Biofilm Production in Borehole Water Sources and their Susceptibility to Antibiotics and Antibiofilm effect of Noni Foliar extracts

Abstract

Background: Bacteria produce biofilm in a variety of systems; water, implant devices in humans and living tissues. They can be persistent and express virulent factors, increased resistance to antimicrobials and human immunity. Plant-based extracts have shown promising outcomes in inhibiting quorum sensing system and modulation of biofilm formation with lesser side effects. Thus, foliar extracts of Noni plant was the subject of such trial as an antibiofilm/antibacterial agent.

Objective: Investigate borehole water sources from three communities in Port Harcourt Local Government Area (PHALGA) for biofilm producers (BPs), antibiogram as well as antibiofilm effect of Noni (*Morinda citrifolia*) foliar extracts.

Method: Microbiological protocols were adopted to isolate and identify bacteria whereas Congo red agar was used to detect biofilm producers (BPs). Antibiogram of BPs were done (with various antibiotics) as well as antibiofilm/antibacterial effect of Noni foliar extracts.

Results: Bioassays detected the biofilm producers (BPs) as *Staphylococcus aureus, Klebsiella pneumoniae, Bacillus and Serratia* species. The bioburden were 55.0±2.5CFU/mL and 51.0±0.2CFU/mL for total heterotrophic bacteria and coliform counts from Ọbaziolu and Rumuelechi communities respectively. Rumuelechi borehole water had the highest percentage relative abundance of *S. aureus* (80%) and *Bacillus* (66.7%) whereas Ọbaziolu had *Serratia* (66.7%) and negative/zero for *S. aureus* and Ọrora had the least of all the isolated BPs. Antibiogram data revealed that all the BPs were 100% resistant to Cloxacillin and Cotrimoxazole but showed high rate of varying susceptibility to other antimicrobials; *S. aureus* depicted (100%) susceptibility to Gentamicin and Streptomycin; *Bacillus* to Tetracycline; *Serratia* to Augmentin and *K. pneumoniae* to Gentamicin. All the BPs showed multidrug resistance (MDR) with *Serratia* and *K. pneumoniae* being resistant to 6 and *Bacillus* and *S. aureus* 5-antibiotics respectively. Furthermore, susceptibility test on these BPs indicated that inhibitory activity of Noni foliar extracts were concentration-dependent. At a concentration of 3000mg/mL methanolic extract (MtE) showed the largest inhibition zone of 20.00mm against *B. subtilis* and aqueous extract (AqE) 15.00mm on *Serratia* species and such phenomenon is indicative of broad spectrum activity. Chloramphenicol (control) had the overall largest inhibition zones on the BPs except on *B. subtilis*. The susceptibility of these crude phytocompounds to BPs promises to be a novel and an alternative natural agent to synthetic antibiofilm products.

Conclusion: Biofilm producers were detected in borehole water supply in three communities in Port Harcourt Local Government Area. The predominant BPs were *Staphylococcus aureus*, *Bacillus* and *Serratia* species. The BPs showed multi-antibiotic resistance with the Gram negative bacteria being resistant to more drug-types. Crude phytocompounds of Noni foliar extracts demonstrated broad spectrum activity with promising prospects as likely therapeutic option against biofilm-based infections. Additionally, the degree of multidrug resistance (MDR) depicted by these BPs to conventional antimicrobials is worrisome and demands regular monitoring, environmental sanitation and good hygienic measures to mitigate public health hazards.

Keywords: Biofilm producers; Borehole water; Antibiogram; Noni Foliar extracts; Multidrug resistance.

1. INTRODUCTION

Water is an essential and indispensable commodity to all life-forms. Boreholes which is a ground water or an artificial source of water supply is one of the most commonly used in rural/urban communities and cities in Nigeria. Though, equitable access to safe and affordable potable water in a sustainable manner, has been the policy thrust of governments and international organisations but the challenges still persist globally. Boreholes allow constant supply and access to clean water and must be protected from deterioration and contamination. Reduction in water quality can be attributed to contamination by sewage and other anthropogenic activities at different points of water supply system which may transmit water-borne diseases [1,2,3]. Diarrhoea is one of the most common diseases associated with poor water quality, sanitation and hygiene resulting in high mortality among infants below the age of five. The potential risk for public health in water systems is the transfer of antibiotic resistant bacteria (ARB) and antibiotic resistant genes (ARG) from environment to humans [4,5,6]. Bacteria produce biofilm on submerged water surfaces such as natural aquatic systems, water pipes, living tissues, tooth surfaces, indwelling medical devices and implants [7,8]. Biofilm is a group of bacterial cells imbedded in a matrix or complex bacterial community that has expression of virulence factors, increased tolerance to antimicrobials and human immunity [9-12]. A biofilm can persist up to 10 years in a food processing facility despite regular cleaning and sanitation [13].

