

Antibacterial Activities of *Psidium guajava* (Guava) and *Velvet tamarin* (Icheku) Local Chewing Sticks on *Streptococcus mutans* Isolated from Human Mouth

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Abstract

Globally dental diseases are mainly caused by *Streptococcus mutans*, it is one of the leading causative agents of dental caries worldwide, because of its resistance to conventional antibacterial agents, alternative therapies are used to control resistance of oral pathogens. This research was done to determine the antibacterial activities of *Psidium guajava* (guava) and *Velvet tamarin* (Icheku) chewing sticks on *Streptococcus mutans* isolated from the oral cavity. The study was conducted in Owerri Imo State Nigeria during November-December period. Phytochemical analysis of the plant extracts was done using appropriate techniques. The procedure used for antimicrobial susceptibility test was disk diffusion method. Serial dilutions of *Psidium guajava* (guava) and *Velvet tamarind* (Icheku) extracts were prepared, Muller-Hinton media was used to put together the extract of serial dilutions of *Psidium guajava* (guava) and *Velvet tamarin* (Icheku) and a microbiological procedure were used for visually determining the minimum inhibitory concentration as well as minimum bactericidal concentration. Phytochemical evaluation of the plants' extracts revealed that it contains saponins, tannins, alkaloid, steriods, glycosides and phenol. The results obtained from the antibacterial susceptibility testing of the extracts against *Streptococcus mutans* showed that the zones of inhibition recorded ranged from 18 mm to 27 mm. Ethanol (Soxhlet) extract of Icheku twig showed no zone of inhibition on the isolated organism. The ethanol (soxhlet) extract of the individual *Psidium guajava* (guava) and *Velvet tamarin* (Icheku) has a better antibacterial effect when compared to their aqueous extracts and combined forms. *Psidium guajava* (Guava) and *Velvet tamarin* (Icheku) twigs are made up of composite that is active against *S. mu-*

tans and can be used in oral hygiene. There is a need for further investigation on the plant extracts as the rural poor make use of it because it is cheap, readily available and the rich also use it once they are in the village mostly in Eastern Nigeria. Similarly chewing sticks has been reported to be practiced by 90 of rural population in Nigeria.

Keywords

Antibacterial Activity, Chewing Sticks, *Psidium guajava* (Guava), *Velvet tamarin* (Icheku), *Streptococcus mutans*

1. Introduction

Nowadays, 60% - 90% of the young people worldwide suffer from dental caries [1]. The scientific name for cavities or tooth decay is dental caries, it is the breakdown of teeth caused by acid made by bacteria, the acid they make destroys the tooth hard tissues (enamel, dentin and cementum). This acid is produced by the bacteria when they break down food debris or sugar on the tooth surface and thus a diet high in simple sugar is a risk factor. If mineral breakdown is greater than build up from sources such as saliva, caries results. Proper oral hygiene habits are needed for the control of dental caries due to their multifactorial etiology [2] and [3].

Streptococcus mutans is a facultative anaerobic gram positive coccus commonly found in the oral cavity and is a significant contributor to dental caries and the main microorganism associated with caries and dental plaque [4]. This organism splits the sucrose in food and uses one of the sugars to build its capsule which sticks tightly to the tooth. The bacteria that are trapped in the capsule use the sugar to fuel metabolism [5].

Chewing sticks are often used in Nigeria and Africa in general in maintaining oral hygiene, they are made from roots, twigs or stems of a plant. The preferred are cleaned with water to remove dirt, cut to convenient length which varies from 15 - 30 cm long and filed in a bundle. Chewing sticks obtained from a variety of selected plants are used as traditional method of mechanical oral hygiene by up to 80% of Nigerians. Almas, 2004 have demonstrated chewing stick as effective as toothbrushes and their use has been encouraged by the WHO. Apart from their mechanical effects many of the chewing sticks have been shown to have significant antimicrobial activity against a broad spectrum of microorganisms.

In Middle East and Africa shrubs and local trees with good taste, bitter and bristle are chosen as chewing sticks for their beneficial effects on the supporting tissues and teeth [6]. The comparative benefit and popularity of chewing sticks in the world as an oral hygiene alternative make it a cheap agent for plaque control in our environment. Their taste is having anti-plaque and many other pharmacological properties [7]. Most of these plants' species have antibacterial properties, good flavor, foaminess, hardness and a texture that is friendly on the teeth

and the supporting tissue. Freshly cut specimens are always preferable because they are easily chewed into a brush without scattering.

