Evaluation of the Antibacterial Properties of the Hydro-Ethanolic Leaf and Stembark of *Psidium guajava* (Myrtaceae) on Cariogenic and Periodontopathic Bacteria

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Authors' contributions

This work was carried out in collaboration among all authors. Authors FNA, AMA, FCN and FKPR designed the study, performed the statistical analysis, wrote the protocol and wrote the first draft of the manuscript. Authors TFE and PC managed the analyses of the study and the literature searches. All authors read and approved the final manuscript.

Original Research Article

ABSTRACT

Background: Caries and periodontal diseases are major public health problems. Due to increase in bacterial resistance to antibiotics, there is a need to promote the known potential of plants and search for new anti-infectious substances as therapeutic alternatives to antibiotics.
Aims: This study was aimed at evaluating the antibacterial property of the hydro-ethanolic leaf and stem bark extracts of *Psidium guajava* on cariogenic and periodontopathic bacteria.
Methodology: This was an experimental study that took place in the Laboratory of Chemistry and Microbiology (Clinique Universitaires des Montagne (CUM)) of Bangangté between January to June 2020. They were dried in shade for 3 weeks and ground to obtain a coarse powder. The dried

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powders were macerated for 72 hours in a water-ethanol mixture (30:70). Then, the phytochemical screening and quantification of the total polyphenol content followed. Microdilutions were used to determine the minimum inhibitory concentrations (MIC) of guava. Müller Hinton agar was used for obtaining the minimum bactericidal concentrations (MBC) and the determination of inhibition diameters of the bacteria.

Results: Extraction yields of 17.36g and 42.55g were obtained for leaf and stem bark, respectively. The phytochemical screening revealed the presence of various secondary metabolites among which the total phenol content was quantified at 236.1878 and 255.7682 mg equivalent of gallic acid per gram for leaf and stem bark, respectively. The carious bacteria isolated were: *Streptococcus mutans, Actinomycetes viscosus* and *Lactobacillus acidophilus.*While, the periodontal bacteria were: *Actinobacillus actinomycetemcomitans, Porphyromonas gingivalis, Prevotella intermedia, Fusobacterium nucleatum ,Tannerella forsythensis*. The results obtained showed that the combined extracts of leaf and stem bark had an activity higher than that of the stem bark which in turn had a higher activity than the leaves.

Conclusion: Non-invasive independent predictors for screening esophageal varices may decrease medical as well as financial burden, hence improving the management of cirrhotic patients. These predictors, however, need further work to validate reliability.

1. INTRODUCTION

Nature has been a source of medicinal agents for thousands of years. Recently, a lot of attention is being focused on producing medicines and products that are natural. Medicinal plants and minerals are major actors in traditional, complementary and alternative medicine (TCAM).They are being used in the prevention and treatment of human diseases; caries and periodontal diseases being major oral health problems [1].

WHO defines oral health as a state of being free from chronic mouth and facial pain, oral and throat cancer, oral infection, sores, gum diseases, tooth decay, tooth loss and other diseases and disorders that limit an individual's capacity in biting , chewing, smiling, and psychological wellbeing [2]. Oral health can be obtained by the practice of a good oral hygiene which includes proper brushing , flossing , use of interdental cleaners, tooth pastes, eating a balanced diet and regular dental check-up [2]. Whose absence exposes the individual to mouth odour and pathologies such as dental caries and periodontal diseases [2].

Dental caries are a major public health problem globally and is the most widespread non communicable disease according to WHO [2]. They are expensive to treat, consuming 5-10% of healthcare budget in high income countries [2].Tooth decay affects an estimated 60-90% of school children and nearly 100% of adults worldwide according to the WHO global oral health data base [3]. They have a multifactorial aetiology, it has been suggested to be triggered by 3 major factors; host susceptibility, convenient environment for bacteria growth and the type of microorganisms [4]. For instance, free sugars found in the diet are the essential dietary factors in the development of dental caries in susceptible host [2]. Bacterial such as *Streptococci mutans, Actinomycetes viscosus* and *Lactobacilli acidophilus* are well known cariogenic oral bacterial [4].

Periodontal diseases are infectious and inflammatory diseases of the tissues surrounding the teeth; gum, cementum, alveolodental ligament and alveolar bone. They have been recognised as a major health problem worldwide. It has a prevalence in industrialised countries 30-50% in adults [5]. In Africa, it has a prevalence of ;33% in Ghana, 27.5% in Nigeria, 30% in Senegal [6].Their initiation and progression are significantly related to the proliferation of certain pathogenic bacteria, bacterial toxins and the inflammatory response of host or substrate [7]. These bacteria present in dental plaque are mostly anaerobic bacterial among which *Actinobacillus actinomycetemcomitans (Aa), Porphyromonas gingivalis (Pg), Prevotella intermedia (Pi), and Tannerella forsythensis (Tf)* have shown to be associated with the onset and progression of periodontal diseases [8].

Although antibiotics are routinely used to prevent systemic infections originating from the oral cavity, they are not recommended for regular prevention of dental plaque formation because of

risk that bacteria will develop resistance to them [7]. However, the conventional treatment of these diseases includes mechanical debridement and the use of chemotherapeutic agents. Despite the successes registered by the conventional methods of treatment, effective treatment of these diseases are challenging due to: the inadequate supply of drugs, the prohibitive cost of treatments, the side effects of several allopathic drugs, and the development of resistance to currently used drugs due to self-medication and monotherapy in bacterial infection control [9]. In Cameroon, it is estimated that 3 out of 20 patients are able to buy prescribed drugs in hospitals [10]. In attempt to overcome the limitations of these conventional methods of treatment, the implementation of novel treatment inspired by nature has gained increasing interest [11].

In recent years, the use of plant extracts as well as other forms of medicinal treatment have resurfaced and gained popularity [7]. According to WHO, over three- quarters of the world population rely on plants and their extracts for healthcare needs [9]. Various natural products such as garlic , aloe vera and herbs have been used effectively to treat oral diseases in Ayurveda , in the form of mouth washes and tooth pastes [7]. *Psidium guajava* Linn (guava) is one of such plants that has been used to manage various systemic conditions, enhance oral hygiene and consists of bioactive substances [7]. Anterior studies done on the plant shows that it has antidiarrheal, antimicrobial, anti-parasitic, antitussive, antioxidant, anticancer and anti-hyperglycaemic effects [7]. Hence, the present study consist of evaluating the antibacterial properties of this plant on major carious and periodontal bacteria, specifically by identifying the secondary metabolites of this plant by chemical screening, then determine the MIC, the MBC and the MIC/MBC ratio so as to promote its use in the prevention and treatment of caries and periodontal diseases.