Out of an estimated 16million deaths recorded annually, 65-80% of these cases are linked to bacterial communities that proliferate by forming biofilms [14-17]. Biofilm-linked persistent infections are difficult to treat due to resident MDR microbes as well as the numerous and complex survival strategies utilized [18-22]. The communication of individual cells for the formation of biofilms is fundamental; therefore, blocking this quorum sensing (QS) process is an

important goal for the control of biofilm infections and represents a new method of combating antimicrobial resistance [23,24]. Several investigators, have identified the disruption of biofilms by inhibiting the QS system using many natural and synthetic molecules with potential therapeutic approaches [11,25,26,27]. Following the prominent impacts of biofilms implicated in infectious disease and spread of MDR as well as healthcare cost, it is pertinent to discover new antibacterial agents that can regulate biofilm formation and development. Evidence-based researches have demonstrated that natural products from plants had antimicrobial and chemo-preventive properties with lesser side effects in modulation of biofilm formation and QS inhibition activities [28,29].

A variety of phytocompounds (such as polyphenols, tannins, alkaloids, saponins, etc) have been earlier reported [28,30] to inhibit the QS mechanism of BPs in diverse ways; by blocking the QS autoinducers, inhibition of QS signalling molecules, proteolysis of transcription factors associated with QS [31,32,33]. Others are Quorum quenchers, inhibition of bacterial adhesion and suppression of genes, interfering with accessibility to nutrients essential for adhesion and bacterial growth, anti-adhesive properties and decreasing the content of biofilm exopolysaccharides [34-38]. As such natural products had continued to be a prolific source of agents against biofilm producing/biofilm-based pathogens [39,40]. Phytochemical extracts of Noni foliage have been reported to demonstrate strong antimicrobial activity [41,42] but there is paucity of information regarding antibiofilm effect and was therefore, used as the treatment agent against BPs in this study. Based on these accumulated reasons, Noni foliar extracts (NFEs) was used on BPs isolated from borehole water supply after subjecting them to antibiotic treatments. Furthermore, the data generated from this research demonstrates fundamental evidence that

NFEs have the potential to be explored as preventive agent or therapeutics against biofilm-based infections.

2. MATERIALS AND METHODS

2.1 Description and sample collection sites

Drinking water samples were collected from borehole taps from three different communities, viz; Ọbaziolu, Rumuelechi and Ọrora respectively, in Nkpolu-Orowurokwo, Port Harcourt, Rivers State, Nigeria. These communities are the business hub of Port Harcourt. The borehole tap water samples were allowed to run/flow for 5minutes and sterile plastic bottle was used for collection. A total of nine (9) water samples were collected from three different locations/sites of the communities at an interval of three days. Noni foliage samples were collected from Dilomat farm in Rivers State University (RSU). Fresh Noni (*M. citrifolia*) foliage/leaves were obtained from the Dilomat farm in Rivers state University. They were identified in Plant Science Biotechnology Department by a Plant Taxonomist, Dr. (Mrs) M.G. Ajuru, Rivers state University, Port Harcourt. Both samples were taken to the Department of Microbiology, RSU for analyses.

2.2 Bacteriological analysis

Total heterotrophic bacterial count was determined by direct inoculation of 0.1ml of 100ml of each water sample into solidified nutrient agar and spread plated in duplicates. Eosin methylene blue and MacConkey were used for enumeration of the *Escherichia coli* and coliforms. Centrimide agar and *Salmonella Shigella* agar were used to enumerate the growth of *Pseudomonas* and *Salmonella* and *Shigella* species respectively. The plates were incubated in duplicates at 37^{o}C for 24hours, except Eosin methylene blue plates which was incubated at 45^{o}C for 48hours for faecal coliforms. Discrete colonies of bacteria were isolated and subcultured. The

purified cultures were stored in the refrigerator at 4□. Identification of these isolates followed cultural, morphology and biochemical and sugar fermentation tests [43,44].

2.3 Screening for biofilm production

Bacterial isolates were screened for biofilm production by inoculating them onto Congo red agar (CRA) and incubated at 37°C for 24 hours. The formation of black crystalline colonies indicates a positive test for biofilm production.

