**Original Research Article**

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**PHYTOCHEMICAL ANALYSIS AND ANTIBACTERIAL ACTIVITY OFMETHANOLIC STEM EXTRACT OF BOMBAX BUONOPOZENSEP.BEAUV (SILKCOTTON TREE)**

**ABSTRACT**

The methanolic stem extract of B. buonopozense with documented ethno-medicinal applications were fractionated in different solvents (n-hexane, chloroform, ethyl acetate, n-butanol and water) and subjected to phytochemical screening and antibacterialactivity against some disease-causingmicroorganisms including Gram positive and Gram negative (Staphylococcus aureus, Salmonellaspp and Escherichia coli).Phytochemical screening revealed the presence of alkaloids, saponins, saponin glycosides, tannins, hydrolysable tannins, steroids and triterpenoids, flavonoids, phenols and volatile oils.Each fractionsdemonstrated antibacterial activity against all the organisms tested. MIC values of each fraction revealed strong inhibition against all the organisms. The methanolic stem extract of B. buonopozense in chloroform, water, ethyl acetate, n-hexane and n-butanol showed the following MIC values;Staphylococcus. A. 6.25, 25, 12.5, 6.25, and 12.5. E. coli 100, 100, 50, 6.25 and 12.5. Salmonella spp. 3.125, 12.5, 25, 3.125 and 12.5 respectively. This study shows that the extract posses’ antibacterialproperties which can be used as alternatives to conventional antibiotics.

**KEYWORDS:***Bombax buonopozense*, antibacterial activity, Diosquinone, Phytochemical constituents, Inhibition, *Staphylococcus aureus, Escherichia coli*, MIC.

**INTRODUCTION**

Natural products, such as plant extracts either as pure compounds or as standardized extracts, provides unlimited opportunities for new drug discoveries because of the unmatched availability of its chemical diversity. The use of plant extracts in the treatment of diseases has become an important interest over the years. This is as a result of the fact that microorganisms are developing resistance to many drugs and as such created situation where some of the common and less expensive antimicrobial agents are losing effectiveness 1, 2.In view of this, there is an urgent need to find the alternative to chemotherapeutic drugs in disease treatment particularly those of plants origin which are easily available and have considerably less side effects3, 4.

In the past, humans used plant to treat common infectious disease and even long before mankind discovered the existence of microbes, the idea that certain plants had healing potential was well accepted5. Researchers are increasingly becoming involved in the screening of such plants with the aim of establishing their potential antimicrobial effects and identifying the compounds responsible for their antimicrobial properties6.Specifically, the medicinal value of this plant lies in some chemical substances that produce a definite physiological action on the human or animal body7. The most important of these bioactive constituents which are mainly: secondary metabolites are alkaloids, flavonoids, tannins and phenolic compounds8,9. These phytochemicals are toxic to microbial cells. Medicinal plants generally contain a number of compounds which may be potential natural antibacterial for the treatment of common bacterial infections. Plant derived medicines are relatively safer than synthetic alternatives, offering profound therapeutic benefits and more affordable treatments10.

*B. buonopozense* (Bambacaceae) is commonly known with local names (Hausa: gurjiya, Igbo: Akpu, Nupe: Kutukpachi; Yoruba: Ogbolo). It is a large tropical tree that grows up to 40 min height with large buttress roots that can spread 6 m down11. The individual leaf has entire margin and quite large, measuring from 8 to 23cm in length by 3 to 7.5cm in width with the under sides of the leaf being conical buds which contain many seeds that are 5 to 6mm in length, all of which have a cotton-like fiber covering12. It is a wild plant whose edible floral parts is used as vegetable by the inhabitants of North Central, Nigeria and as medicine due to its nutritive and therapeutic properties11. The plant is widely distributed in West African countries such as Ghana, Gambia, Cote D’ivoire, Nigeria and others. Hot decoction of the dried stem bark of the plant is taken orally for the treatment of malaria in Ghana. The bark is used to treat chest pain in Gambia. Decoction of the leaves is used to manage stomach ulcers and burns in Ghana. Aqueous extract of the leaves is claimed to be effective in the treatment of diarrhea and dysentery. The immature fruits are prepared as an emollient for skin, decoction of the young leaves is used as a warm bath for febrile children (Irvine F. R, 1961). The grounded bark is taken by pregnant women to increase lactation; the extract from the bark is drunk on the head for dizziness. The gum resin from the bark is pulverized, mixed with oil and used to manage skin disease7.

