species requires understanding their ecological niches and maintaining the quality of their aquatic environments (Akpaniteaku and Emmanuel, 2017) [6]. Studies have also shown that some freshwater crab species have narrow temperature tolerance and cannot survive in brackish or saline water, which further limits their spread and makes them useful as indicators for environmental monitoring (Ng *et al*., 2021). Freshwater crabs, especially those from less explored river systems like the Ebonyi River Basin, are increasingly being studied for their biochemical constituents and therapeutic potentials.The Ebonyi River Basin is home to the *Potamon ebonyicum* (Fig 1), a species that plays an essential role in the local ecosystem and is frequently consumed by inhabitants and tourists (Akpaniteaku *et al*., 2019) [4]. While research has largely focused on its pathogenic exposure, the unique biology of *P. ebonyicum*, especially its vulnerability to environmental stressor and bioaccumulation of substances, suggests its potential as a reservoir of bioactive molecules (Akpaniteaku and Udeozor, 2018) [3, 7]. Bioactive compounds such as peptides, sterol, carotenoids, and fatty acids from crabs have shown significant pharmacological effects, including antioxidant, antiinflammatory, and antimicrobial activities (Manivannan *et al*., 2022) [12].

Majority of the pharmaceutical companies are engaging in isolation of bioactive compounds from the marine organisms making the structure and function of the compounds become known in drug development advances in research have raised the issue of looking into natural products from the freshwater ecosystem which have beneficial effects on health and wellbeing of humans (Chaven and Patil, 2021). Regular consumption of freshwater crab by humanbeings make it absolutely necessary to assess the health implication of their components. And it is very important to evaluate the bioactive compounds in *Potamon ebonyicum*, which has been serving as source of protein and other essential elements in Ebonyi River Basin. The study may contribute to accessibility and development of local material for the pharmaceutical industry.

Microbiological surveys in the Ebonyi River Basin have highlighted the susceptibility of *P. ebonyicum* to bacterial contamination, notably with species like *Pseudomonas aeruginosa* and *Staphylococcus aureus* (Akpaniteaku *et al*., 2019) [4]. These findings not only indicate the need for public health caution but also suggest the presence of microbial interactions that may stimulate the production of defensive bioactive metabolites within the crab. Despite this, there is a significant lack of studies investigating the bioactive compounds inherent in this freshwater crab species. Given the increasing global interest in natural products for pharmaceutical development, studying bioactive components in *P. ebonyicum* could contribute to novel drug discovery and nutraceutical development. This study, therefore, aims to isolate and characterize bioactive

compounds from the freshwater crab species in the Ebonyi River Basin, thereby enhancing our understanding of the biomedical potential of indigenous aquatic organisms in the country.

Fig 1: *Potmon ebonyicum*

Fig 2: *Potmon ebonyicum* Ventral view (Female)

Fig 3: *Potmon ebonyicum* Ventral view (Male)

Materials and Methods

Crab used in this study was collected from Ishieke community in the periurban area of the river basin with no mining and quarry activities. Adult species was collected for the bioactive analysis. Male and female crabs (Fig. 2 and 3) weighing 34.81 to 38.49g and 24.78 to 33.21g respectively were sampled. After collection, they were transferred alive to the laboratory and subjected to mercy killing by mechanical stunning.

1. Chitin Production

Whole Crab was transferred to the laboratory and dried in crucibles before being transferred to an electric oven for complete drying, and to obtain the sample in powder form. The sample was ground into powder with pestle and mortar and subjected to standard methods which involved deproteinization, demineralization and deacetylation. Dried 73.31g of the male crab and 58.03g of the female crab were deproteinized by treating in 200ml of 1.5N NaOH and the mixture plugged under agitation at 80°C for 3hrs. The mixture was washed with distilled water until it achieved a neutral pH of 7.0. It was immersed in ethanol for 10mins for further bleaching and the resulting Chitin was dried in an oven at 70°C for 3hrs. Weighed into round bottom flasks containing 200ml of 0.5M HCl. The reactor was carried out at room temperature under agitation at 250rpm for 6 hours afterwards, the demineralize samples were filtered and washed with distilled water until neutral PH. They were by immersing in ethanol for 10mins and dried in an oven at 70°C for 3 hours.

