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MICROBIOLOGICAL ASSESSMENT AND ANTIMICROBIAL PROPERTIES OF CALABASH CHALK

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ABSTRACT
Deliberate consumption of clay is common among Africans and in South Asia. Apart from the traditional practices involving the use of clay, nanoclays such as calabash chalk have found use in nanomedicine, essentially in the form of nanocomposite when exfoliated and organically modified. Microbial evaluation of commercially available calabash chalk showed a heterotrophic count ranging from 1.0–2.1 x 10^4 CFU/g, frequency of organisms isolated included; *Staphylococcus* sp. (36%), *Escherichia coli* and *Citrobacter* sp. (20% each), *Bacillus* sp. (16%) and *Clavibacter* sp. (8%) with the following fungal isolates; *Aspergillus* sp., *Penicillium* sp., *Fusarium* sp. (25% each), *Mucor* sp., *Saccharomyces* sp. (10% each) and *Rhizopus* sp. (5%). Antibacterial activity of the calabash chalk against selected pathogens was determined at various concentrations using well-in-agar diffusion and disk diffusion methods. Concentrations of 100mg/ml and 50mg/ml had the highest inhibitory activities on all the test isolates, concentrations from 6.25mg/ml to 25mg/ml had very little or no inhibitory activity on the test isolates. *Streptococcus* sp., *Candida* sp. and *Klebsiella* sp. were the organisms with the highest zone of inhibitions among the test isolates.

KEYWORDS: Calabash chalk, antimicrobial activity, pathogens.

INTRODUCTION

Calabash chalk is a naturally occurring substance composed of Aluminum silicate hydroxide from kaolin clay group with the basic formula $-Al_2Si_2O_5(OH)_4$ (Dean *et al.*, 2004, Ekosse and Jumbam, 2010). It is readily available commonly as Calabar stones, La Craie or Argile in French, Ndom and Nzu in most parts of Southern part of Nigeria (Kelle *et al.*, 2014). It is usually sold in blocks, pellets and powder forms (Dean *et al.*, 2004). The calabash chalk is grounded into a fine powder and applied to the face as facial powder and antiperspirant which makes the face to remain dry (Popoola *et al.*, 2013). Calabash chalk is also commonly ingested in Africa and has been reported to contain important nutrients such as; phosphorus, potassium, magnesium, copper, zinc, manganese and iron. The locals consume this chalk in order to extract these minerals for body need; it is a common practice among pregnant women to alleviate the symptoms of morning sickness (Abrahams *et al.*, 2013), as well as young children, however the fact about the nutritional value of this substance is still unknown (Ekong *et al.*, 2013). Studies have also established that deliberate consumption of clay is common among the Tanzanians and Kenyans in the eastern part of Africa, as well as, Senegal, Mali and Nigeria in West Africa, and South Asia (Bisi-Johnson *et al.*, 2010; Abrahams *et al.*, 2013). Apart from the traditional practices involving the use of clay, nanoclays such as calabash chalk have found use in nanomedicine, essentially in the form of nanocomposite when exfoliated and organically modified. Nanocomposites have been shown to exhibit antimicrobial activities against bacteria, spores and viruses (koper *et al.*, 2002; Huang *et al.*, 2005). Williams *et al.* (2011) reported that French green clay materials have been previously used as cure for skin diseases such as necrotizing fasciitis caused by *Mycobacterium ulcerans*.

The probable presence of highly toxigenic organisms such as *Clostridium perfringens, C. tetani,* and *C. botulinum,* which occur in soil habitats have been inferred, generating safety concerns and counteracting the probable positive usage of clay as nanomaterial. This study was carried out to determine the microbial composition and antimicrobial activity of the calabash chalk on common pathogenic microorganisms.

MATERIALS & METHODS

Sample collection

Ten kilograms of calabash chalk was collected from Choba, Ruuosi, Oil Mill, Mile I, Mile III and Oyigbo markets in the Port Harcourt metropolis of Rivers state. The samples were packaged in polythene bags and transported to the Microbiology laboratory of the University of Port Harcourt for analysis

Sample preparation and Analysis

The chalk samples were air dried and ground with a sterile mortar and pestle to achieve a smooth consistency.