This study was designed to evaluate fractionated methanolic extract of *Bombax buonopozense*(stem) for antibacterialand phytochemical screening against multi-drug pathogenic organisms to ascertain its potentiality in treating infections caused by micro-organisms.

**MATERIALS AND METHODS**

**COLLECTION OF SAMPLES**

The fresh stem of *Bombax buonopozense* were collected and identified by a taxonomist Mr. Ozioko of INTERCEED Nsukka.The stem of *bombax buonopozense*was washed and air dried for two weeks.The dried stem was ground to powder and stored in an air tight polyethylene bag ready for extraction.

**PREPARATION OF PLANT EXTRACTS/EXTRACTION**

The method in our previous work was used with little modification 3. Extraction was done by cold maceration with 95% methanol with continuous stirring and agitation, extracts were removed intermittently and fresh solvent added to ensure for neat extraction, this was done for 72hrs. After 72hrs, the extract was pooled together and filtered using Whatman 2 filter paper. The extract was evaporated to dryness in vacuum using rotary evaporator at 400C± 0.1. The extract wassubsequently weighed and stored in glass sample bottles at 40C.

**TEST ORGANISMS FOR MICROBIAL ANALYSIS**

Clinical isolates of *Staphylococcus aureus*(Gram positive), *Salmonella*spp(Gram positive), and *Escherichia coli*(Gram negative) obtained from the microbiology department, Enugu State University Teaching Hospital (ESUT) Enugu were used for this study. The organisms were sub cultured in nutrient broth at 370C for 6h prior to antibacterialtesting.

**SOLVENTPARTITIONING/FRACTIONATION**

The methanolic stem extract of *Bombax buonopozense* were partitioned using solvent extraction method.Five solvents were used in solvent partitioning (water, n-hexane, chloroform, ethyl acetate and n-butanol).The extractwas uniformly dispersed in 100ml of water and stirred with a magnetic stirrer to achieve uniform mixture or homogeneity, it was then poured into a separating funnel, then aliquots of n-hexane was added, the separating funnel was agitatedbefore being allowed to settle.Heavier lower layer (aqueous) was carefully tapped off and then the upper layer (n-hexane) was tapped off subsequently, this was done continuously until the n-hexane aliquots were reasonably clear, it was tapped off and stored. The same procedure was repeated for other immiscible solvents (chloroform, ethyl acetate and n-butanol), leaving behind in the separating funnel, the water portion of the extract, which was also collected and stored in reagent bottle at 40C.

**PHYTOCHEMICAL SCREENING**

The extract was subjected to qualitative phytochemical screening according to standard methods using the n-hexane, chloroform, ethyl acetate, n-butanol and water fractions of the extract. This was done to determine the presence of saponins, Alkaloids, Saponin Glycosides, Tannins, Hydrolysable tannin, steroids and Triterpenoids, flavonoids, Anthraquinone, Phenols, glycosides and volatile oils.

The tests are as follows:

**TEST FOR SAPONINS**

1. 2.5cm3 of each fraction of extracts was vigorously shaken with 10cm3 of water for 2 minutes in a test tube. 2cm3 of olive oil was then added. It was observed for persistent frothing and emulsion formation and result recorded.
2. 1ml of each fraction of extracts was treated with 1% lead acetate solution. Formation of white precipitate indicates the presence of saponins.

**TEST FOR SAPONIN GLYCOSIDES**

2.5cm3 of mixture of Fehling’s solution A and B was added to 2.5cm3 of each fraction of extracts in a test tube and observed for bluish green precipitate and observation recorded.

**TEST FOR STEROIDS AND TRITERPENOIDS (LIBERMANN BURCHARD TEST)**

2cm3 of acetic anhydride was added to 2cm3 of each fraction of extract in a test tube and cooled well in ice. 3cm3 concentrated sulphuric acid was carefully added and a change from violet to blue to green colour was observed and recorded.

