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Phytochemical Investigation and Anti-Microbial Activity of *Clausena anisata* on Cariogenic and Periodontopathic Bacteria

Ashu Michael Agbor[1*], Kuete Pierre Fotsing[2], Suzy Hermine Dountio[1], Ntungwen Charles Fokunang[3] and Sudeshni Naidoo[4]

[1]Université des Montagnes, Dental School, Bangangté, Cameroon.
[2]Faculty of health and biomedical sciences, Université des Montagnes, Cameroon.
[3]Department of Toxicology, Faculty of health and biomedical sciences, University of Yaoundé, Cameroon.
[4]Department of Community Dentistry, University of the Western Cape, South Africa.

Authors' contributions

This work was carried out in collaboration among all authors. Authors AMA, KPF, NCF and SN contributed to the design of the study as well as acquisition of data, its analysis and interpretation and was involved in the drafting of the manuscript. Author SN made substantial contributions to the conception and design and in the drafting and revision of the manuscript. All authors read and approved the final manuscript.

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ABSTRACT

Clausen aanisata is used in African traditional medicine to treat a variety of infectious and parasitic diseases. Infectious diseases are a major public health problem especially in developing countries where resistance to commonly used antibiotics is growing exponentially. The objective of this study was to evaluate the antibacterial activity of *Clausena anisata* on cariogenic and periodonthopatic bacteria. This was a laboratory experimental study using macerated hydroethanolic extracts of the

**Corresponding author: E-mail: agborasm@yahoo.com;*

stem and leaves of *Clausena anisata* in a water-ethanol mixture (30:70) for 48 hours. Phytochemical screening revealed flavonoids, tannins, alkaloids, coumarins, phenols, triterpenes and saponosides, these being more abundant in the leaves. The antibacterial activity of the hydroethanolic extracts was evaluated against different clinically isolated strains of three Gram negative *(Aggregatibacter actinomycetemcomitan, Fusobacterium nucleatum and Prevotella intermedia)* and two Gram positive (Streptococcus mutans and Latobacillusspp) bacteria. Polyphenols found more in the leaves extract was estimated at 62.8 mgTAE/g against 36.8 mgTAE/g in the stem bark extract. The study revealed that the leaves extract had bactericidal and bacteriostatic activity on isolated strains of *Aggregatibacter actinomycetemcomitan* and *Fusobacterium nucleatum* being the most susceptible with MICs of 50 mg/ml whereas the stem bark showed bacteriostatic activity on all isolated strains with *Aggregatibacter actinomycetemcomitan* being the most susceptible with MIC of 50 mg/ml. The antimicrobial action of the plant could be exploited in formulation of mouth washes and other medications in the management of oral infections like periodontitis and tooth dacay.

Keywords: Phytochemical analysis; antibacterial activities; Clausena anisata; leaves; stem bark; hydroethanolic extract.

1. INTRODUCTION

Clausina anisata is a shrub that grows in Africa forest used in African traditional medicine to treat a vast number of infections including parasitic infections like flatworm infestations, taeniasis and schistosomiasis. It has been reported to be used for eye complaints, influenza, respiratory ailments, heart disorders, hypertension, abdominal cramps, constipation, gastro-enteritis, hepatic diseases causing bad breath; malaria, fevers and pyrexia, boils, rheumatism, arthritis, other inflammatory conditions like headaches, body pains [1]. In cattles it is used in the management of toothaches, swollen gums, convulsions and some other mental disorders; impotence and sterility; blood tonic; dysentery in cattle [1].

Periodontal diseases and dental caries are the two most prevalent oral infections affecting mankind worldwide [2, 3]. Bacterial plaque is the main culprits of dental caries and periodontal diseases. Effective prevention of these infections can be achieved by mechanical removal of dental plaque by proper tooth brushing and flossing. However, the majority of the population particularly aged individuals may not perform mechanical plaque removal sufficiently and thus antimicrobial mouth rinses such as Triclosan and Chlorhexidine may be used to limit these two plaque-related oral infections [2].

There are a large variety of antibiotics and antiseptics against these dental germs, although antibiotics are used routinely to prevent systemic infections originating from the oral cavity, they are not recommended for regular prevention of dental plaque formation because of the risk that bacteria will develop resistance to them [2].