2.4 Assessment of antibiotic susceptibility

Antibiotic susceptibility tests carried out according to Kirby-Bauer' disc diffusion (DD) method. Each overnight bacterial isolate at 37□ for 18h was mixed with physiological saline to make a suspension comparable to the 0.5 McFarland turbidity standard which represents □ $x10^8$ CFU/mL. The suspended isolates were spread asceptically using a swab on Mueller-Hinton agar (MHA) and antibiotic multidiscs were placed on the surface of the culture plate. The resistance of bacterial isolates to particular antibiotics was reported based on the inhibition zones [45].

The antibiotics used are shown in Table 1.

Table 1. Antibiotics used against bacterial isolates and quantity

Antibiotics		Quantity
Cloxacillin	(CXC)	30 µg
Gentamicin	(GEN)	10 µg
Amoxycillin + Clavulanic acid	(AUG)	30 µg
Erythromycin	(E)	30 µg
Tetracycline	(TET)	25 µg
Streptomycin	(STR)	30 µg

Ofloxacin	(OFL)	5µg
Chloramphenicol	(CH)	20 µg
Cotrimoxazole	(COT)	25 µg

2.5 Determination of phytochemical components of Noni foliar extract

Noni foliar leaves were shade-dried for 25days and pulverised with sterile mechanical blender. Fifty gramme of the powder was soaked in 500ml of distilled water for 24hours and filtered with Whatman No.1 filter paper. The filtrate was then evaporated using a hot oven at 50^{o}C the resulting filtrate was the aqueous extract (AqE). Similarly, the same quantity of pulverised foliage was soaked in 200ml of 80% methanol for 48 hours, then filtered and evaporated at 50^{o}C and the resultant substance was methanolic extract (MtE). After evaporation 20ml of Dimethyl sulphoxide (DMSO) was used as diluent for various extracts. Standard methods for phytochemical determination of alkaloids, flavonoids, saponins and tannins were employed [30.46,47].

2.6 Statistical analysis

Data generated from this study were analysed using the Statistical Package for Social Sciences (SPSS vs 22).

3. RESULTS

A total number of 21 isolates were obtained from the sampled borehole water sources, out of which 19 of them were biofilm producers (BPs) and two non-identified BPs. The bacterial species identified were; *Staphylococcus aureus*, *Bacillus cereus*, *B. subtilis*, *Klebsiella peumoniae* and *Serratia* species and belonged to four genera. The bacterial load at the different communities are presented in Table 2. Ọro-ọbaziolu (Ọbaziolu) had the highest heterotrophic bacterial counts followed by Rumuelechi and Ọro-ọra (Ọrora) least. Rumuelechi had very high coliform counts compared to the rest of the communities. Ọbaziolu community had higher faecal counts than those of the other communities (2.0±0.7 CFU/mL) respectively. All the communities were negative for total *Salmonella-Shigella* and *Pseudomonas* counts from the sampled borehole water sources.

Table 2. Bacterial load (CFU/ml) of borehole drinking water sources from the 3-communities in Nkpolu-Oroworukwo

Location	THBC	TCC	TSSC	TFCC	TPC
Ọbaziolu	55.0±2.5	4.0±2.1	-	3.0±0.8	-
Rumuelechi	27.0±9.9	51.0±0.2	-	2.0±0.7	-
Ọrora	11.0±13.4	3.0±0.8	-	2.0±0.7	-

Legend: THBC- Total heterotrophic bacterial count, TCC- Total coliform count, TSSC- Total *Salmonella Shigella* count, TFCC- Total faecal coliform count, TPC- Total *Pseudomonas* count.

The percentage relative abundance of BPs of the borehole water sampled from the 3-communities are displayed in Figure 1. Rumuelechi borehole water samples harboured all the BPs at relatively higher percentage than those of other communities. Ọrora samples were negative for *Klebsiella* and *Serratia* species whilst Ọbaziolu's were negative for *Staphylococcus aureus*. Rumuelechi had the highest percentage relative abundance of biofilm producing species of *S. aureus* and *Bacillus* whereas Ọbaziolu had the highest of *Serratia* BPs. Both Rumuelechi and Ọbaziolu are at par in terms of biofilm producing species of *Klebsiella* which was absent from borehole water sampled from Ọrora community. The observance of an appreciably high percentage relative abundance of BPs (a virulent factor) was not quite surprising because of overcrowding/high population density and poor environmental sanitation and hygiene practices. The results further confirms that the four genera isolated from boreholes generally has a strong ability to form biofilm *in vitro* which may cause harm to public health security.