**TEST FOR GLYCOSIDES (GENERAL)**

1. Dilute sulphuric acid (2.5cm3) was added to 5cm3 of fractions of extract in a test tube and boiled for 15 minutes. Then 2cm3 of 10% sodium hydroxide and 5cm3 of mixed Fehling’s solution A and B were added. The formation of brick red precipitate is a positive test.
2. The fractions of extracts werehydrolyzed with HCL for few hours on a water bath and the hydrolysate was subjected to Bontrager’s test to detect the presence of glycosides.
3. **Bontrager’s Test:** Hydrolysate was treated with chloroform and then the chloroform layer was separated. To this equal quantity of dilute ammonia solution was added. Ammonia layer acquires pink colour, shows the presence of glycosides.

**Test for Tannins**

1. **Ferric Chloride Test:** To 1-2ml of each fraction of extracts a few drops of 5% aqueous fecl3solution was added. A violet colour formation indicates the presence of tannins.
2. **Lead acetate test:** In a test tube containing about 5.0ml of each fraction of extracts a few drops of 1% lead acetate was added. A yellow precipitate indicates the presence of tannins.

**TEST FOR HYDROLYSABLE TANNINS**

4cm3 of 10% ammonia solution was added to 4cm3 of each fraction of extracts in a test tube and shaken very well and observed for the formation of an emulsion and the result recorded.

**TEST FOR FLAVONOIDS**

1. **Shinoda’s Test:** In a test tube containing 0.5ml of each fraction of extracts, 5-10 drops of diluted HCL and small piece of magnesium (magnesium ribbon) were added and the solution was boiled for a few minutes. A reddish pink colour indicates the presence of flavonoids
2. **Alkaline Reagent Test:** To 1.0ml of each fraction of extracts, few drops of dilute sodium hydroxide were added. An intense yellow colour produced in the extracts which becomes colourless on addition of a few drops of dilute acid indicates the presence of flavonoids.

**TEST FOR ALKALOIDS**

1. **Dragendroff’s Test:** To 1ml of each fraction of extracts 4ml of HCl was added. To the acidic medium 1ml of Dragendroff’s reagent was added. An orange or red precipitate produced immediately indicates the presence of alkaloids.
2. **Wagner’s Test:** Two drops of Wagner’s reagent was added to 2cm3 of each fraction of extracts in a test tube and observed for a deep brown precipitate and observation recorded.
3. **Mayer’s Test:** Three drops of Mayer’s reagent was added to 2cm3 of each fraction of extracts in a test tube and observed for a reddish precipitation or colouration.
4. **Tannic Acid Test:** Two drops of 10% (W/v) tannic acid was added to 2cm3 of each fraction of extracts in a test tube and observed for a cream colouration and observation recorded.

**VOLATILE OIL TEST**

Six drops of ferric chloride solution were added to a mixture of 2cm3 of each fraction of extracts in a test tube and 2cm3 of 90% (v/v) ethanol. The resulting mixture was observed for green coloration and the result recorded.

**TEST FOR PHENOLS**

To 3ml of each fraction of extracts, 3ml of 5% w/v ferric chloride solution was added. The blue-black colour indicates the presence of phenol

**TEST FOR ANTHRAQUINONES**

5ml of each faction of extracts was hydrolyzed with dil. Sulphuric acid (H2So4) and extracted with benzene. 1 ml of dilute ammonia was added to it. Rose pink colouration indicated the positive response for anthraquinones.

**ANTIMICROBIAL SCREENING**

The agar diffusion technique as described by13was used to determine the antibacterialactivity of the plant extract. To test antibacterial activity of the stem extract of *Bombax buonopozense* 0.8g of extract was dissolved in DMSO and then varying concentration of the extract (100mg/ml, 50mg/ml, 25mg/ml, 12.5mg/ml, 6.25mg/ml, 3.125mg/ml and 1.562mg/ml) were obtained. Standard inoculums of 1.5x108 cells which matched, 0.5 McFarland standard was spread on the surface of a sterile Muller Hinton agar plates in duplicates. A sterile, 6mm cork borer was used to make a hole on the Muller Hinton agar plates in which 0.1 ml each of the plant extracts were added. The plates were incubated at 370c for 24hrs. The antimicrobial activity was detected by measuring zones of inhibition in millimeters.