2. Chitosan production

Deacetylation of the Chitin was achieved by reacting Chitin with 200ml of 12.5m NaOH. The reaction mixture was cooled down and kept frozen at 4°C in a refrigerator for 12hrs.

Afterwards the temperature of the mixture was raised to 115°C, and the reaction agitated at

250 rpm for 6 hours. The resulting chitosan was filtered washed with distilled water until neutral pH and dried in an oven at 70°C for 3 hours, cooled in a dessicator and stored in a container for characterization.

3. Peptide production

3.1. Standard Preparation

This preparation was achieved by dissolving 3g of standard peptide powder in 10ml of phosphate buffer and stir for 5 minutes. Diluted further in separate beakers using phosphate buffer solution to yield 10mg/ml, 40mg/ml, 60mg/ml, 80mg/ml and 100mg/ml. Absorbance of the standard solution was measured at 210nm and graph of absorbance plotted against concentration to get the calibration graph in Figure 4.

3. 2. Sample preparation

One (1)g of the produced male and female crabs chitosan samples were each dissolved in 10mg/ml of 0.2m NaoH and stir at 50 degrees for 30 mins. The solution was cooled and the pH adjusted to 4.5 and the absorbance read at 210nm.

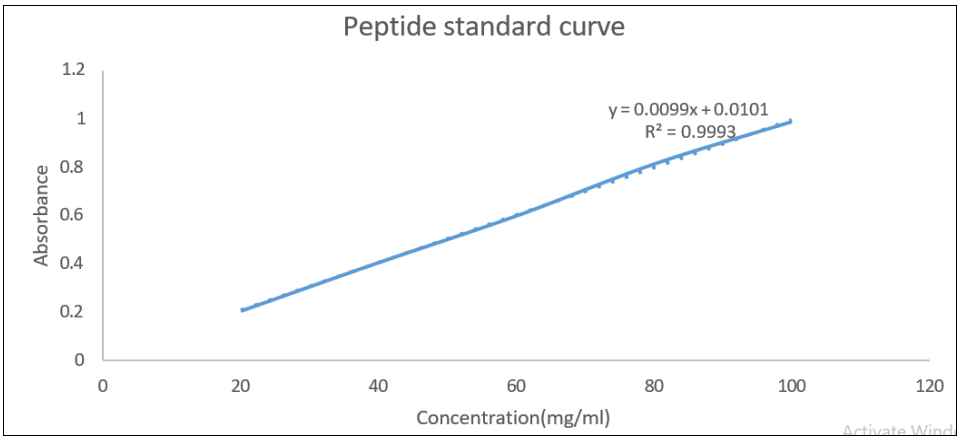

Fig 4: Standard curve for peptide analysis on the chitosan of male and female crab (*Potamon ebonyicum*)

Peptide content calculation using the formular; y=0.0099x + 0.0101 Where y = Absorbance X = Concentration

1. Male crab

Absorbance = 0.018 Therefore, peptide concentration; 0.061=0.0099x + 0.0101

$$x = \frac{018-0.0101}{0.0099} = 0.797\text{mg/m}$$

2. Female crabs

Absorbance = 0.013 Therefore, peptide concentration; 0.043=0.0099X + 0.0101

$$x = \frac{013-0.0101}{0.0099} = 0.292\ \text{mg/ml}$$

Polysaccharides production

1. Standard Preparation

This preparation was achieved by taking 100mg of glucose standard into a 250ml beaker and adding 5ml of 2.5N Hcl. Boiled in a hot bath for 3hrs at 100°C. Cooled and neutralized with sodium carbonate powder. Filtered and made up to volume with 100ml of distilled water. Distilled further in separate beaker to yield 0.02mg/ml, 0.04mg/ml, 0.06mg/ml, 0.08mg/ml and 0.1mg/ml. One(1) ml of phenol solution and 5ml of 96% H2SO4 were added to each of the beakers. The beakers were placed in a water bath and boiled at 25-30°C for 20 minutes and observed for development of a green colour. The absorbance of the standard solutions was measured at 490nm and a graph of absorbance was plotted against concentration to get the calibration graph in Figure 2