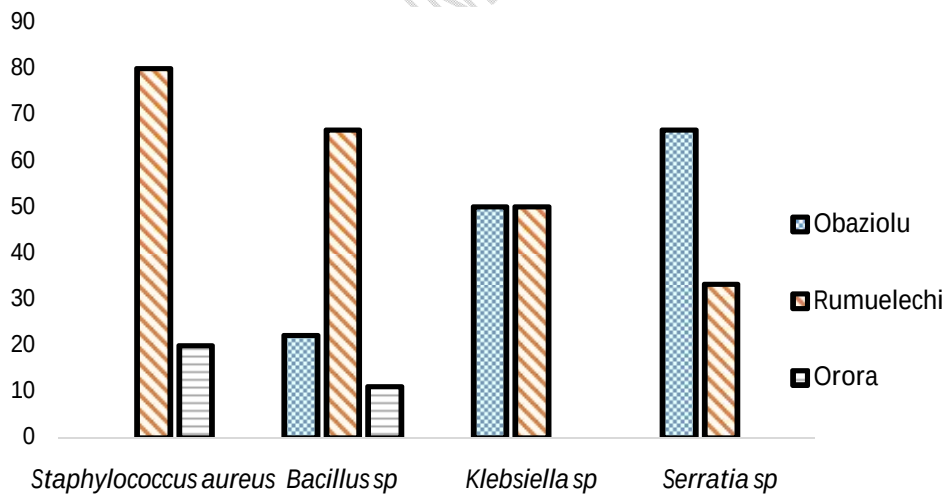

Figure 1. Percentage relative abundance of biofilm producers in borehole water sampled in three communities in Nkpolu-Oroworukwo, PHALGA.

The antibacterial resistance and susceptibility data of the 7 bacterial isolates to nine antimicrobial agents are shown in Table 3. These 7 bacterial isolates were selected based on very strong biofilm production ability. These isolates showed the highest resistance rate to Cloxacillin followed by Cotrimoxazole, Streptomycin, Ofloxacin, Chloramphenicol and Tetracycline. *S. aureus* depicted highest susceptibility (100%) to Gentamicin and Streptomycin; *Bacillus* to Tetracycline; *Serratia* to Augmentin and *K. pneumoniae* to Gentamicin. Only *S. aureus* and *K. pneumoniae* were susceptible to Gentamicin. All the bacterial species showed multidrug resistance (MDR) with *Serratia* and *K. pneumoniae* being resistant (100%) to 6-antibiotics and *Bacillus* and *S. aureus* 5-antibiotics respectively.

Table 3. Antibacterial Resistance and Susceptibility Profiles of 7 of the bacterial isolates to 9 antimicrobials

Antibiotic class	Antimicrobial	**Staphylococcus*	**Bacillus*	*^Serratia*	*^K. pneumoniae*
		Resistance n(%)			
β –lactams	CXC	2(100)	2(100)	2(100)	1(100)
	AUG	2(100)	1(50.0)	0(0.00)	1(100)
Macrolides	ERY	0(0.00)	2(100)	2(100)	1(100)
Aminoglycosides	GN	0(0.00)	1(50.0)	2(100)	0(0.00)
	STR	0(0.00)	2(100)	2(100)	1(50.0)
Fluoroquinolones	OFL	2(100)	0(0.00)	2(100)	1(50.0)
Tetracyclines	TET	2(100)	0(0.00)	1(50.0)	1(100)
Chloramphenicol	CH	0(0.00)	2(100)	2(100)	1(100)
Sulphonamides	COT	2(100)	2(100)	1(50.0)	1(100)
Susceptibility (100%)					
	GEN	2(100)			1(100)
	AUG			2(100)	
	STR	2(100)			
	TET		2(100)		

Legend: CXC = Cloxacillin; ERY = Erythromycin; GN = Gentamycin; AUG = Amoxicillin – Clavulanic acid; OFL = Ofloxacin; STR = Streptomycin; TET = Tetracycline; CH = Chloramphenicol; COT = Cotrimoxazole. * = Gram-positive bacteria; ^ = Gram-negative bacteria.

The phytochemical constituents of *M. citrifolia* foliar extracts are displayed in Table 4. Flavonoid had the highest composition (mg/g) followed by saponin and the least was tannin. Virtually all these phytocompounds have been reported to possess antibacterial agents [30,47] which undoubtedly contributed to their inhibitory/antibiofilm activity (Figure 2).