2. METHODS

This was an in-vitro experimental study that took place at the biochemistry and Microbiology laboratories of Université des Montagne's, teaching hospital or Clinique Universitaire des Montagne's (CUM) of Bangangté-Cameroon between Januarys to June 2020. The study was carried out on samples of carious lesions collected from 10 patients who presented with

dental caries and periodontal diseases at the University de Montagne's dental clinic. The patients selected were adults who presented in the clinic with obvious dental cavities and periodontal diseases, diagnosed and confirmed dental caries and periodontal diseases by a dentist. Patients on antibiotics, antibacterial mouthwashes and other forms of alternative medicines were excluded from the study.

2.1 Materials

The *Psidium guajava* leaf and stem bark required for this study was harvested from a matured guava tree in Bangangté in the Western region of Cameroon and was identified at the Cameroon National Herbarium and registered under the number 450228/HNC. The leaves and stem bark of *Psidium guajava* were dried at room temperature away from sun light (in shade) for 3 weeks. This action was conducted in order to preserve the structure of the constituents of the plant. Then the dried stem back and leaves were ground using a mechanical blender until a coarse powder.

The extracts were prepared by macerating the 334 g of dried guava leaves and 591g of dried guava stem bark in 1500ml of solvent using an ethanol: water (70/30, v/v) mixture at room temperature for 24h. Then, the supernatant was filtered using Whatman N°1 filter paper and the residue was further extracted twice by same process. After 72 h, the filtrate was concentrated in a rotary vacuum evaporator at 40°C for 3 days, respectively. The resulting filtrate clear and dark brown. It was stored in clean sealed bottles in a cool, dark place and protected from light.

2.2 Phytochemical Screening of Extracts

Chemical tests were carried out by using standard procedures to identify the preliminary phytochemical screening following the methodology of Harbone (1973) [12].

2.2.1 Test for flavonoids: Shinoda test (conc HCL+ Magnesium chips) [12]

5 ml of dilute ammonia solution was added to a portion of the hydroethanolic extract of each plant extract (2ml) followed by addition of concentrated H_2SO_4 (few drops). A yellow colouration observed in each extract indicated the presence of flavonoids. The yellow colouration disappeared on standing.

2.2.2 Test for phenols [12]

Inside a test tube, 5mg of extract is dissolve in 2ml ethanol, followed by the addition of three (3) drops of 10% (V/V) Iron chloride III. The appearance a greenish blue color indicated the presence of phenols.

2.2.3 Test for tannins [12]

0.5 g of each extract was introduced into 2ml of distilled water. The mixture was boiled in a water bath for 3 minutes. Then, 200µl of 0.1% ferric chloride was added. The appearance of a brownish green or a blue-black coloration indicated the presence of tannins.

2.2.4 Test for alkaloids: Mayer's test [12]

To a 5ml of each extract, a drop of Mayer's reagent was added by the side of the test tube. A creamy or white precipitate indicates the test is positive.

2.2.5 Test for coumarines [12]

3 ml of 10% NaOH was added to 2 ml of each extract. The formation of yellow colour indicates the presence of coumarins.

2.2.6 Test for saponins [12]

In a test tube containing 5ml of distilled water, 5mg of each extract was dissolved and then boiled for 5munites. After cooling, the contents of each test was agitated vertically and allowed to stand for 1 minute. The appearance of persistent foam of more than one centimeter indicates the presence of saponins.

2.2.7 Test for anthocyanins [12]

2 ml of aqueous extract is added to 2 ml of 2N HCl and ammonia. The appearance of pink- red to blue-violet indicates the presence of anthocyanins.

2.2.8 Test for terpenoids (Salkowski test) [12]

Five (5) ml of each extract was mixed in 2 ml of chloroform, and concentrated H_2SO_4 (3 ml) was carefully added to form a layer. A reddish brown colouration of the inter face was formed to show positive results for the presence of terpenoids.

2.3 Determination of Polyphenols by the Folin-Ciocalteu Method

The method used is Folin-Ciocalteu, described by Singleton and Rossi. This method is based on the reduction in alkaline medium of the phosphotungstic and phosphomolybdic mixture of Folin reagent by reducing groups of phenolic compounds, leading to the formation of products of blue colour reduction whose intensity is proportional to the amount of polyphenols present in the sample. These have an absorption maximum at 765nm [13].

Procedure: 200 µl of Folin (10 fold dilutions) was added to 100 µl of each extract. After 2 min, 1ml of Sodium carbonate solution (20%) was added. After 30 min of incubation at room temperature and in the dark, the absorbance of the mixture was read at 765 nm. The optical density (OD) values were considered to be compliant if for a correlation coefficient R^2 was as close as possible to 1 [13].

The quantification of the total polyphenol content was done in function of a linear calibrated curve (y = a x + b) realised by the standard "gallic acid" at different concentrations (25-125 mg/l) in the same conditions as the standard. The results were expressed in milligrams equivalent of gallic acid per gram of extract (mg EQ/g extract).

2.4 Collection of Bacteria Samples

2.4.1 Collection of carious bacteria

The collection of carious bacteria was done after diagnosis of the disease. The detection of the carious disease was done by visual examination and tactile sensation [14]. The diseased tooth after diagnosis was cleaned, air dried and isolated using salivary cotton rolls. The sampling was carried out using a sterile dental excavator and immediately discharged into a tube containing the medium of transportation. The transport medium used was the brain heart infusion broth.

2.4.2 Collection of periodontal bacteria

The choice of the site of the sampling was made after the analysis of the usual signs of the disease: bleeding of the surrounding gingiva, loss of attachment, and pocket depth [15].

Sample was taken at least two hours after any food, beverage or brushing. The supra gingival plaque was removed with curettes, cotton rolls, and sterile saline. After cleaning, the selected

site was isolated from saliva using salivary cotton roll and air dried. A sterile paper point was introduced into the pocket until resistance was felt using sterile dental tweezers. After 10 to 20 seconds the paper point was removed. The tip was introduced into tube containing medium of transportation (BHI).