Dialium guineense also known as *Velvet tamarind* (or, black velvet), is a tall tropical, fruit bearing tree, it belongs to the family of Fabaceae and sub-family of Caesalpinioideae. *D. guineense* is a seasonal fruit, the stem is used as chewing stick, this stick contains saponin which adds cleaning effect to the teeth and at the same time removes plaques and caries on the teeth of users. In Nigeria, the twigs are used because they contain bioactive compounds that are made up of saponins, tannins, alkaloids and flavonoids. Chewing sticks from *Dialium guineense* when used are very efficient, effective, and reliable for cleaning teeth. The teeth of chewing stick users are usually devoid of dental plaque which makes their teeth strong, clean and fresh [8].

Psidium guajava (Family Myrtaceae) which is commonly known as guava is a tropical fruit cultivated in many tropical and sub-tropical regions. The plant is used traditionally for medicinal purposes and treatment of various human ailments. It is rich in antioxidants compounds and contains a high level of ascorbic acid. The important active constituents are saponins, tannins, flavonoids and alkaloids, these chemicals are responsible for their effectiveness when used as twig [9]. In Nigeria *Psidium guajava* chewing sticks are used in mechanical and chemical cleaning of oral tissue and it is efficient and effective. The teeth cleaned with *Psidium guajava* chewing sticks are usually devoid of tartar and other stains from the teeth, provide enamel barrier, whitens teeth, mineralize dental tissue, increase salivary flow, fresh and devoid of dental plaques and caries [10].

The present study was set to determine the antibacterial activities of *Psidium guajava* (guava) and *Velvet tamarin* (Icheke) chewing sticks on *Streptococcus mutans* isolated from human mouth.

2. Materials and Methods

2.1. Study Area

Owerri municipal is a local government area in Imo State, Nigeria. It has an estimated population of above 127,213 as of 2006 (National and State Population Census, 2006) and is approximately 58 square kilometres in an area with latitude and longitude of 5°28'34.7160"N and 7°1'33.0708"E. Its elevation is around 71 meters in height which is equal to 233 feet.

2.2. Ethical Approval

Scientific and Ethical permit/clearances were obtained from Medical Centre Federal Polytechnic Nekede in Owerri municipality. Written informed consent was given to parents or guardians of children that participated in the study.

2.3. Study Population

The study population includes human of all ages and sex. The saliva samples

from recruited participants were as a result of random sampling of the general population. Volunteers were recruited with their consent and in the case of children, the consent of their parents. This research was done between November and December.

2.4. Samples Collection

Saliva Samples was collected from a consenting adult volunteer at Federal medical Centre Owerri, Imo State. Criteria used in the collection of the saliva samples include:

- 1) The patient was told when to collect the saliva (which is from 8 - 10 a.m. if possible) and the subject was asked to refrain from drinking, eating and oral hygiene procedures for at least an hour prior to the collection.
- 2) The subject was given distilled drinking water and be asked to rinse their mouth well for a minute and then expectorate or swallow the water.
- 3) Five minutes after this oral rinse, the subject was asked to spit into a 2ml sterile tube. Encourage the subjects to place the tube on ice while collecting the saliva.
- 4) Approximately 2 ml volume of saliva was collected.
- 5) The specimen was returned to the laboratory immediately for processing. Processing was done within an hour window of time [11].

2.5. Isolation of the Test Bacterium

The media used for isolation of the organism is Mitis-salivarius Bacitracin (MSBA) and it was prepared according to the manufacturer's instruction.

A milliliter of each sample collected was spread on MS-agar plates using sterile spreading glass. Cultures were incubated anaerobically, using anaerobic candle jar, for 48 hrs. at 37°C.

2.6. Identification of Isolates

Colonies grown on MS-agar medium were spread on the Mitis-salivarius Bacitracin agar (MSBA) plates and incubated anaerobically for two days. Subcultures were repeated several times in order to obtain pure cultures. The isolates will also be identified and characterized using the following tests: gram staining, catalase and haemolysis tests [12] and [13]. Results will be compared to those of reference strain of the organism in Bergey's Manual of Determinative Bacteriology 9th, 1994.