Table 4. Phytocompounds of *Morinda citrifolia* (Noni) foliar extract in (mg/g)

Parameter	Quantity (mg/g)
Flavonoid	17.75
Saponin	5.71
Alkaloid	2.74
Tannin	0.40

The inhibitory zones of BPs with different concentrations of Noni extract are presented in Figure 2. Generally, the activity of the extracts were concentration-dependent. The largest zone of inhibition from this study occurred with the highest concentration. At 3000mg/mL of aqueous extract (AqE) the largest inhibition zones (15.00mm) was observed on *Serratia* sp and the least was on *S. aureus* (12.5mm). At the same concentration methanolic extract (MtE) showed the largest inhibition zones of 20.00mm against *B. subtilis*, followed by *S. aureus* and *K. pneumoniae* and the least was *Serratia* sp (10.00mm).

Chloramphenicol (control) had the largest inhibition zones on *B. cereus* (21.00mm) and least on *K. pneumoniae* (15.00mm). However, MtE of Noni had much beneficial effect on *B. subtilis* in terms of inhibition activity almost comparable with the control and portends a bright future as an antibiofilm for mitigating the health risks posed by BPs. The ability of

these extracts to inhibit the growth of Gram positive and Gram negative bacteria is indicative of their broad-spectrum activity.

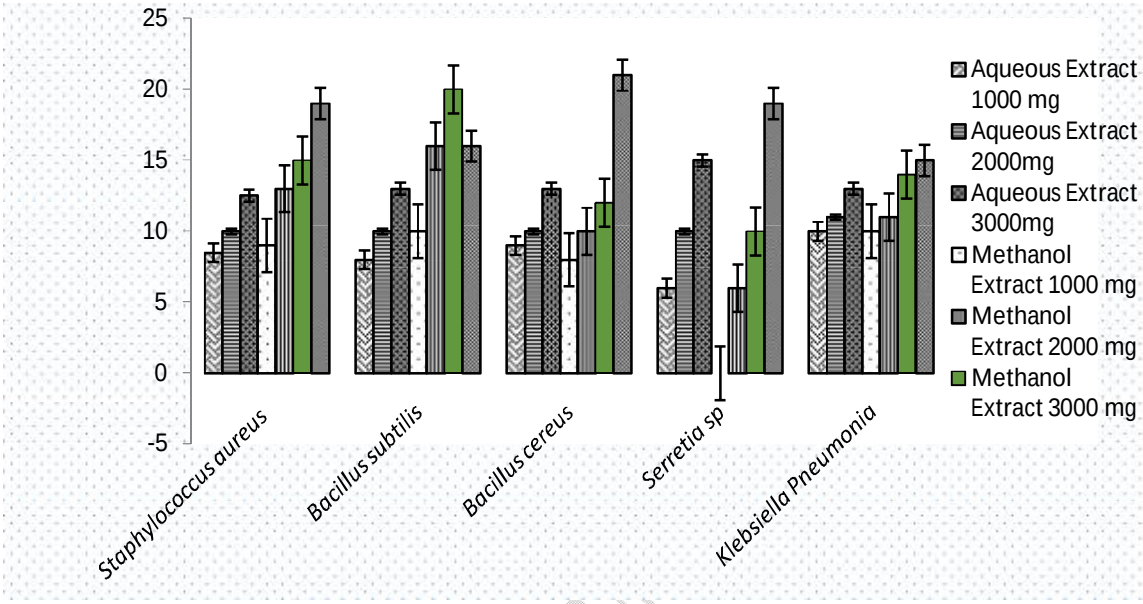

Figure 2. Inhibitory zones of test bacteria with different concentrations of Noni foliar extracts.

4. DISCUSSION

The heterotrophic bacterial and faecal coliform counts as well as biofilm forming *Serratia* were highest from borehole water sampled from Ọbaziolu community. Those from Rumuelechi community had a higher percentage relative abundance of bacteria that forms biofilm than from other communities. Bacterial types isolated from these communities are similar to those reported in Khana LGA, Rivers State except for *Klebsiella pneumoniae* and *Serratia* species [48]. Undoubtedly, these communities are part of Diobu, a densely populated area in Port Harcourt LGA with poor environmental sanitation and hygienic practices. Similar observations of relative abundance of *S. aureus*, *Bacillus* and *Klebsiella pneumoniae* as well as *Serratia* species had been reported in boreholes [5,48]. The absence of *Escherichia coli* a major faecal coliform may be due to the fact that the locations of the boreholes were far from sewage, refuse dump, depth of boreholes, confined in premises and commercialisation, such that the tank does not retain the water for a long time which is in aberration with the high *E. coli* counts earlier reported in rural communities in Rivers State [48]. These bacteria may be potential opportunistic pathogens of several pathologic conditions in humans and animals.