Enumeration of micro-organisms

Ten fold serial dilutions of all the samples were carried out and 0.1ml each of selected dilutions was plated using the pour plate method (Cheesbrough, 2005). Enumeration of total viable count was done using plate count agar (Oxoid, CM325, UK). Yeast and mould counts were done on Sabouraud dextrose agar (Oxoid). All cultures were

incubated at 37^{O}C for 24hours while yeasts and mould counts were incubated at 25^{O}C for 3 to 5 days.

Identification of Isolates

Isolates were stored on nutrient agar slants at 4^{O}C for further confirmatory tests which included IMVIC test, carbohydrate utilization, and reaction on TSI, gelatin liquefaction, nitrate reduction, urease production and motility. Wet mount of the fungal isolates were prepared in lactophenol cotton blue and examined under low power binocular microscope and compared to the published morphological characteristics of fungi (Watanabe, 2010).

Antimicrobial Activity Evaluation

Test isolates

Pure cultures of *Escherichia coli*, *Bacillus cereus*, *Pseudomonas aeruginosa*, *Staphylococcus aureus*, *Streptococcus* sp., *Klebsiella pneumonia* and *Candida albicans*, were obtained from the University of Port Harcourt Teaching Hospital, Rivers State.

Determination of antimicrobial activity of calabash chalk (Nzu).

Well- in- Agar Method

The bacterial inoculum of the test isolates was uniformly spread using sterile cotton swab on a sterile Petri dish of Muller Hillton agar. Dilutions of the calabash chalk yielding concentrations of 100mg/ml, 50mg/ml, 25mg/ml, 12.5mg/ml and 6.25mg/ml were added to each of the 5 wells (7 mm diameter holes cut in the agar gel, 20 mm apart from one another). The plates were incubated for 24h at 37^{0}C, under aerobic conditions. After incubation, confluent bacterial growth was observed. Inhibition of the bacterial growth was measured in mm. Test were performed in duplicates.

Disc Diffusion Method

Same number of subsequent dilutions of the calabash chalk was performed as described above. 7 mm filter paper discs (Whatman, no. 3) were impregnated with 20 ml of each of the different dilutions. The discs were allowed to remain at room temperature until complete diluent evaporation and kept under refrigeration until ready to be used. The disks were placed Muller Hilton agar containing bacterial inoculum and incubated at 24h at 37^{0}C. Tests were performed in duplicates.

Determination of the minimum inhibitory concentration

The broth dilution method was used. A stock solution of 20 mg/10ml was prepared. 1ml of nutrient broth was dispensed into test tubes and sterilized by autoclaving at 121°C at 15 psi for 15 min. The calash chalk was serially diluted from the stock solutions to obtain varying concentrations. The concentrations were; 1, 0.5, 0.25 and 0.125 mg/ml. 0.1 ml of each test isolate was inoculated into the various test tubes containing varying concentrations and then, incubated at 37°C for 24 h. Culture plates were adjudged as free of growth (-), slight growth (+), moderate growth (++) or heavy growth (+++).

RESULTS & DISCUSSION

Table 1.0 shows the mean total heterotrophic count (THC) of the chalk samples collected from the various markets. The heterotrophic count ranged from 1.0 – 2.1 x 10^4 CFU/g, with the sample from Oyigbo having the least THC (1.0 x 10^4 CFU/g) and sample from Mile I having the highest THC (2.1 x 10^4 CFU/g). The heterotrophic count recorded is similar to the findings of Popoola *et al*., 2013; Kelle *et al*., 2014 where heterotrophic count ranged from 1.5 – 2.0 x 10^4 CFU/g.

