Determining the phytochemical properties and antibiogram of different chewing stick plants on selected*streptococcal* species isolated from the oral cavity

Abstract

Chewing sticks are tiny twigs obtained from plant stem which measures from 12-25cm long and tied in bundles of 5- 10. Over the years, some plant stems (twigs) have been known and used as effective tooth cleaning agent long before the production of toothpaste, mouthwash and mouthsprays. This study aims at determining the phytochemical properties and antibiogram of different chewing stick plants on two streptococcal species isolated from the oral cavity. *Streptococcus pyogenes* and *Streptococcus mutans* were isolated from oral swabs of patients attending Rivers State University TeachingHospital and properly identified to species level. The pure isolates were further subjected to the antimicrobial effect of the aqueous and ethanolic extracts of the chewing stick plant: *Vernonia amygdalina*,*Jatropha curcas*, *Massularia acuminate* and *Phyllanthus mullerianus*. A qualitative phytochemical screening and quantitative analysis were carried out on the plant's stem to determine the presence of antimicrobials. Percentage of antimicrobials present in the plant stem ranged from 33.3% - 66.7% with *Jatropha curcas*, *Massularia acuminate* and *Vernonia amygdalina* as the highest and *Phyllanthus mullerianus*the least. The antibiogram of the isolates to conventional gram-positive antibiotics were determined by disc diffusion method. *Streptococcus pyogenes* had 75% sensitivity and 25% resistance to the antibiotics while *Streptococcus mutans* showed 50% sensitivity, 16.7% intermediate and 33.3% resistance to the antibiotics. The findings of this investigation on the antibacterial activity of the aqueous and ethanolic extracts of the chewing stick plants on the test isolates demonstrated that the ethanolic extract of *Massularia acuminate* has the highest zone of inhibition of 15.5 ±0.71mm at 100mg/ml and the least 8±0mm *Phyllanthus mullerianus*. Effect of the ethanolic extract of *Jatropha curcas* and *Vernonia amygdalina* ranged from 0 - 9.5±0.71mm showing little effect on the test isolates. The aqueous extract ranged from 8±5.7 - 9.5±0.71mm at 100mg/ml with *Massulara acuminate* as the highest and the lowest zones of inhibition. This finding indicates that *Massulariaaccuminata* has the ability to suppress the growth of dental plaque forming *Streptococcus mutans* and *Streptococcuspyogenes*. Chewing sticks are more affordable and easily accessible, they could be recommended in community oral health programs.

Key: dental plaque, chewing stick, antibiogram, streptococcal species

Introduction

Dental caries is one of the most prevalent oral infections that people have experienced in modern times, and it is a dangerous condition that affects the teeth (Moses *et al.,* 2011). Acids generated when food particles ferment on teeth are what cause tooth decay (Silk, 2014). Some bacteria's such as *Staphylococcus aureus, Streptococcus mutans, Lactobacillus acidophilus* etc. are in charge of creating dental plaque, which is the buildup of a thick, white film on teeth, by interacting with saliva and leftover food particles in the mouth (Marsh and Devine, 2011). As a result, proper dental health education and the promotion of traditional and conventional methods of teeth cleaning should be encouraged in underdeveloped nations with insufficient dental facility supply. The abrasive properties of toothpaste help to eliminate dental plaque and food particles from teeth while also improving oral health. Due to the active element fluoride, it also prevents gingivitis, or gum disease, and tooth decay (Limeback& Robinson, 2012). Prior to the invention of toothpaste, chewing sticks were the primary natural dental care item used by early people. Nonetheless, studies looking at the general effects of chewing sticks on dental health have identified several naturally occurring chemical components in chewing sticks that support healthy oral hygiene (Al-Bayati and Sulaiman, 2018). Chewing stick extracts are currently used as a flavoring and active ingredient in toothpaste (Sudhir *et al.,* 2018).Chewing sticks, is a traditional toothbrush, constructed from bigger plant stems or from slender twigs. Chewing sticks are used by people to brush their teeth; they are available in the market in bundles of five to ten sticks, each of which is roughly 25 centimeters in length. Some chewing sticks are still covered in bark, which people form into toothbrushes with their teeth (Almas, 2001). The chewing stick plants used in this study are *Vernonia amygdalina,Jatropha curcas, Massularia acuminata* and *Phyllanthus muellerianus*.*Vernonia amygdalina* is a member of the daisy family, it usually reaches a height of 2.6–16.4 ft, with elliptical leaves that can reach 20 cm (7.9 in) in length and rough bark Ijeh and Ejike (2011). *V. amygdalina* is known in English as "bitter leaf" due to its bitter flavor (Farombi and Owoeye 2011).This plant's twigs and sticks are chewed on for dental hygiene in Nigeria, as the stems are utilized in Uganda to create soap. The physic nut is known scientifically as "*Jatropha curcas*." With its implied medical applications, the Greek terms jatr¬os (doctor) and troph´e (food) are the source of the genus name Jatropha (Abdulla *et al.*, 2011). This plant is a member of the Euphorbiaceae family, which includes drought-resistant shrubs and trees that are widely found in semi-cultivated and wild settings throughout Africa, India, South East Asia, Central and South America (Mart´ınez*et al.,* 2006). The bark and stem of *J. curcas*possesses phytochemical properties such as saponins, steroids, tannins, glycosides, alkaloids, and flavonoids(Igbinosa*et al.,* 2009). *Massularia acuminata* (Rubiaceae) known as orinijebu or pakoijebu in Yoruba-Western Nigeria, can grow to aheight of five meters in length.*Massulariaaccuminata*possess some antimicrobial properties capable of inhibiting the