The collected carious and periodontal samples were placed in a transport medium. The transport medium used was the brain heart infusion broth. In order to minimize oxygenation of samples collected, mineral oil was added into the tubes containing brain heart broth.

2.5 Culture and Bacteria Identification

Once in the laboratory, the bacteria samples found in the transport media (brain heart infusion) were mixed using a vortex. The samples collected from the 10 patients were then seeded into the agar plates containing the culture media (fresh blood agar, MRS agar) using an inoculating loop (platinum wire). The seeding method used was streaking. Streaking was done by making back and forth movements in a zigzag manner using an inoculating loop. The loop was sterilized from one bacteria sample to another by passing it over a Bursen burner flame.

Given that bacteria responsible for periodontal diseases are strict anaerobes, samples collected in the periodontal pockets were inoculated and placed in anaerobic jars. Carious bacteria being facultative anaerobes didn't require anaerobic jars. The bacteria samples were then incubated at 37°C for 24 hours.

After 24 hours incubation time, the bacteria colonies present were then sub cultured by transferring some cells from the previous culture to a fresh culture media. These fresh culture media were then incubated at 37°C for 24 hours to obtain pure colonies. We then proceeded to identify the bacteria found in the pure colonies. Bacteria identification as done following standard methods such as Gram staining [16] and biochemical tests.

2.6 Identification of Bacteria [16]

2.6.1 Macroscopic test

The macroscopic test was done be appreciating, the size, shape, structure, and coloration of the colonies on the culture media.

2.6.2 Microscopic test

Gram tests were carried out on samples which permitted to identify the size and the shape of the bacteria. They were categorized according to their shape (rod-shaped, coccus shaped, or cocco-bacilli), and how they Gram stained (Gram positive or Gram negative). This was seen under a light microscope at objective 100 with immersion oil.

2.7 Gram Staining Technique [16]

Gram staining procedure divided the bacteria samples into two groups based on their cell wall composition: Gram positive and Gram negative. The process was as follows:

Fixation of the clinical material (bacteria samples) to the surface of microscopic slide was done by heating. It was followed by application of the primary stain (crystal violet). Crystal violet was allowed to remain on the slide for 60 seconds. It stained cells purple. Then Crystal violet on slide was washed off using distilled water. The slide was then flooded with Gram's iodine solution and allowed to remain on slide for 60 seconds. Slide was washed off using distilled water. The slide was then decolorized with alcohol for 10 seconds. It was rinsed immediately using distilled water. The slide was counter stained with safarin for 60 seconds. It was finally rinsed with distilled water and allowed to air dry.

The bacteria were considered Gram -positive when purple-stained, or Gram- negative when pink stained.

2.8 Biochemical Test

2.8.1 Catalase test

This test was used to identify organisms that produce the enzyme catalase. This enzyme deoxidises hydrogen peroxide by breaking it down into water and oxygen gas. A small amount of bacteria was brought in contact with a drop of hydrogen peroxide. A positive result was indicated by the presence of bubbles resulting from the production of oxygen gas [16].

2.8.2 Mannitol motility agar test

This is a differential medium, used to identify organisms that are capable of using up mannitol

as a food source by producing acidic by-products of fermentation that lowered the pH of the media. A positive result was shown by the red phenol turning yellow. It was also used to determine whether the organisms are motile by use of flagella. A positive test was seen by the entire tube being turbid, indicating that the organisms are motile [16].

2.9 Kliger's Iron Agar (KIA)

This is a differential medium. It tested for organisms' abilities to ferment glucose and lactose to acid and acid gas end products. It also permitted the identification of sulphur reducers. The first differential ingredient was glucose. Organisms capable of fermenting this sugar used it up and liberated acidic by products that turned phenol red indicator yellow. When lactose was the organisms' food source, the fermentation of this sugar produces acid by products and the media remained yellow. Gas produced as a result of glucose or lactose fermentation, was revealed by the presence of fissures in the agar. KIA tubes also detected the production of hydrogen sulphide, seen as a black precipitate [16].

2.10 Gelatinase Hydrolysis Test

This test was used to determine bacterial production of gelatinase enzymes that liquefy gelatin. Gelatine stripes were immersed in saline solution containing bacterial samples. A positive result was observed when the gelatin liquefied, while a negative result showed no liquefaction [16].

2.11 Evaluation of Antibacterial Activity of *Psidium guajava* Leaf and Stem Bark

2.11.1 Preparation of bacteria inoculums

18-24 hours old colonies of each bacteria strain after identification was collected using a platinum loop. They were then introduced into 5ml of sterile physiological saline (NaCl 9/1000) and the turbidity was adjusted to that of the 0.5 Mc Farland scale, corresponding to the concentration of 1.5×10^8 Colony Forming Units / ml (CFU / mL). This was done near a Bursen burner flame to avoid contaminations [17].

2.11.2 Susceptibility test

The antibacterial activity was carried out using disc-diffusion method. Petri dishes containing MHA of 60mm were inoculated with the bacteria inoculum containing 1.5×10^8 Colony Forming Units / ml (CFU / mL) using a platinum loop. Sterile 6mm discs were placed on the surface of the medium. A concentration of 100mg/ml of leaf extract, stem bark extract and a combination of both extracts were prepared. 15µl of each plant extract was placed on the each sterile disc. The plates were then incubated for 24 hours at 37°C, priory placing plates containing periodontal bacteria in anaerobic jars. The zone of inhibition was recorded in millimetres (mm) [18].

2.12 Sensitivity Test

2.12.1 Determination of the minimum inhibitory concentration (MIC)

2.12.1.1 Operating mode

The minimum inhibitory concentration (MIC) of the extracts was determined using broth dilution technique. A stock solution was prepared at 100mg/ml of each crude extract (leaves, stem bark and a combination of both). Two fold serial dilutions of the extracts were prepared by adding 2ml of 100mg/ml of the extract into the first test tube containing 2ml of Mueller Hinton broth, thus producing solution containing 50mg/ml of the extract. The process continued serially up to test tube No. 10, hence producing the following concentrations; 50, 25, 12.5, 6.25, 3.125, 1.56, 0.78, 0.39, 0.195, 0.097 mg/ml. 15µl of 0.5 ml of 10^8 McFarland equivalent standards of test organisms were introduced into the test tubes and incubated at 37°C for 24 hours. After incubation the test tubes were observed for growth by checking for turbidity. The experiment was conducted in triplicate [18].