Traditional medicine is commonly used by various populations around the world. In Europe the rate of traditional medicine visits varies from one country to another. Plants which form the bases of African traditional medicine have been reported to treat many oral health infections [3-6].

Among these medicinal plants is the genus *Clausena (Rutaceae),* represented by 20 species in India. *C. anisata* is a forest undergrowth shrub commonly called 'Horsewood' or the more descriptive Africans common name 'Perdepis'. Recent studies have shown that different species of this plant (*Cymbopogon citratus, Clausena anisata, Ocimum gratissimum, Ocimum basilicum, Lippia multifora, Eugenia caryophyllata* and *Menthapi perita*) have antimicrobial activity against many oral bacteria [2].

A review of literature shows a death on the study on antibacterial activity of *C. anisata* against oral pathologies in Cameroon. The aims of this study was to carry out a chemical characterization of hydroethanolic extracts of *C anisata* leaves and to evaluate its in vitro antibacterial activity on cariogenic and periodontopathic oral microorganisms.

2. METHODOLOGY

This was an experimental study carried out between December 2018 to June 2019 at the Chemistry Laboratory of Université des Montagnes where the plant extraction process

and phytochemical analysis was performed and Laboratory of Microbiology, Université des Montagnes Teaching Hospital or Cliniques Universitaires des Montagnes (CUM) where the antibacterial activities were tested.

Included in the study were patients with dental caries and periodontitis consulting at the Dental Clinic of the "Cliniques Universitaires des Montagnes" (CUM) who gave their consent. Patient on antimicrobial treatment, mouthwash, traditional medicine were excluded. A total of 10 patients were recruited in the study.

2.1 Study Materia

The *Clausena anisata* plant was harvested from Wabane, a locality of Magha, department of Lebialem, South West Region of Cameroon and was identified at the Cameroon National Herberium and registered under the number N°19 846/SRFCam.

The bacterial strain used were isolated from patients who came to consult at the Dental Clinic at Université des Montagnes.

2.2 Drying and Grinding of Vegetable Material

The leaves and stem bark of *Clausena ansata* harvested were dried at room temperature and protected from light for about two weeks for the leaves and one month for the stem bark. This action was conducted to conserve the chemical constituents of the plant. The leaves and bark were ground into fine powder by means of an electric grinder.

2.3 Preparation of the Plant Extracts

The plant extract was obtained by macerating 300 g of leaves powder in 2000 ml 30:70 hydroalcoholic solution and 500 g of stem bark powder in 3000 m of 30:70 hydroalcoholic solution for 48 hours. The macerate obtained was filtered with a Whatman N°1 filter paper. The filtrate obtained was concentrated by a rotary evaporator at 35°C and then dried in an oven at 55°C for 48 hours to obtain the raw extracts.

The steps followed in the laboratory analyses of the plant extracts are represented in Fig. 1.

The masses of the hydro-ethanolic extracts obtained were determined and the yield calculated with respect to the initial masses according to the formular below.

$$\text{Yield } = \frac{M\ ass\ of\ dry\ extract}{M\ ass\ of\ powder} X\ 100$$

2.4 Phytochemical Screening of the Plant

Phytochemical screening chemical tests were carried out on hydro-ethanoic extract of *C. anisata* using standard procedures to identify the constituents as described by Harborne, Sofowora and Trease [7-9] as follows.

Flavonoids: To 4ml of the hydro-alcoholic extract (2ml of extract+2ml of Methanol) put in a test tube, we added 1ml of concentrated hydrochloric acid (HCL) and some magnesium chips. A staining according to the type of genin indicates the presence of flavenoids. Flavones: orange, Flavenones: red cherry, Flavanols: red purplish

Phenols: Inside a test tube, 5mg of extract was dissolved in 1ml ethanol. Then three [3] drops of 10% (V/V) Iron chloride III were added. The appearance of greenish blue color characterized the presence of phenols.

Tannins: 0.5 g of extract was introduced into 2ml of distilled water, the mixture was then heated in a water bath for 3minutes. 200µl of 1% ferric chloride was added. The presence of tannins was characterized by the development of a greenish or blue-black coloring.