2.7. Plant Twigs Collection and Identification

Local identification of the plants *Psidium guajava* (guava tree) and *Velvet tamarin* (Icheke tree) and collection of their twigs were done during field walks at Okwelle Irete Owerri, Imo State with the help of key informants and a guide. Taxonomical identification was done with the help of a botanist and voucher specimens stored at the Federal Polytechnic Nekede Owerri.

2.8. Extraction and Sterilization of Aqueous Extracts of the Plant Twigs

Preparation of extracts was done according to the method of (14), with a few modifications as follows: fresh twigs from *Psidium guajava* (guava tree) and Velvet tamarin (*Icheku* tree) was crushed using a wooden mortar and pestle and allowed to dry under shade for 3 weeks and a sterile manual grinder was used to crush the twigs into powder.

2.8.1. Aqueous Extraction of Plant Twigs

Aqueous extracts of the two plant twigs was prepared by adding 10 gm of each plant twig powder to 100 ml of deionized distilled water. The extraction process was allowed to boil for 30 minutes. Occasional shaking of extraction flasks was also done to facilitate the process. (In this method, it is assumed that the extraction process progresses until there is saturation of the aqueous phase). The different extractions were filtered to get extract of 10% concentration of each type of plant twig. Finally, the filtered extracts were sterilized by passing each through a bacterial membrane filter (0.45 μm pores, Ministart[®], Satorious, UK) under positive pressure. The filtrates were then labeled and stored under refrigeration (2°C) awaiting antibacterial tests.

2.8.2. Soxhlet Extraction

This was done using 95% ethanol in a Soxhlet apparatus for the two plants [15].

2.9. Phytochemical Analysis of *Psidium guajava* (Guava) and *Velvet tamarin* (Icheku)

The aqueous and soxhlet extracts obtained from the twig of the plant were subjected to phytochemical test using standard methods [15].

2.9.1. Test for Saponins (Frothing Test)

3 ml of each extract was shaken vigorously for about 5 min; it was allowed to stand for 30 sec and observed for frothing which is indicative of the presence of saponins.

2.9.2. Test for Tannins (Ferric Chloride Test)

2 drops of 5% FeCl_3 were added to 1 ml of the extract. A greenish precipitate indicated the presence of tannin in the four extracts.

2.9.3. Test for Glycosides

10 ml of 50% H_2SO_4 was added to 10 ml of each extracts in a test tube. The mixture was heated in boiling water for 15 minutes. 10 ml of Fehling's solution was added and the mixture was boiled. A brick red precipitate was observed in all the samples, showing presence of glycosides.

2.9.4. Test for Alkaloids

2 Drops of Mayer's reagent was added to 1 ml of each extract in a test tube and observed for a creamy precipitate indicative of the presence of alkaloid.

2.9.5. Test for Steroids (Salkowski's Test)

5 drops of concentrated H_2SO_4 was added to 1 ml of each extract. A red colouration was observed for each extract showing the presence of steroid.

2.9.6. Test for Flavonoids

1 ml of 10% NaOH was added to 3 ml of the extracts. A yellow colouration showed the presence of Flavonoids in each extract.

2.9.7. Test for Phlobatannins (Hydrochloric Acid Test)

2 ml of the extract was added to dilute hydrochloric acid and observed for a red precipitate formation that indicated the presence of phlobatannins.

2.10. Preparation of Culture Plates

Ampoule containing pure forms of *S. mutans* were obtained. Culture plates for *S. mutans* were prepared, by inoculating the content of the ampoule in nutrient agar at 37°C for 12 h. Growth obtained from agar plates was transferred to nutrient agar for testing the antimicrobial activity of the extracts and mouthwash.

2.11. Antimicrobial Susceptibility Testing

The disc diffusion (agar well) technique as described by (16) was adopted for this study to evaluate the antibacterial activity of the plant extracts. 0.2 ml aliquot of each of each of the extract was aseptically dropped into agar wells (of 6 millimetres in diameter) bored on already inoculated nutrient agar plates containing the test organism (*Streptococcus mutans*) and appropriately labelled. The nutrient agar plates were then incubated at 37°C for 24 hours for the development of zones of inhibition or its absence. The zones of inhibitions were measured with a meter rule.