2. Sample preparation

Hundred (100)mg of the male and female crabs chitosan samples were taken in 250ml beakers, and 5ml of 2.5N HCL added and boiled in a hot water bath for 3hrs at 100°C. Cooled and neutralized with sodium carbonate powder. Filtered and made up to volume with
100ml of distilled water. Diluted further to yield 0.1 mg/ml. One(1)ml of phenol and 5ml of
H2SO4 were added to the sample. The beakers were placed in a water bath and boiled at 2530°C for 20 minutes and observed for the development of green colour. Absorbance of the sample was measured at 490nm and the concentration of polysaccharides presents in the sample extrapolated from the standard curve.

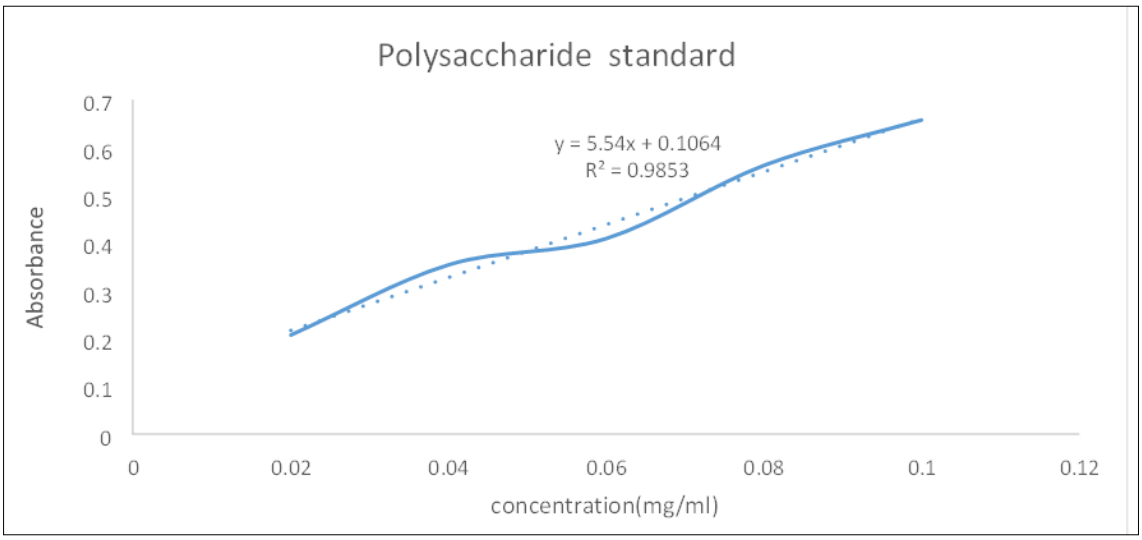

Fig 5: Standard curve for polysaccharides concentration analysis on the chitosan of male and female crabs (*Potamon ebonyicum*)