2.12.2 Determination of minimal bactericidal concentrations (MBC)

2.12.2.1 Operating mode

From each tube that did not show visible growth (those at or above MIC), the contents were streaked onto Mueller Hinton agar plates using an inoculating loop. The plates were then incubated at 37°C for 24 hours. The MBC was recorded as the lowest concentration of the extract that had less than 99% growth on the agar plates.

2.12.3 Evaluation of the ratios MBC / MIC

The MBC/MIC ratios confirm the bacteriostatic or bactericidal nature of a substance. When these ratios are greater than or equal to 4, the substance is said to be bacteriostatic; if these ratios are less than 4, the substance is considered bactericidal. If they are equal to 1, then it is said to be "absolute bactericidal" [17].

2.12.4 Measurement of the inhibition diameters

The measurement of the inhibition diameters using the solid medium diffusion method was performed from the MIC and MBC concentrations for each strain. This test was performed on each strain (8) thrice.

2.12.5 Procedure

Sterile disc 6mm in diameter cut from Whatman paper n°1, impregnated with 15µl of the different extracts of known concentration solutions (MICs and MBCs), were gently deposited on the surface of Mueller Hinton agar previously seeded by streaking with bacterial inoculum using a platinum loop. Simultaneously, an amoxicillin antibiotic disc (30µg) was deposited. Upon application the different extracts and amoxicillin diffuse from the disc in a uniform way in the agar. After 15 minutes at room temperature followed by incubation in the oven at 37°C for 18 to 24 hours, the disks were surrounded by circular zones of inhibition corresponding to an absence of culture. The negative control consisted of a disc impregnated with MHB only [17].

2.13 Statistical Analysis

Data was captured into Microsoft excel spreadsheet, analyzed in SPSS and presented in the form of tables and figures. Bivariate analysis (t- test) was carried out to compare the inhibitory diameter of the hydro-ethanolic extracts of the leaf, stem bark and combination of leaf and stembark to that of amoxicilline. Descriptive and inferential statistics such as unpaired t-test and ANOVA were employed to compare between the groups. $P < 0.05$ was considered statistically significant.

3. RESULTS AND DISCUSSION

3.1 Results

3.1.1 Extraction yield

The yield of the hydroethanolic (30:70) extracts of leaves and stem bark of guava were 17.39g and 42.5g respectively.

3.1.2 Phytochemical screening

The results of the chemical screening of *Psidium guajava* leaves and stem bark shows the presence of metabolites such as alkaloids, saponins, phenols, flavonoids, antraquinones, terpernoids, coumarines and tannins. are more abundant than others. Further details are presented in Table 1.

3.1.3 Quantification of total polyphenol content

The method of quantification by Folin-Cioecalteu described by SINGLETON and ROSSI was validated by the verification of the linearity and repeatability. A linear regression curve was plotted using the regression equation $y=0.0286x + 3E-05$ $R^2 = 0.9778$

From the gallic acid calibration range, the total polyphenol content of 236.187 and 255.7682 mg of gallic acid equivalent per gram of crude extract was obtained for the leaves and stem bark of *Psidium guajava* respectively.

3.1.4 Identification of the bacteria samples

The identification the carious and periodontal bacteria used for the study are summarised in Tables 2 and 3.

Table 1. Phytochemical constituents of *Psidium guajava* leaf and stem bark extracts

S	Phytochemicals	Leaf extract	Stem bark extract
1	Alkaloids	+	++
2	Saponin	++	+++
3	Phenol	++	+++
4	Flavonoids	++	+++
5	Antraquinone	+	+
6	Terpenoid	+	++
7	Coumarines	+	+
8	Tannin	++	+++

Key: +++ = abundant,++= fairly present,+= mildly present -- = absent

3.1.5 Identification of carious bacteria

The diagnosis of the clinical assessment of patients revealed patients presenting with dental caries and complication of dental caries such as acute and chronic pulpitis. The identification of the carious bacteria samples based on their macroscopic characteristics, microscopic characteristics and using standard biochemical test is presented in Table 2.

The identification reveals that carious bacteria are Gram-positive immobile bacteria. They test negative to catalase and gelatinase and positive to mannitol and Kligger.

3.1.6 Identification of periodontal bacteria

The diagnosis of the patients revealed patients presenting with cases such as localised and generalised gingivitis and chronic periodontitis. The identification of periodontal bacteria samples based on their macroscopic characteristics, microscopic characteristics and using standard biochemical test is presented in Table 3.

The identification of the periodontal bacteria reveals that, they are immobile Gram-negative bacteria. They test positive to mannitol and Kligger. They test negative to calatase and gelatinase except *Fusobacterium nucleatum* which is gelatinase positive.

3.1.7 Antibacterial activity of *Psidium guajava*

Minimum inhibitory concentration (MIC), minimum bactericidal concentration (MBC) and the MBC/MIC ratio.

Table 2. Morphological and biochemical identification of Carious bacteria

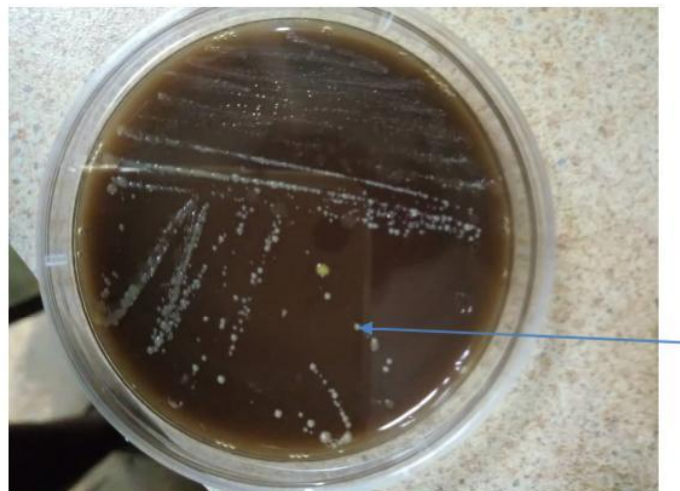|	Parameters	Sample1	Sample2	Sample3
Macroscopic Characteristics:	Form	Ovoid	Round	Round
	Colour	Whitish	Yellowish	Whitish
Microscopic Characteristics:	Mobility	Immobile	Immobile	Immobile
	Gram	Positive	Positive	Positive
	Form	Coccus	Rod-shaped	Rod-shaped
	Morphology	Long chains	Isolated	Isolated
Biochemical Tests	Catalase	Negative	Negative	Negative
	Gelatinase	Negative	Negative	Negative
	Mannitol	Positive	Positive	Positive
	Kligger	Positive	Positive	Positive
Bacteria:		*Streptococcus mutans*	*Actionomyces viscosus*	*Lactobacillus acidophilus*