**DETERMINATION OF MINIMUM INHIBITORY CONCENTRATION (MIC)**

This was determined using broth dilution method as described by13. The minimum inhibitory concentration was defined as the lowest concentration of the compound to inhibit the growth of microorganism.Varying concentrations of the extracts (100mg/ml, 50mg/ml, 25mg/ml, 12.5mg/ml, 6.25mg/ml, 3.125mg/ml and 1.562mg/ml) were prepared. An amounf of 0.1ml of standardized test organisms was inoculated into the tubes containing the different concentrations of the extract and controls were equally set up by using solvents and test organisms without extract. These were incubated for 24hrs at 370c. The tube with least concentration of extract without growth after incubation was taken and recorded as the minimum inhibitory concentration.

**DATA ANALYSIS(STATISTICAL ANALYSIS)**

The tests were carried out in triplicates, the data obtained were subjected to one-way ANOVA using statistical package for social science (SPSS), version 23. Means were separated using Least Significant Difference (L.S.D), considered statistically significant at P < 0.05.

**RESULTS**

**PHYTOCHEMICAL SCREENING**

Phytochemical analysis of the fractionated methanolic stem extract of *Bombax-buonopozense* were conducted using the fractions of each of the solvent. Table 1 shows the positive and negative reactions of the extract fraction to the following secondary metabolites: Alkaloids, Flavonoids, Tannins, Saponins, Saponin glycosides, hydrolysable tannins, phenols,anthraquinones, steroids/triterpenoids, volatile oils and glycosides.The result of phytochemical analysis of the stem extract is shown in Table 1.

**Table 1: Phytochemical profile of the fractions of *Bombax buonopozense* stem extract**

|  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- |
|  | | **Solvents** | | | | |
| **S/N** | **Phytoconstituents** | **n-hexane** | **Chloroform** | **Ethyl acetate** | **n-butanol** | **Water** |
| 1 | Alkaloids | **+++** | **+++** | **++** | **\_** | **\_** |
| 2 | Saponins | **\_** | **\_** | **+++** | **+** | **+++** |
| 3 | Saponin glycosides | **\_** | **\_** | **+** | **+++** | **+++** |
| 4 | Tannins | **+** | **\_** | **\_** | **\_** | **+** |
| 5 | Hydrolysable tannins | **+++** | **++** | **+** | **\_** | **+** |
| 6 | Steroids & triterpenoids | **+** | **+** | **\_** | **\_** | **\_** |
| 7 | Flavonoids | **\_** | **\_** | **+** | **\_** | **\_** |
| 8 | Anthraquinone | **\_** | **\_** | **\_** | **\_** | **\_** |
| 9 | Phenols | **\_** | **+** | **+++** | **++** | **\_** |
| 10 | Glycosides | **\_** | **\_** | **\_** | **\_** | **\_** |
| 11 | Volatile oils | **\_** | **++** | **\_** | **+** | **\_** |

***Key---(+) = suspected/slightly present, (++)= present, (+++)= very present, (-)= absent***

**DETERMINATION OF ANTIMICROBIAL ACTIVITIES OF THE EXTRACT**

The result of the antibacterial activity of the fractions of methanolic extract of *B*. *buonopozense* against the test organisms, namely, *staphylococcus aureus*, *Escherichia coli* and *salmonella spp*are showed in tables 2 to 6 while table 7 shows the result of minimum inhibitory concentration of the extract on the test organisms. The extract showed varying degrees of growth inhibition against the isolates.

**Table 2: Antibacterial activity of aqueous (water) fraction of *Bombax-buonopozense* stem extract**

|  |  |  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- | --- | --- |
| **Test Organisms** | **Different concentration (mg/ml)/zones of inhibition (mm)** | | | | | | | |
| 100 | 50 | 25 | 12.5 | 6.25 | 3.125 | 1.56 |
| *Staphylococcus aureus* | **12**±1.73 | **12**±0.57 | **6**±1.73 | **4**±1.15 | **2**±0.57 | **0.0**±0.00 | **0.0**±0.00 |
| *Escherichia coli* | **5**±0.57 | **3**±0.57 | **0.0**±0.00 | **0.0**±0.00 | **0.0**±0.00 | **0.0**±0.00 | **0.0**±0.00 |
| *Salmonella spp* | **14**±0.57 | **12**±1.73 | **10**±1.15 | **5**±1.73 | **2**±0.57 | **0.0**±0.00 | **0.0**±0.00 |

Results represent **mean**± standard error (n = 3).