TABLE 1: Mean Total Heterotrophic Bacteria Count of Calabash chalk Samples

Sample Source	Mean CFU/g (10^4)
Choba	1.6
Rumosi	1.4
Oil Mill	1.6
Mile I	2.1
Mile III	1.3
Oyigbo	1.0

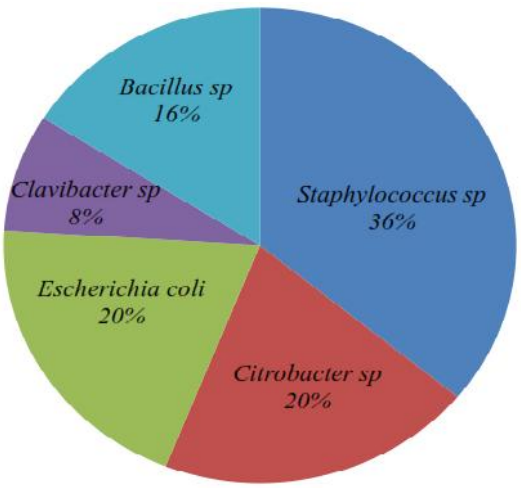

FIGURE 1: Frequency of Occurrence of Bacteria Isolates

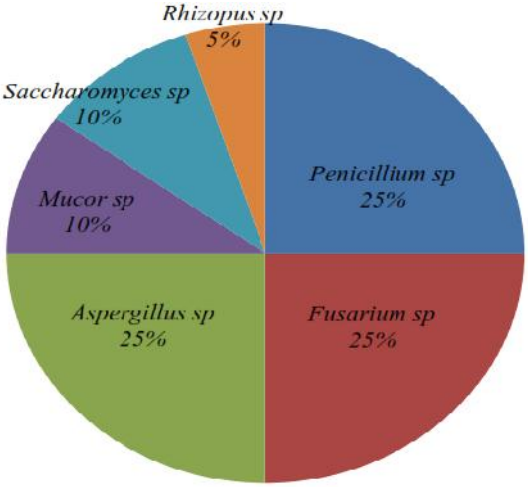

FIGURE 2: Frequency of Occurrence of Fungal Isolates

Fig 1.0 shows the frequency of occurrence of isolated bacteria, *Staphylococcus* sp. was the highest (36%), *Escherichia coli* and *Citrobacter* sp. (20% each), *Bacillus* sp. (16%) and *Clavibacter* sp. was the least occurring bacteria (8%). This was slightly contrary to the report by Okunola *et al*. (2013) where predominating bacteria identified from the high total count were *Staphylococcus aureus* and *Micrococcus acidophilus*. *Aspergillus* sp., *Fusarium* sp and *Penicillium* sp had an occurrence of 25%, *Mucor* sp. and *Saccharomyces* sp. had an occurrence of 10% each while *Rhizopus* sp. was the least occurring fungus with 5% as shown in Fig 2.0, the fungi identified in this study are similar to those reported by Okunola *et al*. (2013); Popoola *et al*. (2013) in similar studies. Pregnant women and children consume clay most likely for the purpose of beneficial bacteria as reported by Kikouma *et al*. (2009), it is important to be careful of opportunistic bacteria that can become infective. Organisms such as *Aspergillus* sp., *Staphylococcus* sp. and *Escherichia coli* identified in this study are known to be pathogenic.

Fig 3.0 shows that calabash chalk concentrations of 100mg/ml and 50mg/ml had the highest inhibitory activities on all the test isolates, concentrations from 6.25mg/ml to 25mg/ml had very little or no inhibitory activity on the test isolates. *Streptococcus* sp., *Candida* sp. and *Klebsiella* sp. were the organisms with the most zones of inhibitions among the test isolates, these organisms have been implicated in a variety of human infections (Ibrahim *et al*., 2009; Okigbo and Mmeka, 2008). The results show that the well-in-agar diffusion method recorded the most potent inhibitory activity on the test isolates. The results are similar to the reports of Popoola *et al*. (2013); Cleidson *et al*. (2007), where calabash chalk concentration of 100mg/ml broth had significant inhibitory activity against *Staphylococcus aureus, S. epidermidis* and *S. pyogens*. The probable mechanism by which calabash chalk function as an antibacterial has previously been reported. The presence of pyrite in some samples may be contributed to bactericidal action (Cohn *et al*., 2006). *Escherichia coli* killed by aqueous leachates of antibacterial clay indicated elevated intracellular concentrations of iron and phosphorous relative to controls as observed by Williams (2011).