2.12. Determination of Minimum Inhibitory Concentration (MIC)

For the MIC test, 2 g of each of the extract was dissolved in four millilitre (4 ml) of peptone water; this gives 500 mg/ml. Also, 0.8 g of the same exudate was placed in 4 ml of peptone water to obtain the concentration of 200 mg/ml. Thereafter, two fold serial dilutions was carried out from the 200 mg/ml concentration by transferring 2 ml of the 200 mg/ml concentration to 2 ml of peptone water contained in a test tube and homogenized properly. This procedure of transferring 2 ml of the tube to 2 ml of peptone water contained in the subsequent tubes was continued until the eighth tube. The following concentrations were thereafter obtained: 500 mg/ml, 250 mg/ml, 200 mg/ml, 100 mg/ml, 50 mg/ml, 25 mg/ml, 12.5 mg/ml, 6.25 mg/ml and 3.13 mg/ml. Having obtained the different concentrations and dilutions, three drops of overnight broth cultures of the test organisms were inoculated into the dilutions in each case of the test organisms [17]. The tubes were then incubated at 37°C for 24 hours. The lowest concentration of each of the exudates that inhibited the growth of the test organisms were recorded as the MIC.

2.13. Test for Minimum Bacteriocidal Concentration of the Extracts

Tubes showing no visible growth from the MIC test were sub-cultured onto sterile nutrient agar plates and incubated at 37°C for 24 hours. The lowest concentration of the extracts yielding no growths recorded as the MBC.

3. Results and Discussions

3.1. Identification of Isolate

Table 1 reported the isolation of *Streptococcus mutans* and the result is in line with the finding of Ryan and Ray, (2010) which states that *Streptococcus mutans* is a facultative anaerobic cocci-shaped, gram positive bacteria commonly found in the oral cavity and has a major role in tooth decay formation. Thus, *Streptococcus mutans* are considered the main causative microorganism associated with dental caries that plays a major role in tooth decay [18].

3.2. Phytochemical Analysis of the Plant Extracts

Phytochemical analysis of the plants extracts revealed the presence of tannins, saponins glycosides, alkaloid, steriods, and phenol (**Table 2**). These phytochemical constituents depicts the antimicrobial effects of the plants and these results support earlier findings on the efficacy of the plant extracts and also confirmed

Table 1. Cultural morphology and biochemical characteristics of the *Streptococcus mutans*.

Morphological Motility Characteristics Test	Gram Reaction	Oxidase Test	Catalase Test	Citrate Test	Coaguase Test	Motility Test
Milkish raised, non-mucoid colonies on MSBA with alpha haemolysis on Blood Agar	Gram positivecocci in chains	-	-	-	-	-

Key: - = Negative; + = Positive; MSBA = MitisSalivarius Bacitracin Agar.

Table 2. Phytochemical screening of the plants extracts.

Phytochemical	Extracts			
	G_e	I_e	G_{aq}	I_{aq}
Saponins	+	+	+	+
Tannins	+	+	-	+
Glycosides	+	+	-	+
Alkaloid	+	+	+	+
Steriods	+	+	+	+
Flavanoids	-	-	-	-
Phlobatannins	-	-	-	-

Keys: G_e = Ethanol (Soxhlet) extract of Guava twig; I_e = Ethanol (Soxhlet) extract of Icheku twig; G_{aq} = Aqueous extract of Guava twig; I_{aq} = Aqueous extract of Icheku twig; + = Present; - = Absent.

the rationale for the medicinal use of the studied plants [19]. The results from this study reveals that the ethanol extract of plants extracts contain more of the constituents when compared with the aqueous extracts.

3.3. Antibacterial Susceptibility Test

Table 3 shows the result of the antibacterial susceptibility testing of the extracts against *Streptococcus mutans*. The zones of inhibition recorded ranged from 18 mm to 27 mm. Aqueous extract of Icheku twig showed no zone of inhibition on the isolated organism indicating that it didn't inhibit its growth. The activity of the ethanol extract of guava and icheku twig was more pronounced (27 mm) than that of aqueous extract of Guava and Icheku twig (23 mm); this could be because of high active compounds present in the extract. The result in this work shows that there is variation in the degree of antibacterial activities of the extracts. The variation in the antibacterial activities is presumed to be due to difference in the quantity of compounds present in those plant extracts and the extracting solvents. Similar variations have been reported by [20] and [21].