Fig. 1. Arrow points at Lactobacillus acidophilus on MRS agar

Table 3. Morphological and biochemical identification of periodontal bacteria

	Parameters	Sample1	Sample2	Sample3	Sample4	Sample5
Macroscopic	Form	Round	star-shaped	circular	Round	Round
Characteristics:	Colour	deep red	Whitish	Violet	Grey	Yellowish
Microscopic	Mobility	Immobile	Immobile	immobile	Immobile	Immobile
Characteristics:	Gram	Negative	Negative	negative	Negative	Negative
	Form	Rods	Coccobacillus	Rods	Rods	Rods
	morphology	Chains	Chains	isolated	Isolated	Chains
	Catalase	Negative	Negative	negative	Negative	Negative
Biochemical	gelatinase	Negative	Negative	positive	Negative	Negative
Tests:	Mannitol	Positive	Positive	positive	Positive	Positive
	Kligger	Positive	Positive	positive	Positive	Positive
Bacteria:		*P.g*	*A.a*	*F.n*	*P.i*	*T.f*

Key: P.g: Porphyromonas gingivalis, A.a: Aggregatibacter Actinomycemcomitan, F.n: Fusobacterium nucleatum, P.i: Prevotella intermedia, T.f: Tannerella forsythia

Fig. 2. Fusobacterium nucleatum on columbia agar

Table 4. Determination of the MIC, MBC and the MBC/MIC ratio of carious and periodontal bacteria on the leaves extract of *Psidium guajava*

Bacteria	MIC (mg/ml)	MBC (mg/ml)	MBC/MIC
Streptococcus mutans	12.5±0	12.5±0	1
Actinomycetes viscosus	12.5±0	25±0	2
Lactobacillus acidophilus	12.5±0	25±0	2
Porphyromonas gingivalis	12.5±0	50±0	4
Aggregatibacter actinomycemcomitans	12.5±0	50±0	4
Fusobacterium nucleatum	12.5±0	50±0	4
Prevotella intermedia	12.5±0	50±0	4
Tannerella forsythia	12.5±0	50±0	4

The values of the minimum inhibitory concentration (MIC), the minimum bactericidal concentrations (MBC) and the ratio MBC/MIC as well as the standard deviation of the hydroethanolic extracts of the leaves, stem bark and the combination of leaves and stem bark of *Psidium guajava* on the carious and periodontal bacteria strains are presented in the Tables 4, 5 and 6 respectively.

Leaves: The MIC of the leaf extract is 12.5mg/ml and MBC ranges from 12.5 to 50mg/ml for all the

bacteria isolates. *Streptococcus mutans* is the most susceptible of the bacteria with MIC and MBC of 12.5mg/ml. Its MBC/MIC ratio of 1 reveals that it is absolutely bactericidal. *Actinomycetes viscosus* and *Lactobacillus acidophilus* have both have MBC/MIC ratio of 2 revealing bactericidal to the leaf extract.

Stem bark: The MIC of the stem bark extracts of *Psidium guajava* ranged from 6.25 to 12.5mg/ml, while the MBC vary from 6.25 to 50mg/ml for the bacteria isolated. The MBC/MIC ratio of 1 and 2 reveal a bactericidal activity of the stem bark extract on *S. mutans, A. viscosus* and *L.acidophilus* respectively.

Table 5. Determination of the MIC, MBC and the MBC/MIC ratio of carious and periodontal bacteria on the stem bark extract of *Psidium guajava*

Bacteria	MIC (mg/ml)	MBC (mg/ml)	MBC/MIC
Streptococcus mutans	6.25±0	6.25±0	1
Actinomycetes viscosus	6.25±0	12.5±0	2
Lactobacillus acidophilus	6.25±0	12.5±0	2
Porphyromonas gingivalis	12.5±0	50±0	4
Aggregatibacter Actinomycemcomitans	6.25±0	25±0	4
Fusobacterium nucleatum	6.25±0	25±0	4
Prevotella intermedia	6.25±0	25±0	4
Tannerella forsythia	6.25±0	25±0	4

Table 6. Determination of the MIC, MBC and the MBC/MIC ratio of carious bacteria on the combination of leaves and stem bark extract of *Psidium guajava*

Bacteria	MIC (mg/ml)	MBC (mg/ml)	MBC/MIC
Streptococcus mutans	3.125±0	3.125±0	1
Actinomycetes viscosus	6.25±0	6.25±0	1
Lactobacillus acidophilus	6.25±0	6.25±0	1
Porphyromonas gingivalis	6.25±0	12.5±0	2
Aggregatibacter actinomycemcomitans	3.125±0	6.25±0	2
Fusobacterium nucleatum	3.125±0	6.25±0	2
Prevotella intermedia	6.25±0	6.25±0	1
Tannerella forsythia	6.25±0	6.25±0	1

3.1.8 Combination of Leaves and Stem Bark

The combination of the leaves and stem bark extracts of *Psidium guajava* varied from 3.125 to 6.25mg/ml for MIC. MBC values ranged from 3.125 to 12.5mg/ml for the bacteria isolated. The MBC/MIC ratio of 1 and 2 reveals an absolute bactericidal activity and a bactericidal activity of the combined extract on the isolated bacteria samples.

Determination of the inhibition diameters of MIC, MBC and amoxicillin: The values of the inhibition diameters of the minimum inhibitory concentrations (MIC), minimum bactericidal

concentrations (MBC) of the leaves, stem bark and an combination of leave and stem bark of *Psidium guajava*, amoxicillin and distilled water (positive control) on carious and periodontal bacteria are presented in Tables 7, 8 and 9.

Leaves: *Streptococcus mutans and Lactobacillus acidophilus* are more susceptible to the leaves extract of *Psidium guajava*. The values range from 12 to14.5 mm for MIC and 13 to 15 mm for MBC of the leaves extract depending on the bacteria strain. The activity registered by amoxicillin (8-11 mm) is lower than that obtained by the stem bark extract.