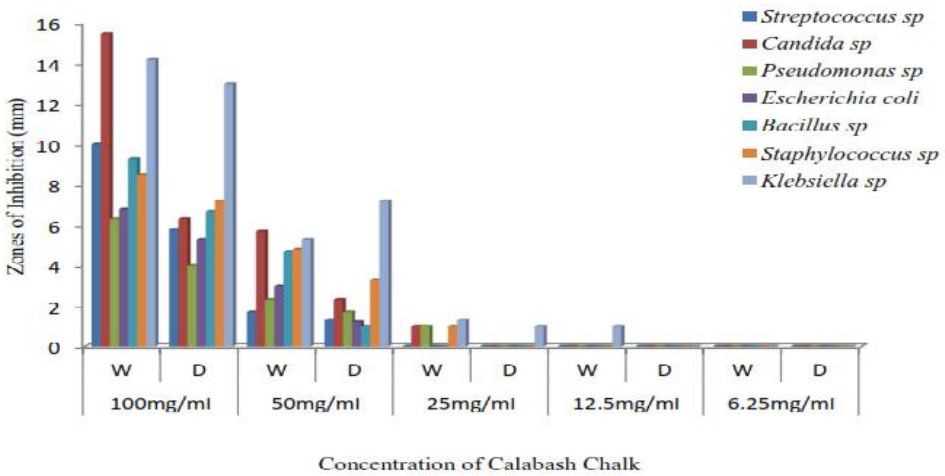

FIGURE 3: Means of inhibition growth diameter obtained by diffusion methods (well- in-agar (W) and disc diffusion variants (D)) using different concentrations of Calabash chalk against selected pathogens

Table 2.0 shows the Minimum inhibitory concentrations of calabash chalk on the test isolates. Calabash chalk concentration of 1.0mg/ml inhibited growth of *Streptococcus* sp., *Candida* sp., *Staphylococcus* sp. and *Klebsiella* sp. While calabash chalk concentration of 0.5mg/ml inhibited the growths of *Streptococcus* sp. and *Candida* sp. only, there was no inhibition of the growth of *Pseudomonas* sp., *Escherichia coli*, and *Bacillus* sp. and concentrations less than 0.5mg/ml had no inhibitory effects on all test isolates, this result is in agreement with the findings of Poopola *et al*. (2013), in which calabash chalk concentration of 10^{-1}mg/ml had the highest inhibitory effects on *Staphylococcus aureus* and *Streptococcus pyogens*. The inhibitory effects recorded in this study are little when compared to related studies of other natural products as reported by Cleidson *et al*. (2007).

TABLE 2: Minimum Inhibitory Concentrations of Calabash Chalk of the test isolates

Test Isolates	Concentration (mg/ml)			
	1.0	0.5	0.25	0.125
Streptococcus sp	-	-	+	++
Candida sp	-	-	+	++
Pseudomonas sp	+	++	+++	+++
Escherichia coli	+	++	+++	+++
Bacillus sp	+	++	+++	+++
Staphylococcus sp	-	++	+++	+++
Klebsiella sp	-	+	++	+++

CONCLUSION

Although microbial analysis of the calabash chalk samples in this study have shown the presence of potentially pathogenic microorganisms, this is not enough to condemn this natural product. It is important to note that calabash chalk exhibited considerable antibacterial activities against potentially pathogenic microorganisms such as *Staphylococcus aureus*, *Escherichia coli*, *Klebsiella* sp. and *Candida* sp. This study shows that calabash chalk can be exploited as an external antibacterial agent; however it must be applied cautiously, due to its relatively high heterotrophic count.

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