Polysaccharides content calculation

Using the formular; y=5.54x + 0.1064 Where y = Absorbance X = Concentration

1. Male crab

Absorbance = 0.498

Concentration; 0.498= 5.54x+ 0.1064

$$x = \frac{541-0.1064}{5.54} = 0.0784\times1000=78.40\text{mg/ml}$$

2. Female crab

Absorbance =0.567 0.567 = 5.54x+0.1064

$$x = \frac{569-0.1064}{5.54} = 0.0836\times1000=83.60\text{mg/ml}$$

Sample preparation alkaloids and lipids profile

1. Extraction of oil from chitosan

250ml round bottom flasks was dried in oven at 105-110°C for about 15 minutes and transferred into a dessicator and allowed to cool. The 250ml flask was filled with 100ml n-hexane solvent 5g of the chitosan samples (male and female) each were weighed and inserted into the thimble of sochlet apparatus with cotton wool underneath to serve as filter. The apparatus was assembled on the boil flask of the sochlet apparatus and allowed to stand on electric hot plate at temperature of 60-75°C, and allowed to reflux about 4 times for five repeated extraction. Extract from the flask was collected and emptied into rotatory evaporator at temperature of 40-60°C to separate the n- hexane solvent from the extracted oil. The extracted oil was collected and stored in a container for florisil clean up.

2. Florisil clean up for alkaloids and lipids profile

An hydrous $NaSO_4$ (0.5g) was added to 1.0g of activated florisil (magnesium silicate) (60100nm mesh) on an 8ml column plugged with glass wool packed column was filled with 5ml n- hexane for conditioning. Stopcock was opened to allow N- hexane run out until it reached top of the $NaSO_4$, into a receiving vessel whilst tapping gently the top of the column till the extracted chitosan oil was transferred on to the column with disposable Pasteur pipette.

Eluate was collected into a sample vial for alkaloids and lipids profile analysis using gas chromatography with flame ionization detection (GC-FID).

3. Quantification of alkaloids and lipids profile

The analysis of alkaloids and lipids profile constituents were performed on buck M910 Gas chromatography equipped with a flame ionization detector (FID). A Restek 15 meter M×T-I column (15m×250nm×0.15nm) was used. The injector temperature was 280°C with splitless injection of 2ml of sample and a linear velocity of 30cms-1, helium 5.0pa.s was the carrier gas with a flow rate of 40ml/min. The oven operated initial at 200°C, it was heated to 330°C at a rate of 3°C min-1 and was kept at this temperature for 5min. The detector operated at a temperature of 320°C. The bioactive components were determined by the ratio between the area and mass of internal standards and the area of the identified compounds. The concentration of the different bioactive components was expressed in ug/ml

4. Data analysis

Data were collected on moisture content chitosan yield, polysaccharides, peptide, alkaloids and lipids contents. Correlation analysis was performed with data generated from the moisture content of the male and female crab. One way analysis of variance (ANOVA) using spss software version 25 was performed with the data generated on the bioactive.

Results

Analysis of Moisture Composition

The moisture content of the male and female crabs was analysed.Table 1: shows the mean moisture content of the male and female freshwater crab (*P. ebonyicum*) used for the chitosan production. The male crab had a higher mean weight than the female crab. Conversely, the female crab had a higher mean moisture content compared to male. There was significant difference ($P<0.05$) in mean moisture content (approximately 5.8% higher in the female than the male). The variance within both groups (male and female) was moderate, suggesting the difference was likely meaningful.

Table 1: Moisture Composition of male and female freshwater crab (*Potamon ebonyicum*)used for chitosan extraction in Ebonyi River Basin

Sex	Mean Weight	Mean Moisture	Variance
Male	36.655	58.95	2.41
Female	29.201	64.75	3.42

Production and characterization of chitosan

The chitosan was extracted from the Chitin of the male and female freshwater crab (*P. ebonyicum*) for the bioactive compounds analysis. Results of the extraction and characterization are shown in Figures4 to 8. The Chitin of the female crab yielded more chitosan than that of the male crab. The chitosan was about 8.8% more concentrated in the female than in the male crab. More polysaccharides was produced from the female crab than the male crab and Concentration difference between both gender was 5.20mgl-1. Bioaccumulation of peptide was more in the male crab than in the female crab, 0.797 and 0.292 ugml-1 respectively. There were also more alkaloid and lipid in the male crab (0.0057 and 0.0298 Mg ml-1) than in the female crab (0.0046 and 0.0298 Mg ml -1).

