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Antimicrobial Activities of Extracts of *Macrosphyra longistyla* against Gram-Positive Oral Biofilm-Formers from School Children in Southwestern Nigeria and Toxicity Studies Using Brine Shrimps

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Abstract

The world will benefit from more effective antimicrobial agents against oral conditions arising from the actions of biofilm forming bacteria. Also, information is lacking on the oral biofilm-forming bacterial diversity in Southwestern Nigeria. In this study, we isolate and characterize oral biofilm producing bacteria in the oral cavities of schoolchildren in Southwestern Nigeria. We also investigate the antimicrobial properties of *Macrosphyra longistyla* extracts against the biofilm-formers and the toxicity of potent extracts. Samples were obtained from 109 schoolchildren aged 4 - 14 years from Lagos, Oyo and Osun States. Agar well diffusion technique was used in the antimicrobial susceptibility testing. Toxicity testing was done using brine shrimps (*Artemia salina*). Biofilm-formers in this study are *Klebsiella sp.*, *Streptococcus sp.*, *Staphylococcus sp.*, and *Micrococcus sp.* Ethanol leaf extracts had the highest activity against all biofilm-producing bacteria. Ethanol stem bark extract, which elicited activity against *Klebsiella* only, was found to be less toxic than the ethanol leaf extract. *Staphylococcus* showed >10 mm susceptibility to the ethanol and aqueous extracts of *Macrosphyra longistyla*. *Streptococcus* and *Micrococcus* were susceptible to the antimicrobial actions of the ethanolic leaf extracts. Although the ethanol extracts of the leaves had lower minimum inhibitory concentrations than the ethanol extracts of the stem bark, toxicity studies showed ethanol extracts of the stem-bark to be more toxic than the ethanol extracts of the leaves. In conclusion, ethanolic extracts of *Macrosphyra longistyla* show potential as sources of antimicrobials against gram-positive, oral biofilm-forming bacteria.

Keywords

Biofilms, Plant Extracts, Toxicity, Bacteria, Susceptibility, Antimicrobial

1. Introduction

The presence and growth of microorganisms in the oral cavity contributes to the pathogenesis of diseases in the mouth and in internal organs. Oral diseases caused by biofilms include caries and periodontal disease. Biofilms are defined as aggregation of cells of microorganisms embedded in an extracellular matrix composed of single or multispecies organisms [1] [2] [3]. These conditions arise due to prolonged exposure to causal microorganisms in individuals with poor oral healthcare as children or adults [4] [5]. The microorganisms in the oral cavity can get disseminated to internal organs of the body. Internal diseases originating from the activities of oral biofilm-producing microorganisms include gastrointestinal diseases cardiac issues, and some rare forms cancers. Due to the need to prevent infectious diseases, and to identify causal agents of certain diseases, the detection of biofilm-producing bacteria can serve as a way to identify markers of underlying diseases in the host. One of the ways biofilm-producing bacteria cause harm in hosts is by the disruption of the gut microflora by the ingestion of infectious agents [6] [7]. Sources of related diseases that arise include microorganisms spread via the fecal-oral axis. This explanation seems to throw light on the importance and strategic nature of the mouth, as an axis for the flow of both harmful and unharmful or commensal bacteria introduced into the human body [8] [9]. This is one of the reasons why caries has been termed the most contagious disease. There are also investigations into the link between oral-associated biofilm-producing bacteria and diseases of the brain, by the notion that such microorganisms can cross the brain-barrier. The disruption of the gut microbiome by dento-gingival microorganisms also provides a link to an understanding of the link between oral-associated microorganisms and stress-mediated illnesses [10].

When microorganisms produce biofilms, they are shielded from the action of antimicrobial agents [11]. They also adopt mechanisms that accelerate destruction of cells of the mucous membranes of the mouth. In periodontal diseases, there is possibility, in severe cases, where gums and surrounding tissues and bones are affected [12] [13]. Various associations exist among bacterial species involved in oral biofilm formation or progression. They may be antagonistic towards each other by competing for nutrients or by feeding on another. They may also be commensal and they may co-cause multi-bacterial infections. There are numerous biofilm-producing bacteria in the oral cavity. The prevalence of multi-drug resistant biofilm-producing bacteria with or without pathogenicity islands is an emerging study in the biological sciences [14]. The need therefore arises to address the problems and effects of oral biofilm-producing bacteria.

The first step in the control of these pathogens would be to isolate and characterize the microorganisms responsible for this situation. In the microbiology laboratory, this would traditionally involve obtain oral samples (plaque, saliva or epithelial swabs) and incubation including the growth of the microorganisms on agars fortified with indicators that detect the presence of biofilm-producing bacteria. Subsequent steps will involve antimicrobial activity with therapeutic agents which are known or which are novel [15].