Table 7. Determination of the inhibition diameters of the MIC, MBC of the leaves extract of *Psidium guajava*, MBC of amoxicillin and distilled water on carious and periodontal bacteria

Bacteria strains	MIC (mm)	MBC (mm)	MBCAX (mm)	DW (mm)
Streptococcus mutans	14.5±0.7	14.5±0.7	8.00±0	0±00
Actinomycetes viscosus	14±1.4	14.5±0.7	8.00±0	0±00
Lactobacillus acidophilus	14.5±0.7	15±0	11±0	0±00
Porphyromonas gingivalis	12±0.7	13±0	8.00±0	0±00
Aggregatibacter Actinomycemcomitans	13±0	13.5±0.7	8.00±0	0±00
Fusobacterium nucleatum	12±0.7	13±0	8.00±0	0±00
Prevotella intermedia	12.5±0	13±0.7	8.00±0	0±00
Tannerella forsythia	12±0.7	13±0	8.00±0	0±00

Key: Ax: amoxicillin, DW: distilled water

Table 8. Determination of the inhibition diameters of the MIC, MBC of the stem bark extract of *Psidium guajava*, MBC of amoxicillin and distilled water on carious and periodontal bacteria

Bacteria strains	MIC (mm)	MBC (mm)	MBC Ax (mm)	DW(mm)
Streptococcus mutans	15.5±0.7	15.5±0.7	8.00±0	0±00
Actinomycetes viscosus	14.5±0.7	15±0	8.00±0	0±00
Lactobacillus acidophilus	15.5±0.7	16.5±0	12±0	0±00
Porphyromonas gingivalis	12.5±0.7	13.5±0	8.00±0	0±00
Aggregatibacter actinomycemcomitans	14±0	14.5±0.7	8.00±0	0±00
Fusobacterium nucleatum	12.5±0.7	14±0	8.00±0	0±00
Prevotella intermedia	13±0	13.5±0.7	8.00±0	0±00
Tannerella forsythia	13±0.7	13.5±0	8.00±0	0±00

Key: Ax: amoxicillin, DW: distilled water

Table 9. Determination of the inhibition diameters of the MIC, MBC of the combination of leaves and stem bark extract of *Psidium guajava*, MBC of amoxicillin and distilled water on carious and periodontal bacteria

Bacteria strains	MIC (mm)	MBC (mm)	MBC Ax (mm)	Dw (mm)
Streptococcus mutans	16±00	16.5±00	8.0±00	0±00
Actinomycetes viscosus	15±00	15.5±00	8.0±00	0±00
Lactobacillus acidophilus	16±0.7	17±0.7	12.0±00	0±00
Porphyromonas gingivalis	15.5±0.7	16±0	8.00±0	0±00
Aggregatibacter actinomycemcomitans	17±0	18±0.7	8.00±0	0±00
Fusobacterium nucleatum	15±0.7	16.5±0	8.00±0	0±00
Prevotella intermedia	16±0	16.5±0.7	8.00±0	0±00
Tannerella forsythia	15.5±0.7	16±0	8.00±0	0±00

Key: Ax: amoxicillin, DW: distilled water

Stem bark: *Streptococcus mutans* and *Lactobacillus acidophilus* are more susceptible to the stem bark extract of *Psidium guajava.* The values vary from 12.5to15.5mm for MIC and 13.5 to 16.5 for MBC of the stem bark extract depending on the bacteria strain. The activity registered by Amoxicillin (8-12) is lower than those obtained from the stem bark extract.

Combination of leaves and stem bark: *Aggregatibacter actinomycemcomitans* is the most susceptible to the combined leaf and stem bark extract of *Psidium guajava.* The values of MIC range between 15 to 17mm. The MBC values from 15.5 to 18mm depending on the bacteria strain. The values of amoxicillin (8.0-12.0 mm) are lower than those obtained from the combined extract.

3.2 Discussion

The present study was conducted to evaluate the antibacterial property of the hydroethanolic extracts of the leaf and stem bark of *Psidium guajava* on cariogenic and periodontopathic bacteria. The choice of the plant material was based on its traditional usage in the treatment of oral infections [9,19].

The yield obtained from the maceration of 334 Grams of leaves and 591 Grams of stem bark of *Psidium guajava* was 17.36 and 42.55 respectively. Mohammed et al in 2017, obtained an extraction yield of 1.12 and 5.00 for chloroform and water respectively [20]. A number of factors influenced the yield. A study carried out in India by Velavan et al in 2015 [12] stipulated that the yield of extracts is influenced by different parameters such as the type of solvent. It demonstrated that the hydroethanolic extraction (30:70) has an optimal yield in the extraction of hydro soluble secondary metabolites. This motivated the choice of the solvent used in this study.

Preliminary phytochemical screening of leaf and stem back extract of *P. guajava* showed the presence of alkaloids, saponin, phenol, flavonoids, antraquinones, terpenoid, coumarines, tannins. These findings can be attested to the work of and Abdullah MS et al in Nigeria [18] who also reported similar finding on the phytochemical of guava leaf and stem bark.

The quantification of the total polyphenol content of *P. guajava* leaf and stem bark showed the hydro-ethanolic extract contained 236.1878 and 255.7682 mg equivalent of gallic acid per Gram for the leaf and stem bark respectively. Various studies carried out Nigeria by Ojezele Matthew et al in 2013 and Chibuke Ibe et al in 2014 showed that the antibacterial property of guava is due to the presence of polyphenols [21,22]. Results obtained shows the polyphenol content in the stem bark is higher than those present in the leaves.

The analyses of the antibacterial activity of the hydroethanolic extract of the leaf and stem bark of *P. guajava* reveals that it possesses antibacterial properties. Anterior studies done on the plant shows that it has antibacterial properties due to the presence of terpenoids, saponins and phenolic compounds such as: tanins and flavonoids [1,19]. The antibacterial activity of the hydroethanolic extract could then be associated with the presence of these metabolites. For example, tannins are polyphenolic compounds that bind to proline rich protein that interferes with protein synthesis and has shown to have antibacterial activity. Flavonoids are hydroxylated polyphenolic compounds produced by plants in response to microbial infections to which this aspect has been extensively studied and found to have antimicrobial activity against an array of microorganisms *in vitro*. Their ability has been attributed to their ability to form complexes with extracellular soluble proteins and bacterial cell walls. Terpenoids although mainly used for their aromatic qualities have also been found to be potential agents against inhibiting bacteria. Saponins are glycosides have been found to have inhibitory effects on Gram-positive organism. These groups of secondary metabolites were found in the extracts but one could not rely only on them to explain all the results obtained. Nor is it obvious to anticipate the nature of the interactions that could exist between polyphenols in general, the identified metabolites and those that were not investigated in this study [1].