Correlation and Regression Analysis of Variables in male and female crabs

There was strong negative correlation (r = - 0.094) between weight and moisture content in the male crab. As weight of the male crab increased the moisture content decreased. A strong positive correlation (r = + 0.96) existed between weight of the female crab and the moisture content. As the weight of the female crab increased, the moisture content also increased. A linear correlation (r = 0.9) existed between the key variables (bioactive) in the male and female crab.

Discussion

Bioaccumulation of essential elements (magnesium, iron, zinc, selenium and copper) and non essential elements (lead, cadmium, chromium) in intact male and female *P. ebonyicum* have been reported in theRiver Basin (Akpaniteaku and Okoye, 2018, Akpaniteaku and Udeozor 2018) [3, 7]. Chitosan and bioactive compounds (polysaccharide, peptide, alkaloid and lipids) analysed in the present study may probably be regarded as additional bio resource to the freshwater crab species. Previously, chitosan from the shell of marine and mud crab species have been extracted (Anand *et al* 2014, Mythili and Aysha 2017, Sumaila *et al*. 2020) [8, 13, 17]. The extraction of chitosan from whole freshwater crab in the present study could be a new idea since it was done for the very first time. Laith *et al*. (2017) [11] recommended crab components as the best source of bioactive compounds after shrimp and lobster to provide protein, vitamins, and fatty acid. According to Sumaila *et al* (2020) [17], percentage yield of the chitosan in an unspecified crab species from the Kainji Lake basin, was as low as 15% dry weight. Chitosan yield from the Chitin of the male *P. ebonyicum* in the present study seem to be lower, while that of the female seem to be higher than the percentage yield (Fig 4). Weight and moisture content variation (Table 1)probably indicate potential biological and physiological differences in the male and female crabs, which may impact their bioactive composition. Significantly higher yield of chitosan in the female crab than in the male crab may possibly be attributed to differences in shell thickness, melting cycles and physiological roles. The findings align with report of Ahmed *et al*. (2020) on sex related variation in Chitin yield from crustaceans. Polysaccharides was more abundant in the female, which seems to reinforce its potentials as source of nutraceutical compound. This is in agreement with findings from Manivannan *et al*. (2022) [12] on antioxidantproperties of freshwater crab species. The higher peptide concentration in the male crab than in the female may seem to be the effect of greater muscle mass or

different protein metabolism as suggested by Nguyen *et al*. (2023) [15] in protein hydrolysate from crustaceans. The inverse correlation between weight and moisture in the male, and the positive correlation in the females may seem to suggest a physiological and structural variation. These insights support prior works by Rani *et al*. (2021) [16] and Ng *et al*. (2021) which emphasized influence of sex and habitat conditions on biochemistry of crustaceans.

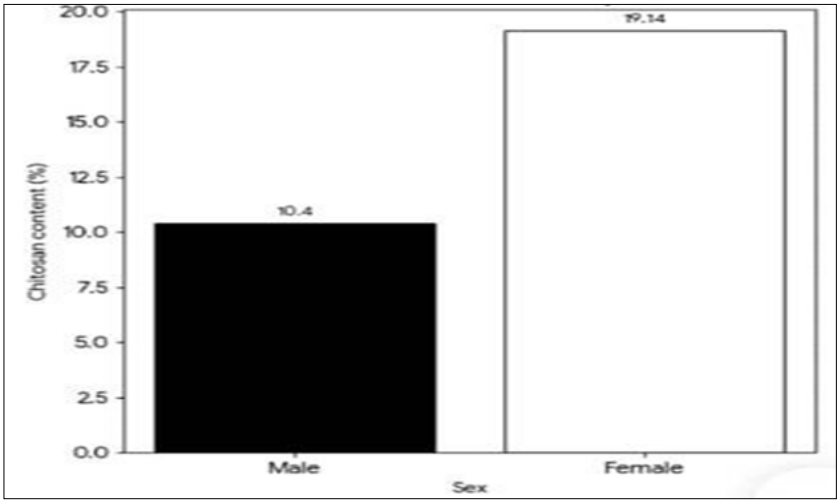

Fig 4: Chitosan concentration in male and female fresh water crab (*Potamon ebonyicum*) in Ebonyi River Basin