growth of some oral microbes. Amongst other health benefits of this plant, it's stem is used in Nigeria as a dental hygiene stick (Ndukwe,2004). *Phyllanthus muellerianus* (Kuntze) Exell is a woody climber or glabrous shrub that is commonly arborescent. It grows in savannah and dry secondary forests, as well as coastal thickets and scrub, and it is widely distributed throughout tropical Africa (Katsayal and Lamai, 2009). It has long been used to treat a variety of illnesses. *Phyllanthus muellerianus* (Kuntze) Excell is a popular medicinal plant found in the tropical regions of West Africa. Known by several names such as "mijiriyarkurumi" in Hausa, "oguazu" in Igbo, "nkanga" in the Efik. The fruits, twigs, and leaves all have antimicrobial properties. Some parts of Nigeria utilize the twigs as chewing sticks (Breytanbach and Malan, 1989).

Aim: phytochemical properties and antibiogram of different chewing sticks on two streptococcal species isolated from the oral cavity.

Materials and Methods

Four different plants used as chewing sticks (*Massularia acuminate, Phyllanthus muellerianus, Vernonia amygdalina,* and *Jatropha curcas*) were gathered from the Rivers State woody savannah known as Elele Community. The chewing sticks were identified at Rivers State University in Port Harcourt, Nigeria, in the department of Plant Science and Biotechnology.

Test Isolates

Clinical specimens were collected from patients of dental clinic in Rivers State University Teaching Hospital, Rivers State. The specimenwere aseptically transported to the microbiology laboratory where it was cultured, isolated and identified using gram staining and biochemical techniques (Aziz, 2020). The streptococcus species was identified to species level by molecular techniques as described by Deiman*et al.,* 2002.

Phytochemical Analysis of the Chewing Stick Plants

flavonoids, tannins, saponins, terpenes, basic alkaloids, and glycosidesanthraquinones, anthocyanosides, reducing sugar and cyanogenic agents were all examined in the plant extract (Sony *et al.*, 2011).

Phytochemical Screening

Alkaloids, glycosides, tannins, saponins, anthraquinones, anthocyanosides, flavonoids, reducing sugars, and cyanogenic agents were all examined in the extract. The plants were gathered in the Nigerian state of Rivers, in the Elele village, a wooded savannah.

Qualitative Method of Analyses

By boiling 20g of the fresh chewing stick in distilled water and filtering the filtrate through a vacuum pump, one could test for anthocyanosides, flavonoids, reducing sugar, saponins, tannins, alkaloids, terpenes, cardiac glycosides, and cynogenic glycosides. The phytochemical screening was subsequently conducted using this filtrate.