Antibacterial activity of the hydroethanolic extract of leaf and stem bark of *Psidium guajava* was evaluated by well agar diffusion method. The extracts showed activity at different concentrations on all bacteria isolates. For the carious bacteria samples isolated (*Streptococcus mutans, Actinomyces viscosus, Lactobacillus acidophilus*), the leaves had an MIC of 12.5 mg/ml and MBC ranging from 12.5 – 25 mg/ml. The stem bark had an MIC of 6.25 mg/ml and MBC ranging from 6.25-12.5 mg/ml. Based on this results, the leaf and stem bark of Ps*idium guajava* showed very low concentration of MIC and MBC. This finding are in conformity with those gotten by Abdullah MS et al in Nigeria in 2019 who stated that *Psidium guajava* leaf and stem bark shows very low concentrations of MIC(6.25-12.5 mg/ml) and MBC(12.5-50mg/ml) [18]. The results of the present study revealed that the stem bark possessed higher antibacterial activity than corresponding leaf extract. This holds true with the results of Abdullah MS et al in Nigeria in 2019 [18]. Elekwa et al, also obtained similar results, stating that the stem bark has a higher antibacterial activity than the leaf [18].

The evaluation of the MBC/MIC ratio of the carious bacteria isolated revealed values of 1 for *Streptococcus mutans* and 2 for *Actionomyces viscosus* and *Lactobacillus acidophilus* for both

leaf and stem bark extract of the hydroethanolic extract of *Psidium guajava*. According to Bonnet et al, when these ratios are greater than or equal to 4, the substance is said to be bacteriostatic, if these ratios are less than 4, the substance is considered bactericidal. If they are equal to 1, then it is said to be "absolute bactericidal [17]. Using this finding, *Psidium guajava* leaf and stem bark extract is said to be bactericidal to *Actionomyces viscosus* and *Lactobacillus acidophilus* since MBC/MIC ratio is 2 and absolutely bactericidal to *Streptococcus mutans* with MBC/MIC ratio 1.

Based on the susceptibility of the carious bacteria isolates to the extracts, the leaves had inhibition diameters ranging from 14 to 15mm, while the values of the stem bark ranged from 14.5-16.5mm for both MIC and MBC. According to Moreira et al in 2005 [23], the susceptibility of the bacteria to the various extracts is classified according to the diameter of the inhibition zones as follows: non-susceptible (-) for the diameter of less than 8 mm, susceptible (+) for a diameter included between 8-13.9 mm, very susceptible (++) for a diameter between 14-19 mm and extremely susceptible (+++) for the diameter of more than 19 mm. The results obtained revealed that the carious bacteria isolated are very susceptible to the leaf and stem bark extracts at different concentration of MIC and MBC. The stem bark revealed to be more efficient than the leaves. For both extracts, *Lactobacillus acidophilus* was the most susceptible of the carious bacteria. *Actionomyces viscosus* was the least susceptible of the carious bacterial isolated.

The periodontal samples isolated using standard macroscopic and biochemical procedures were: *Porphyromonas gingivalis, Aggregatibacter* actinomycemcomitans*, Fusobacterium nucleatum, Prevotella intermedia* and *Tannerella forsythia.* The extracts showed activity at different concentrations on all bacteria isolates. For the leaf extract, the value of the MIC was 12.5mg/ml, while the MBC was 50mg/ml for all periodontal bacterial isolated. The stem bark on the other hand MIC values ranged from 6.25mg/ml (*A. actinomycemcomitans, F. nucleatum, P. intermedia* and *T. forsythia)* to 12.5mg/ml (*P. gingivalis)* and MBC values ranged from 25mg/ml (*A. actinomycemcomitans, F. nucleatum, P. intermedia* and *T.* forsythia*) to 50mg/ml *(P. gingivalis).* The MBC/MIC ratio was 4 for all the periodontal bacteria isolated on both samples. According to Bonnet and al, [17]

presented above, *Psidium guajava* leaf and stem bark are bacteriostatic on all the periodontal bacteria isolated. This is in conformity with results obtained by Reddy and al [7] who stated that guava extracts showed bacteriostatic activity on *P. gingivalis* and *A. actinomycemcomitans.* The results of this present study revealed that the values of MIC and MBC were lower in *A. actinomycemcomitans, F. nucleatum, P. intermedia* and *T. forsythia* compared to that of *P. gingivalis.* Reddy and al obtained similar results in 2018, which reported that MIC values of guava extract were lower in *A. actinomycemcomitans* compared with *P. gingivalis* [7].

Based on the susceptibility of the leaf and stem bark extracts on the periodontal bacteria isolated, the leaves had an inhibition diameter of 12mm (*P. gingivalis, F. nucleatum, T. forsythia*),12.5mm (*P. intermedia*) and 13mm (*A.* actinomycemcomitans) for MIC and 13mm (*P. gingivalis, F. nucleatum, P. intermedia, T. forsythia*) to13.5mm(*A. actinomycemcomitans*) for MBC. While the stem bark had values of 12.5mm (*P. gingivalis, F. nucleatum*), 13mm (*P. intermedia, T. forsythia*) and 14mm (*A. actinomycemcomitans*) for MIC and 13.5 mm (*P. gingivalis, P. intermedia, T. forsythia*), 14mm (*F. nucleatum*) and 14;5mm (*A. actinomycemcomitans*) for MBC. According to the classification of inhibition diameters by Moriera and al [23] presented above, the results obtained reveals that all the periodontal bacteria are susceptible to the leaf extract for both MIC and MBC, while *Porphyromonas gingivalis, Fusobacterium nucleatum, Prevotella intermedia, Tannerella forsythia* are susceptible , *Aggregatibacter actinomycemcomitans* is very susceptible to the stem bark at MIC. At MBC, *Porphyromonas gingivalis, Prevotella intermedia, Tannerella forsythia* are susceptible, while, *Fusobacterium nucleatum* and *Aggregatibacter actinomycemcomitans* are very susceptible to the stem bark. The present study could affirm *Aggregatibacter actinomycemcomitans* to be the most susceptible periodontal bacterial. This goes in line with results obtained by Reddy and al in 2018 in India [7] who concluded that *Aggregatibacter actinomycemcomitans* is more susceptible to guava extracts than *Porphyromonas gingivalis*.[7].