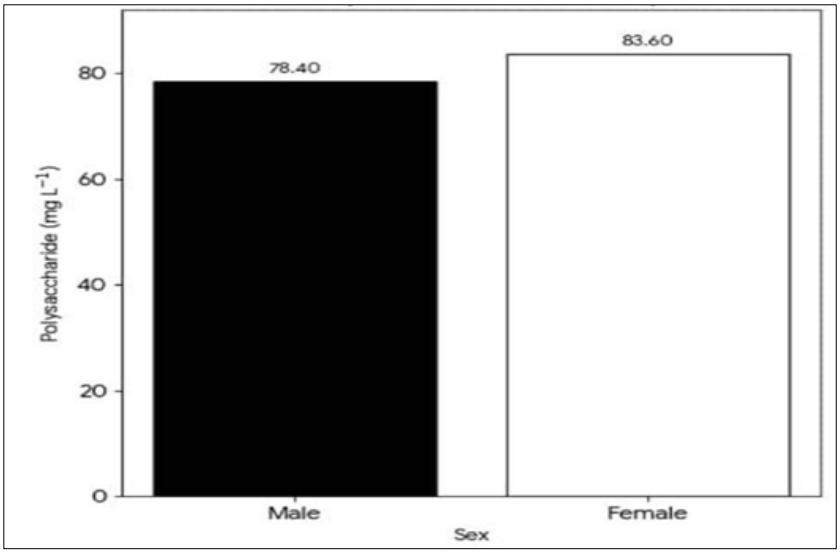

Fig 5: Polysaccharide concentration in male and female fresh water crab (*Potamon ebonyicum*) in Ebonyi River Basin

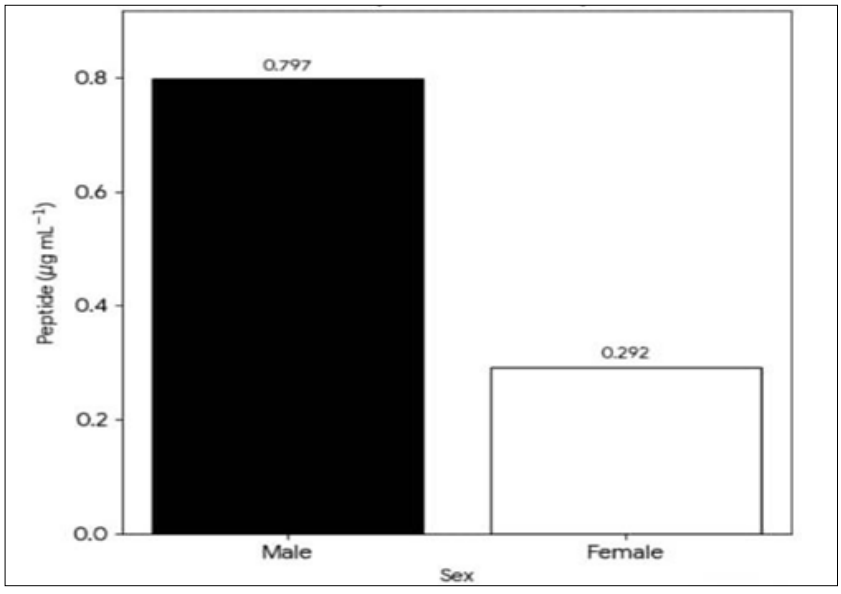

Fig 6: Peptide concentration in male and female fresh water crab (*Potamon ebonyicum*) in Ebonyi River Basin