I. **Test for Flavonoids**

Two milliliters of diluted NaOH were combined with one milliliter of the plant filtrate, and the mixture's color was checked. The color golden yellow suggested the presence of flavonoids. One milliliter of plant filtrate and two milliliters of 10% lead acetate were combined to test for phenolic flavonoids. A brownish precipitate was seen in the mixture, signifying a positive test(Egbuna *et al.,* 2018).

II. **Test for Reducing Sugars**

One milliliter of the plant filtrate was mixed separately with Fehling A and Fehling B; when reducing sugars are present, a brown color is displayed with Fehling B and a green hue is displayed with Fehling A (Egbuna *et al.,* 2018).

III. **Test for Saponins**

After aggressively mixing one milliliter of the filtrate with two milliliters of distilled water, the mixture was let to stand for ten minutes. When foam forms on the mixture's surface and stays there for longer than ten minutes, saponins are present(Gupta *et al.,* 2013).

IV. **Test for Tannins**

When a few drops of 1% FeC1 were applied to two milliliters of the filtrate, a dark green color formed, signifying a successful tannin test (Trease and Evans, 2002).

V. **Test for Anthocyanosides**

A light pink tint in the mixture produced by combining one milliliter of the plant filtrate with five milliliters of diluted HCl indicated a positive test result (Velavan, 2015).

VI. **Test for Alkaloids**

A gram was stirred with 5ml of 1% aqueous HCl on water bath and filtered, few drops of Dragendoff’s reagents were added to 2ml of the extract, an orange-red precipitate was formed indicating the presence of alkaloids (Usman *et al*., 2009).

VII. **Terpenes**

2.0 ml of chloroform was added to 5 ml of aqueous plant extract, the mixture evaporated on the water path and was heated with 3 ml of concentrated H_2SO_4. A gray hue emerged, revealing the terpenoids' entity (Gupta *et al.,* 2013).

VIII. **Test for Cardiac glucosides**

Following the adoption of the killer-kiliani and the legal test, 0.5 grams of the extract were mixed to 2 milliliters of acetic anhydrate and H_2S0_4 (Trease and Evans 2002).

IX. **Test for Cyanogenic glucosides**

This was carried out by subjecting 0.5g of the extract in 10ml of water, filter and add sodium picarateand boil. (Gupta *et al.,* 2013).

Preparation of chewing stick for extraction using aqueous and ethanol solvent

Massularia acuminate, Phyllanthus muellerianus, Vernonia amygdalina, and *Jatropha curcas*were chopped into tiny bits and blended into powder using a sterile food blender. 100g of the powdered chewing sticks were added to 1000ml of ethanol and 1000ml of sterile water respectively, to achieve a 1:10 ratio.The mixture was stored in a sterile, well-capped flask and allowed to sit at room temperature for seven days before being filtered through number 1 Whatman filter paper. The aqueous extract was centrifuged for ten minutes at 2000pm (Umeh *et al*., 2005). Supernatant was transferred to sterile screw-capped vials and refrigerated until needed(Al-koubaisi, 2001). The ethanolic extracts were dried and run through a rotary evaporator, the crystals were placed in sterile sample bottles so they could be used later.

Preparation of Concentrations with the Chewing Stick Extract

The stock (crystals) was weighed and serially diluted into solution with the extraction solvent (DMSO and aqueous respectively), in sterile test tubes labelled 100mg/ml, 50mg/ml, 25mg/ml, and arranged from the highest to lowest concentration of extract desired (Gberikon, *et al.,* 2015).

Preparation of McFarland Standard

A 0.5 McFarland standard was created by combining 9.95ml of 1% sulfuric acid with 0.05ml of barium chloride dihydrate. A visual comparison was made between the standard and a bacterial solution in sterile saline(Cockerill *et al.,* 2012).