Removal of biofilms is by brushing regularly and the use of chlorohexidine [16]. It is important to control the presence of oral biofilms, its progression, and pathogenesis due to microorganisms present in it. This underlies the need to find alternatives to the current aid measures. Oral care will benefit from the creation of any chemical that can prevent caries formation, or the dissolution of oral biofilms. In severe cases, there might be the need to control the biofilms by mechanical removal by a dentist. In order to control the activities of oral biofilm-producing bacteria, there is the race to find novel antimicrobials which can prevent the growth of these bacteria or aid in their removal. Scientists have tried a wide variety of plants and synthetic chemicals for this purpose [17]. It is important to note that in folk medicine, the use of *Macrosphira longistyla* as mouth cleaning device (chewing stick) is common. It is also common to use herbs along with alcohol in the morning to clean the mouth. However, due to hardness of sticks and the possibility of its destruction of gums, it is pertinent to obtain extracts of promising natural products in order to concentrate and characterize them in the race to create novel oral care products with less toxicity. *Macrosphyra longistyla* is common in African folk medicine for use as chewing stick. However, little is documented about its activities against microorganisms, oral microorganisms and oral biofilm-forming bacteria. In our yet-to-be-published preliminary study, we characterized components of the plant and determined its preliminary antimicrobial activity. However, this study characterizes microbiologically, stemming from the fact that it has been found to be effective, its activity against oral biofilm-producing bacteria. We identify some of the bacteria responsible for biofilm-formation in schoolchildren in southwestern Nigeria in order to uncover the strains unique to this environment. Therefore, this study will support ongoing research on bacterial diversity in Southwestern Nigeria and a further investigation into the antimicrobial and toxicity research in the race to discover novel antimicrobials.

2. Material and Method

2.1. Sample Collection and Tests for Biofilm Production

Ethical approvals were obtained from Redeemer's University Directorate of Research Innovation and Partnerships (DRIPs, Redeemer's University's institutional review board), Local Government Education Authority of Lagos State (LGEA), State Universal Education Board in Oyo state (SUBEB), Health Research and Ethics Committee, Ministry of Health, Osun State. Samples were col-

lected after informed consent from parents or guardians. Gargled water samples from the participants (109 healthy children aged 4 - 14 years old) from 4 different schools in Lagos, Oyo and Osun states in southwestern Nigeria were obtained. Subjects gargled potable drinking water and released salivary exudates into sample bottles, ensuring asepsis. This method of sample collection for the purpose of biofilm research is supported by the study by [18]. This is an effective means to achieve our aim. The bottles were then labelled and transported to the laboratory where samples were enumerated and pure isolates were tested for biofilm production. The pour plate method was used to enumerate and obtain pure colonies for identification and characterization. This involved the inoculation of 1ml of gargled water sample onto sterile petri-dish, to which molten nutrient agar (at 45˚C) was added. This petri-dish was then swirled clockwise and anti-clockwise to ensure uniform distribution. The petri dish containing sample was then allowed to set and labelled. Incubation at 37˚C for 18 - 24 h took place and enumeration was done. Pure isolates were then obtained and their 6-hour embryos were stored by streaking in slant method on nutrient agar in bijou bottles, which was corked, labelled and stored at −6˚C for use in subsequent tests. Tests were performed on pure isolates only. Inoculation of each individual representative bacterium on brain-heart infusion agar with Congo-red dye was the method used to determine biofilm production. Black coloration after 18 - 24 h incubation meant biofilm production.

2.2. Identification Biofilm Positive Bacteria

Biofilm producing bacteria were subjected to morphological and biochemical identification tests. Tests included Gram-staining, gas production, sugar fermentation. Sugar fermentation tests were performed with bromocresol agar with yellow pigment production as positive results and bubble for gas production (**Table 1**). Tests were maltose, sucrose, glucose, lactose, mannitol and citrate [19]. Biochemical tests included hydrogen sulphide, Indole, Methyl-red, Voges Proskauer, urea, motility, oxidase, and catalase tests. Incubation was at 37˚C for 24 hours [20].

Table 1. Sugar fermentation of biofilm-producing isolates.

Sample code	Sugar Fermentation Test						
	Maltose	Sucrose	Glucose	Lactose	Mannitol	Citrate	Gas
124_C	[a]+	+	+	+	+	+	+
124_A	+	+	+	+	[b]–	[c]N/A	+
V_3	+	+	+	+	–	N/A	+
33_B	+	+	+	+	+	+	+
VI_3	+	–	–	–	+	–	–

[a]+ positive reaction. [b]– negative reaction. [c]N/A not applicable.