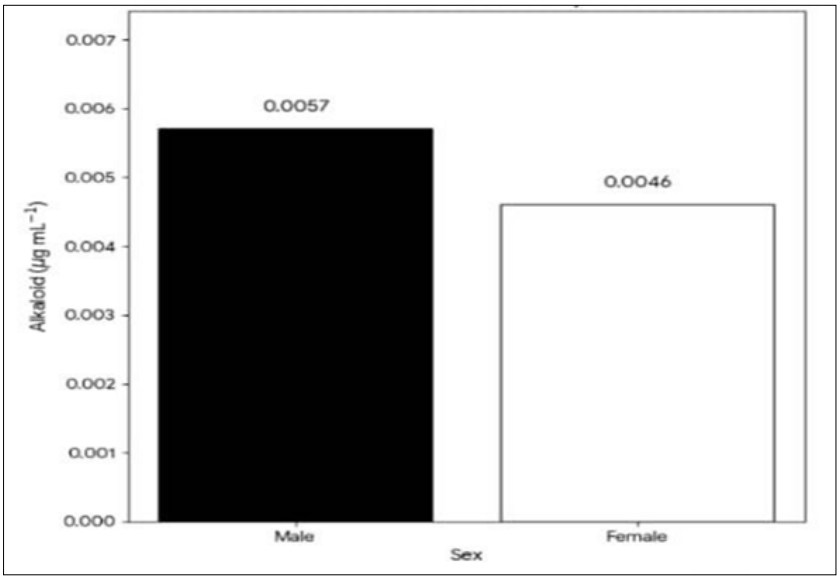

Fig 7: Alkaloid concentration in male and female fresh water crab (*Potamon ebonyicum*) in Ebonyi River Basin

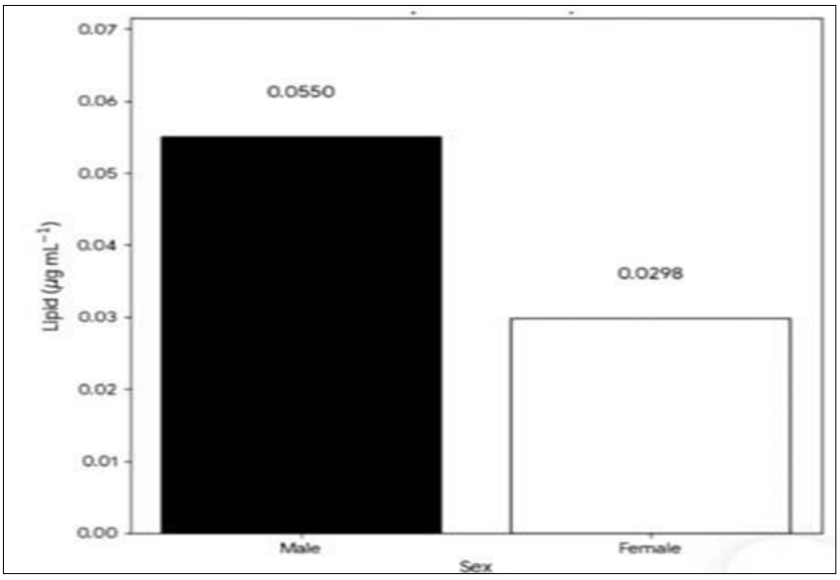

Fig 8: Lipid concentration in male and female fresh water crab (*Potamon ebonyicum*) in Ebonyi River Basin

Conclusion

The study on bioactive compounds from the freshwater crab in Ebonyi River Basin has provided valuable insight into the potential pharmaceutical and nutraceutical applications of the *Potamon ebonyicum*. Extraction and characterization of chitosan from the freshwater crab species revealed significant bioactive properties that could be exploited for medical and industrial purposes. The research work has provided useful information on availability and concentration of the bioactive compounds. It has shown that gender of the P.ebonyicum has relative impact on concentration of the compounds. The findings collectively enhance understanding of sex based biochemical variations in the freshwater crab species, and support the development of strategies for extracting and utilizing bioactive compounds from local aquatic species. It is very important not only from pharmaceutical and health point of view, but it has also a lot of positive impacts on local economy and other aspects of industrial activities in Nigeria.

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