Comparatively, the leaf and stem bark extracts of Psi*dium guajava* from the present study are said to be bactericidal to cariogenic bacteria and bacteriostatic to periodontal bacteria. This

difference in action could be attributed to the cell wall nature since cariogenic bacteria are Gram-positive and periodontal bacteria, Gram-negative. Gram-negative bacteria have an effective permeability barrier, comprised of a thin lipopolysaccharide exterior membrane, which could restrict the penetration of the extruding plant extract. It has been reported that Gram-negative bacteria are usually more resistant to the plant-origin antimicrobials and even show no effect, compared to Gram-positive bacteria which have a mesh-like peptidoglycan layer which is more accessible to permeation by the extracts [1]. Results found in this study were supported in the data reported in literature. Naseer and al in 2018 reported that the antibacterial activity of guava is high against Gram positive bacteria and moderate against the Gram negative bacterial strains [24]. This result is also supported by the findings of Abdullah MS,[18] which revealed that Gram positive bacteria is more susceptible to the leaf extract than Gram negative bacteria.

The antibacterial activity of the combination of *Psidium guajava* leaf and stem bark was evaluated on the carious and periodontal bacterial strains isolated. For the carious bacteria strains, the values of 3.125mg/ml (*Streptococcus mutans*) and 6.25mg/ml (*Actinomyces* viscosus, *Lactobacillus acidophilus* were obtained for both MIC and MBC. The values obtained revealed that the combination of the leaf and stem bark shows very low concentrations of MIC and MBC. This values are lower than those obtained from the leaf and stem bark separately. The MBC/MIC ratio obtained for all the carious bacteria was 1. According to Bonnet and al [17] this reveals an absolute bactericidal nature of the extract on the bacteria isolates. For the periodontal bacteria isolated the values MIC ranged from 3.125mg/ml (*Aggregatibacter actinomycemcomitans*, *Fusobacterium nucleatum*) to 6.25mg/ml (*Porphyromonas gingivalis*, *Prevotella intermedia*, *Tannerella forsythia*) while the MBC ranged from 6.25mg/ml *(A. actinomycemcomitans, F. nucleatum, P. intermedia* and *T. forsythia)* to12.5mg/ml *(P. gingivalis).* The MBC/MIC obtained was 2 *(P. gingivalis, A. actinomycemcomitans, F. nucleatum)* and 1 (*P. intermedia, T. forsythia).* According to Bonnet and al [17] this reveals a bactericidal nature of the combined extracts on *P. gingivalis, A. actinomycemcomitans, F. nucleatum* and an absolute bactericidal nature of the combined extracts on *P. intermedia, T. forsythia.* Thus, the antibacterial activity of the

combined extracts is more efficient than those of the stem bark and leaves separately.

Based on the susceptibility of the combined extracts on the bacteria isolated, the inhibition diameters of the carious bacteria ranged from 15mm to 17mm for both MIC and MBC. While for the periodontal bacterial, the values ranged from 15mm to 18mm for MIC and MBC. According to the classification of inhibition diameters by Moriera and al [23] presented above the inhibition range of 15 -19mm is said to be very susceptible. Hence, the carious and periodontal bacteria isolated are said to be very susceptible to the combined extracts since values range from 15-18mm.

The inhibition diameter registered by the MBC of amoxicillin on the carious bacteria was 8mm for *Streptococcus mutans* and *Actionomyces viscosus* and 11mm for *Lactobacillus acidophilus*, while on the periodontal bacteria an inhibition diameter of 8mm was obtained for all the periodontal isolates. According to Bonnet and al the inhibition diameters of amoxicillin is said to be susceptible with values ≥21mm and resistant with values <16mm [17]. Based on this, the periodontal and carious bacteria isolated are said to be resistant to amoxicillin, given that values are far lower than 21mm (susceptibility value). This resistance could be likened to the abusive prescription of antibiotics, patients not finishing the entire antibiotic course, self-medication by patients and poor infection control in health care settings by the use of monotherapy in the treatment of oral infections [9,10].

Conclusively, the present study was carried to evaluate the antibacterial activity of the hydroethanolic extract of leaf and stem bark of *Psidium guajava* on carious and periodontopathic bacteria. Based on the results obtained and discussed above, the combined extractd of the leaf and stem bark had a higher antibacterial activity than the stem bark which in turn has a higher antibacterial activity than the leaves on the carious and periodontal bacteria isolated.

4. CONCLUSION

The chemical screening of *Psidium guajava* leaf and stem bark showed the plant contains a variety of secondary metabolites such as flavonoids, phenols, quinones, coumarines, tannins, saponins, alcaloides and terponides. The quantification of the total polyphenol content

showed that the stem bark contains more polyphenols than the leaves.

The bacteria were identified by standard microscopic and biochemical test. The carious bacteria samples isolated were: *Streptococcus mutans, Actinomycetes viscosus* and *Lactobacillus acidophilus*. While the periodontal bacteria isolates were: *Actinobacillus actinomycetemcomitans, Porphyromonas gingivalis, Prevotella intermedia, Fusobacterium nucleatum ,Tannerella forsythensis).*

The evaluation of the antibacterial activity of our various extracts by determination of MIC, MBC and the MBC/MIC ratio revealed that *Psidium guajava* exhibited bacteriostatic, bactericidal and absolute bactericidal activities on carious and periodontopathic bacteria.

The determination of the inhibition diameters of these extracts compared to that of the referential antibiotic; amoxicillin was carried out on the tested strains. The hydroethanolic leaf and stem bark extract of *Psidium guajava* had an antibacterial activity clearly superior to that of amoxicillin.

The results obtained showed that the combined extracts of leaf and stem bark had an activity higher than that of the stem bark which in turn had a higher activity than the leaves. The leaf, stem bark and the combined extract of this plant may be a potential source of antibacterial agents and could be used to alleviate the problem of increasing resistance to antibiotics.

5. RECOMMENDATIONS

Multispecies and toxicity studies should be carried and studies on the toxicity of propolis should be carried out.

In vivo studies should be carried out.

The impact of *Psidium* guajava mouth rinse in plaque content and deposition should be studied.

CONSENT

As per international standard, patient's written consent has been collected and preserved by the author(s).

ETHICAL APPROVAL

It is not applicable.

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

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