2.3. Obtaining Plant Extracts

Fresh leaves and stem bark of *Macrosphyra longistyla* (Dc.) Hiern (Family: Rubiaceae; LUH: 9431) were identified by Professor Ernest Durugbo and deposited in the Herbarium of University of Lagos. Plant parts were air-dried at room temperature for three weeks after which they were ground to powder using a homogenizer. Ethanol and aqueous extracts were obtained by introducing 250 g of plant parts in 250 ml solvent. Extracts were then placed into rotary evaporator to reduce liquid content. The extracts were then dried overnight in an oven and weighed to ascertain the yield of each extract.

2.4. Antimicrobial Susceptibility and Minimum Inhibitory Concentration (MIC)

Agar well diffusion technique was used to determine susceptibility of the bacteria to the plant extracts and zones of inhibition were recorded. 18 H bacterial isolates were inoculated into petri dishes containing Mueller Hinton agar using sterile swab sticks. Wells were created using 6mm cork-borers and 100 µL of each extract was dispensed into each well using a micropipette and incubated at 37˚C for 24 h. Inspection was done and zones of inhibition produced by each extract were compared to determine the ones that MIC would be performed on. Minimum inhibitory concentration (MIC) was determined on the susceptible bacteria with zones of inhibition of 15 mm and above by measuring their optical density (OD) at 600 nm and by visual inspection using 96-well microtiter plate. The MIC was given as the minimum concentration of plant extract that was able to inhibit visible growth of the bacteria. Toxicity testing was performed on extracts with higher antimicrobial activity (15 mm and above).

2.5. Toxicity Testing

Artemia salina were hatched in artificial sea water with aeration and an electric lamp above the side of the tank for 24 hours to obtain nauplii. 10 nauplii were then aspirated by pipetting and introduced into each test tube containing serial dilutions (1, 10, 100, and 1000 PPM) of the plant extracts and incubated. Nauplii survivors were counted at 6 hours, 18 hours and 24 hours [21]. Mortality rate was calculated and the data were subjected to statistical analyses to determine the Lethal Concentration (LC_{50}). LC_{50} was determined using the Quest Graph™ LC_{50} Calculator [22].

3. Results and Discussion

3.1. Identification of Biofilm-Producing Isolates

Ethanol extracts of the leaves and stem barks of *Macrosphyra longistyla* show higher activity against these biofilm-producing bacteria than aqueous extracts of same plant. On a broader scale, ethanol extract of the leaves show activity against a higher number of bacteria isolated in this study than ethanol extracts of the stem bark. This seems to justify the combination of this herb with tinctures of

alcohol. *Staphylococcus* showed susceptibility to all extracts used in this study, suggesting that in control of this microorganism, the use of this plant can be a good option. Other microorganisms in this study showed varying degrees of susceptibility to the plant extracts.

Biofilm-producing bacteria isolated in this study were identified biochemically and found out to be *Klebsiella*, *Streptococcus*, *Staphylococcus* and *Micrococcus* (**Table 2**). These bacteria are commonly isolated from studies on the microbiome of the oral cavity [23] [24] [25]. Recently, *Klebsiella* has been a reason of public health concern, not just in Nigeria, but globally. Multi-drug resistant strains of *Klebsiella* have been isolated in nosocomial infections. Hence, it is important to mitigate its actions using novel approaches [26] [27] [28] [29] [30]. Even though gram positive and gram-negative bacteria exist in different associations and have complex relationships in biofilm modes in inhibiting the action of antimicrobials [31], all biofilm-producing isolates were gram-positive. The presence of *Streptococcus* in the oral cavities of schoolchildren in two different states, supports its abundance in studies on the oral cavities of pupils from this region. The presence of *Streptococcus* in oral biofilms presents a concern in its treatment and complications arising from the actions of this bacterium present a global health threat [32] [33] [34]. *Staphylococcus* is commonly isolated as a contaminant in nosocomial and environmental samples [35] [36] [37]. However, its biofilms are a contribution to antimicrobial resistance due to the acquisition of virulence genes and their existence in pathogenicity islands [38] [39] [40]. Drug resistant strains of *Micrococcus* have been identified in hospital and oral samples in Nigeria by [41] and [42].