Determine the efficacy of chewing stick extract on bacterial isolates

To determine the antibacterial test, the modified disc diffusion method was employed. Using the McFarland standard, a well-prepared bacterial suspension was mixed with a few drops of the

isolate. A glass spreader was used to evenly distribute a little amount of the isolate across the whole surface of a Muller Hinton agar plate after it had been carefully prepared. The aqueous and ethanolic extract controls were provided via a disk impregnated with sterile water and 70% DMSO, respectively, while an impregnated disc with the various extract concentrations was positioned equally apart on the plate. For 24hrs the plates were incubated at 37^0C. To assess the antibacterial efficacy, zones of inhibition were measured in millimeters following incubation. Every experiment was carried in duplicate (Sarmad, 2013).

Antimicrobial susceptibility pattern of the isolates using conventional antibiotics

The antimicrobial test was determined using Kirby Bauer's method, often known as the disc diffusion method. Using a sterilized glass spreader, a few drops of the bacterial suspension were equally placed over Muller Hinton plates that had been thoroughly prepared. The typical antibiotic disc was picked and placed in the center of the plate using sterile forceps, and it was then incubated at 37^0C for 24 hours. The zones of inhibition were accurately measured after 24-hour incubation period Fabio*et al.*, 2007.

Statistical Analysis

Version 21 of Anova IBM® SPSS® statistics was used to evaluate the data.

Results

Table 1. Morphological and Biochemical Characteristics of *Streptococus mutans* and *Streptococcus pyogens*Isolates from Clinical Specimens

	Colony Characteristics						Gram Stain Results				Biochemical Tests											
Isolate Code	**shape/ size**	**Elevation**	**Surface**	**Margin**	**Colour**	**Opacity**	**Reaction**	**Shape**	**Arrangement**	**Catalase**	**Oxidase**	**Citrate**	**Capsule**	**Methyl Red**	**VP**	**Motility test**	**Lactose**	**Glucose**	**Fructose**		**Sucrose**	**Suspected Specie**
J4	round (2mm)	Convex	smooth	Entire	grayish-white	translucent	+	cocci	chains	-	-	-	+	-	+	-	A	A	A		A	*S. mutans*
J3	round (2mm)	Convex	smooth	Entire	grayish-white	translucent	+	cocci	chains	-	-	-	+	-	+	-	A	A	A		A	*S. mutans*
J1	round (2mm)	Convex	smooth	Entire	White	translucent	+	cocci	chains	-	-	-	-	+	-	-	A	A	A		A	*S. pyogenes*
J2	round (2mm)	Convex	smooth	Entire	White	translucent	+	cocci	chains	-	-	-	-	+	-	-	A	A	A		A	*S. pyogens*

Table 2. Phenotypic and Genotypic Characterization of Isolates

Isolate Code	Phenotypic Identity	Genotypic Identity	Accession Number
J1	*S. pyogenes*	*S. pyogenes*	JULO01000043.1.

J2	*S. pyogenes*	*S. pyogenes*	CP077685.1.
J3	*S. mutans*	*S. mutans*	AJ243965.1.
J4	*S. mutans*	*S. mutans*	AB294730.1.

Table 3. Virulence features of the isolates

Isolate code	Biofilm	Hemolysis	Motility	Capsule
S. mutans	+	+	-	+
S. mutans	+	+	-	+
S. pyogenes	+	+	-	-
S. pyogenes	+	+	-	-
No(%) virulence	**4(100)**	**4(100)**	**0(0)**	**2(50)**

The outcome of the phytochemical examination completed on *Vernonia amygdalina*, *Massularia acuminate*, *Jathrophacurcasand Phyllanthus muellerianus*Table 4.4 displays a greater and equivalent quantity of antimicrobials present in *Jatropha curcas* and *Vernonia amygdalina* at (66.7% and 66.7%, respectively), with *Phyllanthus muellerianus* having the lowest antibacterial activity at 33.3%. The four chewing stick extracts possessed alkaloids but had neither cardiac glycoside or anthrocynocte.