3.4. Minimum Inhibitory Concentrations (MIC)

The Minimum Inhibitory Concentration (MIC) of the extracts as presented in **Table 4** shows that the aqueous extract of guava twig has the least inhibitory effect on the isolated organism followed by the ethanol (soxhlet) extract of guava and icheku twig (combined) where other extracts except the aqueous extract of icheku twig had the same inhibitory effect. The MIC ranged from 200 mg/ml to 500 mg/ml with the plants extracts. Aqueous extract of Icheku twig was not done because it showed no zone of inhibition on the isolated organism indicating that it didn't inhibit its growth. Similar variations have been reported by 20 and 21. This simply implies that the plant extracts is efficient in inhibiting visible microbial growth but the ethanol extract of guava, icheku and their combined form inhibits the organism at a lower concentration followed by the aqueous extract of guava and icheku and then the aqueous extract of Guava will come last.

Table 3. Antimicrobial susceptibility testing of the extracts against *Streptococcus mutans*.

Plants Extracts	Zone Diameter of Inhibition (mm)	Control	Zone Diameter of Inhibition (mm)
G_e	20		
GI_e	27	Chloramphenical	50
I_e	22		
G_{aq}	18		
I_{aq}	–		
GI_{aq}	23		

Keys: G_e = Ethanol (Soxhlet) extract of Guava twig; GI_e = Ethanol (Soxhlet) extract of Guava and Icheku twig; I_e = Ethanol (Soxhlet) extract of Icheku twig; G_{aq} = Aqueous extract of Guava twig; I_{aq} = Aqueous extract of Icheku twig; GI_{aq} = Aqueous extract of Guava and Icheku twig; – = No Zone.

Table 4. Minimum inhibitory concentrations (MIC) of the plant extracts against *Streptococcus mutans*.

Plants Extracts	MIC (mg/ml)
G_e	200
GI_e	200
I_e	200
G_{aq}	500
I_{aq}	N.D
GI_{aq}	250

Keys: G_e = Ethanol (Soxhlet) extract of Guava twig; GI_e = Ethanol (Soxhlet) extract of Guava and Icheku twig; I_e = Ethanol (Soxhlet) extract of Icheku twig; G_{aq} = Aqueous extract of Guava twig; I_{aq} = Aqueous extract of Icheku twig; GI_{aq} = Aqueous extract of Guava and Icheku twig; *N.D* = Not Done (Since no zone was recorded).

Table 5. Minimum bactericidal concentrations (MBC) of the plants extracts and mouth washes against *Streptococcus mutans*.

Mouth Washes	MBC (mg/ml)
G_e	200
GI_e	500
I_e	200
G_{aq}	500
I_{aq}	N.D
GI_{aq}	500

Keys: G_e = Ethanol (Soxhlet) extract of Guava twig; GI_e = Ethanol (Soxhlet) extract of Guava and Icheku twig; I_e = Ethanol (Soxhlet) extract of Icheku twig; G_{aq} = Aqueous extract of Guava twig; I_{aq} = Aqueous extract of Icheku twig; GI_{aq} = Aqueous extract of Guava and Icheku twig; *N.D* = Not Done (Since no zone was recorded).

3.5. Minimum Bactericidal Concentrations (MBC)

Table 5 shows the results of the minimum bactericidal concentrations of the plant extracts against *Streptococcus mutans*. The result showed that the ethanol (soxhlet) extract of the individual plants used in the study has a better bactericidal effect when compared to their aqueous extracts and combined forms. This implies that both the aqueous and ethanol extract of the plant extracts is efficient in inhibiting visible microbial growth and also kills the microbes.

4. Conclusion and Recommendations

Findings from this work support the use of *Psidium guajava* (Guava) and *Velvet tamarin* (Icheku) twigs in oral hygiene since their potential anti-plaque effect is likely to complement the mechanical plaque-removing property of chewing-sticks and suggests that *Psidium guajava* (Guava) and *Velvet tamarin* (Icheku) twigs contain compounds that are active against *S. mutans*, and merit further investigation as they are possible sources of cheap dental health care for the rural poor.

Conflicts of Interest

The authors declare no conflicts of interest regarding the publication of this paper.

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