**Table 3: Antimicrobial activity of the chloroform fraction of *Bombax-buonopozense* stem extract**

|  |  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- | --- |
| **Test Organisms** | **Different concentration (mg/ml)/zones of inhibition (mm)** | | | | | | |
| 100 | 50 | 25 | 12.5 | 6.25 | 3.125 | 1.56 |
| *Staphylococcus aureus* | **17**±1.15 | **13**±0.57 | **11**±2.88 | **6**±1.15 | **6**±1.73 | **2**±0.57 | **0.0**±0.00 |
| *Escherichia coli* | **6**±0.57 | **5**±1.15 | **2**±0.57 | **0.0**±0.00 | **0.0**±0.00 | **0.0**±0.00 | **0.0**±0.00 |
| *Salmonella spp* | **30**±2.88 | **26**±2.30 | **18**±1.73 | **13**±1.15 | **11**±2.30 | **6**±1.73 | **4**±0.57 |

Results represent **mean** ± standard error (n = 3).

**Table 4: Antimicrobial activities of ethyl acetate fraction of *Bombax-buonopozense* stem extract**

|  |  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- | --- |
| **Test Organisms** | **Different concentration (mg/ml)/zones of inhibition (mm)** | | | | | | |
| 100 | 50 | 25 | 12.5 | 6.25 | 3.125 | 1.56 |
| *Staphylococcus aureus* | **21**±4.61 | **14**±0.57 | **12**±1.15 | **11**±2.30 | **5**±1.15 | **0.0**±0.00 | **0.0**±0.00 |
| *Escherichia coli* | **16**±1.15 | **15**±2.30 | **13**±0.57 | **9**±0.57 | **8**±1.73 | **3**±0.57 | **0.0**±0.00 |
| *Salmonella spp* | **26**±2.30 | **19**±0.57 | **15**±1.73 | **12**±3.46 | **7**±1.15 | **3**±1.15 | **2**±0.57 |

Results represent **mean**± standard error (n = 3).

**Table 5: Antimicrobial activity of n-hexane fraction of *Bombax buonopozense* stem extract**

|  |  |  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- | --- | --- |
| **Test Organisms** | **Different concentration (mg/ml)/zones of inhibition (mm)** | | | | | | | |
| 100 | 50 | 25 | 12.5 | 6.25 | 3.125 | 1.56 |
| *Staphylococcus aureus* | **22**±4.04 | **18**±2.88 | **17**±1.15 | **10**±2.88 | **6**±1.15 | **2**±0.57 | **0.0**±0.00 |
| *Escherichia coli* | **18**±2.30 | **13**±3.46 | **13**±1.15 | **7**±0.57 | **5**±1.15 | **3**±0.57 | **0.0**±0.00 |
| *Salmonella spp* | **25**±0.57 | **21**±3.46 | **17**±2.88 | **11**±1.73 | **10**±2.30 | **4**±1.15 | **2**±0.57 |

Results represent **mean**± standard error (n = 3).

**Table 6: Antimicrobial activities of n-butanol fraction of *Bombax-buonopozense* stem extract**

|  |  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- | --- |
| **Test Organisms** | **Different concentration (mg/ml)/zones of inhibition (mm)** | | | | | | |
| 100 | 50 | 25 | 12.5 | 6.25 | 3.125 | 1.56 |
| *Staphylococcus aureus* | **13**±1.15 | **11**±2.88 | **6**±0.57 | **5**±1.73 | **5**±1.15 | **0.0**±0.00 | **0.0**±0.00 |
| *Escherichia coli* | **10**±1.73 | **7**±0.57 | **7**±1.15 | **5**±1.73 | **2**±0.57 | **0.0**±0.00 | **0.0**±0.00 |
| *Salmonella spp* | **18**±2.30 | **12**±3.46 | **12**±0.57 | **9**±2.30 | **3**±0.57 | **0.0**±0.00 | **0.0**±0.00 |

Results represent **mean**± standard error (n = 3).