Table: 4. Phytochemical properties of the plant extract

Extract code	Ms	Js	Ps	Bs
Phytochemicals				
Flavonoids	+	++	+	+
Reducing sugars	-	-	-	+
Saponins	+	+	-	-
Tannins	++	++	-	++
Anthrocynocte	-	-	-	-
Alkaloids	+++	+++	+++	+++
Terpenes	+	+	++	+
Cardiac glycoside	+	+	-	+
Cynogenic glycosides	-	-	-	-
% antimicrobial present	**66.7**	**66.7**	**33.3**	**66.7**

Key: (+) = positive & present (-) = negative & absent

Key- BS =*Vernonia amygdalina*, MS =*Massularia acuminate*, JS =*Jathrophacurcas*, PS =*Phyllanthus muellerianus.*

Table 5. Percentage of susceptibility, intermediate and resistance of the isolates on the antibiotics.

Antibiotics (conc.)	*S. mutans*	*S. pyogenes*

	N=2			N=2		
	R	**I**	**S**	**R**	**I**	**S**
Cefuroxime (30ug)	**2(100)**	**-**		**-**		**2(100)**
Gentamicine (10ug)	**-**	**-**	**2(100)**	**-**	**2(100)**	**-**
Cefotaxime (30ug)	**-**	**-**	**2(100)**	**2(100)**	**-**	**-**
Ceftriaxone (30ug)	**-**	**-**	**2(100)**	**-**	**-**	**2(100)**
Cefexime (5ug)	**-**	**-**	**2(100)**	**-**	**2(100)**	**-**
Levofloxacine (5ug)	**-**	**-**	**2(100)**	**-**	**-**	**2(100)**
Ciprofloxacin (5ug)	**-**	**-**	**2(100)**	**-**	**-**	**2(100)**
Impenem (10ug)	**-**	**-**	**2(100)**	**-**	**-**	**2(100)**
Azithromycin (15ug)	**-**	**-**	**2(100)**	**2(100)**	**-**	**-**
Ofloxacin (5ug)	**-**	**-**	**2(100)**	**-**	**-**	**2(100)**
Erythromycin (15ug)	**2(100)**	**-**	**-**	**2(100)**	**-**	**-**
Amoxicillin clavunate (30ug)	**2(100)**	**-**	**-**	**2(100)**	**-**	**-**
% R, I & S	**25**	**0**	**75**	**33.3**	**16.7**	**50**

KEY: R = resistant, I = intermediate & S = sensitivity

Table 6. Responseof *S. mutans* and*S. pyogenes*to different concentrations of ethanolic and aqueous extract of the chewing stick plants as shown by their zones of inhibitions.

Plant		Je	Ja	Me	Ma	Pe	Pa	Be	Ba
Isolates	**Conc.**	**Diameter of Zones of Inhibition (mm)**							
S. mutans	**100mg/ml**	**0**	**0**	**15.5±0.71**	**9.5±0.71**	**13±1.4**	**0**	**0**	**0**
	50mg/ml	**0**	**0**	**11±1.4**		**8.5±0.71**	**0**	**0**	**0**
	25mg/ml	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**0**
S. mutans	**100mg/ml**	**9.5±0.71**	**0**	**13.5±0.71**	**8±5.7**	**0**	**0**	**0**	**0**
	50mg/ml	**0**	**0**	**8±5.7**	**0**	**0**	**0**	**0**	**0**
	25mg/ml	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**0**
S. pyogenes	**100mg/ml**	**0**	**0**	**14±2.1**	**9±1.4**	**10.5±0.71**	**0**	**0**	**0**
	50mg/ml	**0**	**0**	**9.5±0.71**	**0**	**0**	**0**	**0**	**0**
	25mg/ml	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**0**
S. pyogenes	**100mg/ml**	**0**	**0**	**10.5±0.71**	**8±5.7**	**8±0**	**0**	**0**	**0**
	50mg/ml	**0**	**0**	**8±5.7**	**0**	**0**	**0**	**0**	**0**
	25mg/ml	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**0**

Key: Je = ethanolic extract of *Jatropha curcas*, Ja = aqueous extract of *Jatropha curcas*Me = ethanolic extract of *Massulariaacuminate*,Ma = aqueous extract of *Massularia acuminate*, Pe = ethanolic extract of *Phyllanthus muellerianus*, Pa = aqueous extract of *Phyllanthus muellerianus*, Be = ethanolic extract of *Vernonia amygdalina*, Ba = aqueous extract of *Vernonia amygdalina*