3.2. Antimicrobial Susceptibility of the Biofilm-Formers to the Plant Extracts

All biofilm-positive isolates were susceptible to the antimicrobial action of ethanol extracts of *Macrosphyra longistyla* leaves with zones of inhibition greater than 15 mm (**Table 3**). However, *Klebsiella* was especially susceptible to the action of ethanol stem bark extract. This effect led to the selection of the isolates for minimum inhibitory concentration (MIC) determination. Our results agree with other similar studies, with the plant exhibiting results similar to the action of standard antibiotics [43] [44]. The lowest MIC was exhibited by 124_C while the highest was exhibited by VI_3 (**Table 4**). Low MIC values indicate to the researcher that such compound shows great potential in drug design as it will be required in low concentration to inhibit the growth of potentially pathogenic bacteria. Antimicrobial research will benefit from further studies on the effect of the extracts on isolates with zones of inhibition which were medium but were not characterized to check their MIC.

3.3. Toxicity of Active Plant Parts

High LC_{50} values indicate is less toxicity, especially than compounds with lower

LC_{50} values. Data on proper scientific studies on the use of this plant in oral care is lacking. This research will therefore add to the limited existing knowledge of this plant. Toxicity assay showed the stem bark of the ethanol extract, with lower LC_{50} value, to be more toxic than the leaf extract (Table 5). Also, the leaf extract showed higher antimicrobial potential than the stem. If this plant's extract should be used at all in oral care, the active compounds must be isolated from the leaf. From the data presented, therefore, it is therefore recommended the stem not be used for oral care due to its toxic nature.

Table 2. Identification of biofilm-producing isolates.

Sample code	Phenotypic Test Performed								
	Gram	H_2S	Indole	[d]MR	[e]VP	Urea	Oxidase	Catalase	Probable organism
124_C	[a]+	+	+	+	+	+	+	+	*Klebsiella sp.*
124_A	+	+	[c]N/A	N/A	[b]–	–	N/A	–	*Streptococcus*
V_3	+	+	N/A	N/A	-	–	N/A	–	*Streptococcus*
33_B	+	-	–	+	+	+	–	+	*Staphylococcus*
VI_3	+	+	–	–	+	N/A	+	+	*Micrococcus*

[a]+ positive reaction. [b]– negative reaction. [c]N/A not applicable. [d]MR Methyl red. [e]VP Vogues Proskauer.

Table 3. Antimicrobial susceptibility results of ethanol and aqueous extracts of *Macrosphyra longistyla*.

Sample code	Extract			
	Ethanol Leaf (mm)	Aqueous Leaf (mm)	Ethanol Stem-bark (mm)	Aqueous Stem-bark (mm)
124_C	15.7 ± 0.54	-	15.6 ± 0.8	-
124_A	15.7 ± 1.97	7.1 ± 0.68	-	12.7 ± 0.12
V_3	15.4 ± 1.00	9.1 ± 0.35	-	6.5 ± 0.09
33_B	15.6 ± 0.10	11.4 ± 0.67	13.5 ± 0.27	10.5 ± 0.57
VI_3	15.8 ± 0.35	10.7 ± 0.58	11.1 ± 0.2	-

Table 4. Minimum Inhibitory Concentration (MIC) of the highly-active plant extracts.

Sample code	MIC	
	Ethanol Leaf (µg/ml)	Ethanol Stem Bark (µg/ml)
124_C	2.5	0.1563
124_A	2.5	N/A
V_3	2.5	N/A
33_B	2.5	N/A
VI_3	>2.5	N/A

N/A not applicable.

Table 5. Toxicity of the active plant extracts.

[d]MSL Conc. Ethanol Leaf (µg/mL)	Effect of extract on Brine shrimps					
	T1 (6 hrs)	T2 (18 hrs)	T3 (24 hrs)	No. of Nauplii Survivors	% Mortality	[a]LC_{50} (µg/mL)
1	10	10	9	29	96.6	
10	8	8	5	21	70	
100	10	9	9	28	93.3	
1000	8	8	6	22	73.3	
						422.05
MSL [b]Conc. Ethanol Stem Bark ([c]ppm)						
1	9	9	9	27	90	
10	10	10	10	30	100	
100	9	9	5	23	76.6	
1000	8	4	2	14	47	
						47.23
Control-Distilled Water (ppm)						
1	10	10	9	29	96	
10	10	9	9	28	93	
100	10	10	10	29	96	
1000	10	10	9	30	100	
						498.58

[a]LC_{50}: lethal concentration. [b]Conc. Concentration. [c]Ppm: Parts per million. [d]MSL: *Macrosphyra longistyla*.

4. Conclusion

Due to the burden and the problems associated with oral biofilms, results from this study become important. Biofilm producing bacteria identified in this study shows diversity in morphological and biochemical characteristics. They vary in their antimicrobial susceptibility pattern to extracts from *Macrosphyra longistyla*. The extracts show *in-vitro* promise in mitigating the effects of these oral biofilm-producing bacteria. It is suggested that the leaves be concentrated and used in drug design.

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Conflicts of Interest

The authors declare no conflict of interest.

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