**Table 7: Minimum inhibitory concentration of chloroform, Aqueous (water), ethyl-accetate, n-hexane and n-butanol fraction of Bombax buonopozense extracts.**

|  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- |
| **Test Organisms** | **Minimum inhibitory concentration (mg/ml)** | | | | |
| Chloroform | Water aqueous | Ethyl acetate | n-hexane | n-butanol |
| *Staphylococcus aureus* | **6.25** | **25** | **12.5** | **6.25** | **12.5** |
| *Escherichia coli* | **100** | **100** | **50** | **6.25** | **12.5** |
| *Salmonella spp* | **3.125** | **12.5** | **25** | **3.125** | **12.5** |

**DISCUSSION**

The results of this study have x-rayed valuable evidence in support of *Bombax-buonopozense* as potent antibacterial agent. The data presented in this study narrowed down the phytochemicals into specific fractions of the extract. Result of the phytochemical screening of *Bombax buonopozense* stem extract showed the presence of these phytochemicals in each of the fractions of the extract.

* n-hexane fraction contains (Alkaloids, tannins, hydrolysable tannins and steroids/triterpenoids)
* chloroform fraction contains (Alkaloids, Hydrolysable tannins, steroids/ triterpenoids, phenols and volatile oils)
* Ethyl acetate fraction contain (Alkaloids, saponins, hydrolysable tannins, flavonoids and phenols).
* n-butanol fraction contains (saponin glycosides, saponins, phenols and volatile oils).
* Water fraction contain (saponins, saponinoglycosides, tannins and hydrolysable tannins).

The result of this study showed that the fractionated methanolic stem extracts of *Bombax-buonopozense* demonstrated reasonable inhibition on the Gram positive bacteria (*Staphylococcus aureus and Salmonella spp*) and the n-hexane, ethyl acetate and n-butanol fractions exhibited reasonable sensitivity on the gram negative bacteria(*Escherichia coli*) while the chloroform and n-butanol fractions has only little sensitivity on the isolates when tested by extracts with high concentrations.

It is believed that the antibacterial activity of this plant is due to the presence of the phytochemicals. Phytochemicals such as Alkaloids, tannins, essential oils, saponins steroids, which are actually the defensive mechanism of the plants against pathogens.The MIC values of the extract was found to have various range, thus indicating that evaluation of MIC is sufficient for measuring bacterial activity12.

The test organisms used in this study are associated with various forms of human infections. *Escherichia coli* are the most numerous aerobic commensal inhabitants of the large intestine. Certain strains cause diarrhea and all can cause infection when they invade sterile sites13, like the urinary tract and wound infections in the elderly and young male often following catheterization or cystoscopy, and it is a secondary invader of ulcers and pressure sores14. *Salmonella spp* typically cause diarrhea and sometimes cause a more serious infection, typhoid fever. *Staphylococcus aureus* constitute a major public health threat, being one of the common causes of hospital and community acquired infections. The demonstration of activity against both Gram positive and Gram-negative bacteria is an indication that the plants can be a source of bioactive substances that could of broad spectrum of activities. Some synthetic drugs cause varying degrees of side effects, hence the need for the development of plant-based compounds which could be useful in meeting the demand for newer drugs with minimal side effects13. Apart from antimicrobial activities, *Bombax buonopozense* extract are also exploited for therapeutic purpose to cure several disorders.

**CONCLUSION**

The methanolic leaf extract of *Bombax buonopozense* was found to possess antidiarrheal, antinoceptic, anti-inflammatory, antipyretic anti malaria activitieswhich however, justifies the scientific use of these plants in traditional medicine in the treatment of infections caused by the test organisms.­­

**LIMITATIONS OF THE STUDY**

This study is limited to Phytochemistry and antibacterialactivity of methanolic stem extract of*Bombax BuonopozenseP.Beauv* (Silk Cotton Tree).

**CONFLICT OF INTEREST**

The authors affirm that there are no conflicts of interest.

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**AUTHOR'S CONTRIBUTION**

UdehValantineChinonyerem

Performed analysis and prepared the manuscript.

Emmanuel Agboeze

Performed analysis and prepared the manuscript

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Supervised the analysis.

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