Out of the 21-bacterial isolates, 19 were biofilm producers/formers (BPs) belonging to four genera *Staphylococcus, Bacillus, Serratia* and *Klebsiella*. Morphologically the isolates indicated higher percentage relative abundance of Gram-positive bacteria than the Gram-negative bacteria. The predominance of BPs in these communities much so with the GPB may not be unconnected with the submerged water pipes, poor sanitation, tank cleaning frequency, water retention time in storage tanks and other user practices as well as the persistence of BPs and their survival strategies [1,7,8,13]. Seven strong BPs were tested against 9 different standard antibiotics of various groups. The results depicted high resistance (100%) to Cloxacillin, Augmentin, Tetracycline, Ofloxacin and Cotrimoxazole for

Staphylococcus aureus but susceptible to Streptomycin and Gentamicin whereas *Bacillus* species showed (100%) resistance to Erythromycin, Streptomycin and Chloramphenicol but (100%) susceptible to Tetracycline. *Serratia* species showed (100%) resistance to Cloxacillin, Erythromycin, Gentamicin, Streptomycin, Chloramphenicol and Ofloxacin but 100% susceptible to Augmentin whereas *K. pneumoniae* was 100% resistant to 5-antibiotics in addition to Erythromycin but 100% susceptible to Gentamicin. Similar findings of MDR phenotype of *S. aureus* and *K. pneumoniae* had been reported from raw milk products and in boreholes respectively [5,49]. The Gram negative bacteria; *K. pneumoniae* and *Serratia* species from this study showed higher drug resistance (100%) to more antibiotics than the Gram positive bacteria (*Staphylococcus aureus* and *Bacillus* species). Such high level resistance to macrolides were adduced to slow permeability to the cell wall and elimination by constitutively expressed efflux pumps [5,50,51]. The macrolides (e.g., erythromycin) prevent protein synthesis by binding to the tRNA binding site on the 50S subunit and causing the tRNA molecules to dissociate from the ribosomes.

The resistance of GPB is often due to mutation or modification (methylation) of the 23S ribosomal RNA subunit, with efflux involvement [52]. The resistance between the GPB to different antibiotics such as β-lactams, fluoroquinolone and sulphonamide for *S. aureus* and macrolide, aminoglycoside and chloramphenicol for *Bacillus* species may be attributed to variation in cell-wall-structural compositions (e.g., the former is a nonspore former and the latter forms spore). Several investigators have found a link between biofilm production and the emergence of antibiotic resistance and that biofilm may contribute to consistency of infection in the environment [53,54]. Due to the public health impact of biofilm producers, increased persistence in infectious disease and spread of MDR, it is needful to discover new antibacterial agents to inhibit biofilm formation and development. This increasing awareness of antibiotic resistance by BPs, against commonly used antimicrobials necessitated the

application of natural agent from plant sources. Many workers have reported that alkaloids, flavonoids, tannins and saponins possess potent antimicrobial activity [28,30,47] and Noni foliar extract happens to be one of such. These phytocompounds may account for the inhibitory activity of Noni foliar extracts on test BPs. The best activity was obtained with methanolic extract on *Bacillus subtilis* (20.00mm) at 3.0 g/mL concentration which was relatively comparable to (21.00mm) of chloramphenicol at 1.0 g/mL on *B. cereus*. Inhibitory differences of plant extracts on GPB and GNB has been reported with more effect on GPB. Such phenomenon was attributed to cell wall differences [47] which was in consonance with our findings, although other antibiofilm mechanisms were not considered or studied. Such potency validates the exploration of this extract as valuable alternative to synthetic antibiotics which are currently becoming less effective on a variety of infectious microorganisms.

5. CONCLUSIONS

This study revealed the presence of biofilm producers in borehole water supply in three communities in Port Harcourt Local Government Area. It also indicated the predominance of *Staphylococcus aureus*, *Bacillus* and *Serratia* species. The isolated BPs showed multi-antibiotic resistance with the GNB being resistant to more drugs. Trials with Noni foliar extracts exhibited concentration-dependent and broad spectrum activity with promising prospects as likely therapeutic option against biofilm-based infections.

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