Discussion

Phytochemical analysis carried out on the chewing stick plant showed that *Massulariaaccuminata*possessed flavonoids, terpens, alkaloids, tanins, and saponins. *Phyllanthus muellerianus* has possesses flavonoids, reducing sugar, terpens, cardiac glucosides, tannins, and alkaloids while *Jatropha curcas*has saponins, flavonoids, terpens, alkaloids, cardiac glucosides and tannins. This result was consistent with the writings of Ikoyi*et al.*, (2023), (Rahu *etal*., 2021). Maximum antibacterial activity against *Streptococcus mutans* and *Streptococcus pyogenes* was demonstrated by the ethanolic extract of *Massulariaaccuminata*, with mean zones of inhibition ranging from 13.5±0.71 to 15.5±0.71 and 10.5±0.71 to 14±2.1 at 100mg/ml, respectively. This aligns with research conducted by Adeleye *et al.*, 2020 citing the highest zones of inhibition on *Staphylococcus aureus* (19.30±0.17 mm) and *Streptococcus mutans* (12.60±0.52 mm) for the ethanolic extract of *Massulariaaccuminata*, which is consistent with some published literatures. The phytochemical constituents of plants are secondary metabolites, which are bioactive components processing the pharmacological activity of plants (Bankole, 2012). *Streptococcus pyogenes* and *Streptococcus mutans*were both inhibited by the aqueous extract of *Massulariaaccuminata*at 100mg/ml, with zones of inhibition ranging from 8±5.7 -9±1.4and 8±5.7 – 9.5±0.71 respectively. Even while the phytochemicals in the chewing stick were extracted at a lower concentration than in the ethanolic extract with the same concentration, sterile water nonetheless worked well as a solvent The presence of the bioactive component cardiac glycoside, which was identified by phytochemical screening, may be the cause of the antibacterial action demonstrated by the extract of *Mussularia acuminata*. In alliance with Ogbe, *et al*., 2022 a higher concentration of the extract utilized may have contributed to the antibacterial zone of inhibition of 16.00±0.30 seen in the *Massulariaaccuminata* aqueous extract against *Streptococcus mutans*. While the aqueous extract showed no zone of inhibition against the test isolates, the ethanolic extracts of *Phyllanthus muellerianus* showed a maximal zone of inhibition ranging from 13±1.41 to 8±0 at 100mg/ml. This is consistent with research conducted by Brusitti*et al*., 2011 declaring that *Streptococcus pyogenes* and *Streptococcusmutans* were very susceptible to the antimicrobial effects of *Phyllanthus muellerianus*, and providing an explanation for the plant's widespread use in Cameroon for the treatment of tetanus and dentistry reasons against *S. mutans*. The test isolates were not inhibited by the ethanolic or aqueous extracts of *Vernonia amygdalina* or *Jatrophacurcas*. This is in contrasts with the findings from a study carried out by Adewumi *et al*., 2014 on the antibacterial efficacy of *Jatropha curcas* seed

and stem extraction and phytochemical screening against several wound infections.Adeoti *et al*., 2021 whereby he discovered *Vernonia amygdalina's* antibacterial efficacy against *Streptococcus* mutans.From the study, *Streptococcus pyogenes* had 54.2% sensitivity and 45.8% resistance to the aforementioned antibiotics. The rate of resistance may result from people using antibiotics frequently, which would cause the organisms to become resistant to the drugs. It may also result from people using mouthwash and toothpaste, which could have interfered with the organisms' ability to grow to their full potential and develop survival mechanisms. However, these organisms have also been found in healthy people who have never received an antibiotic treatment. Because bacteria can undergo mutations, this can also result in an increased rate of medication resistance in the organisms. At different concentrations, *Streptococcus mutans* displayed 37.5% sensitivity, 12.5% intermediate, and 50% resistance to the antibiotics.

Conclusion

According to the findings, *Massulariaaccuminata* and *Phyllanthus muellerianus* had a significant inhibitory effect on the oral pathogens (*S. mutans* and *S. pyogenes*). It is affordable and frequently available; this could be an excellent alternative to toothpaste in rural regions (especially for plant species with great inhibitory activities against cariogenic bacteria).

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