Alkaloids. We searched by precipitation reaction (Dragendroff). To 0.1g of powder we added 1ml of 1% chloridric acid and then we introduced 2 drops of Dragendroff reagent (potassium tetraiodobismutate). The appearance of an orange-red precipitate that comes off at the surface of the solution in the test tube characterized the presence of alkaloids.

Coumarin. In a test tube 0.1 g of the extract was dissolved into 2 ml of distilled water, then 2 ml of sodium hydroxide added. The appearance of a fluorescence blue or greenish yellow characterized the presence of coumarin.

Saponoside. We introduced 5 mg of extract into a test tube containing 5 ml of distilled water, and then boil in a water bath for 5 munites, after which we allowed cooling, then the test tube was shaken vertically for 5seconds and then allowed standing. The appearance of persistent foam of height of more than one centimeter indicates the presence of saponosides.

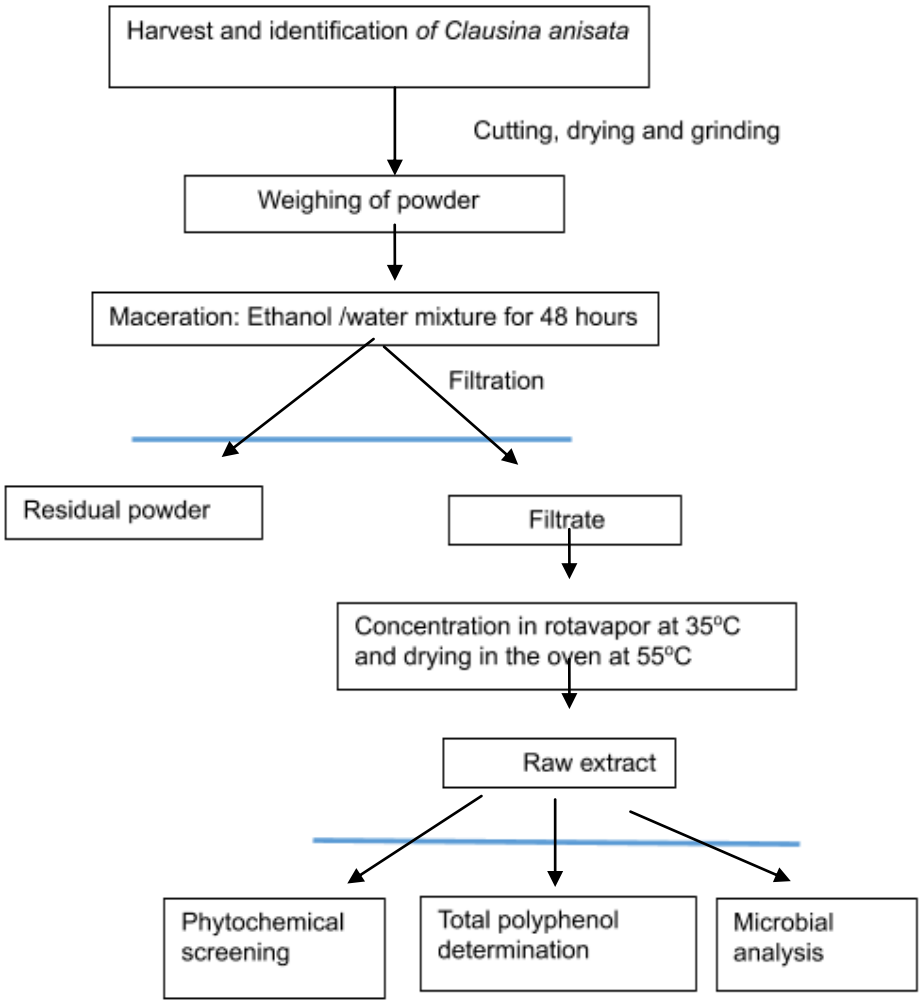

Fig. 1. Laboratoty analyses sequence of the plant extracts

Legend: CALE = Clausenaanisataleaves extract, CASE = Clausenaanisatastem bark extract

Triterpenes. To 0.25 g of extract, we introduced 1.5ml of chloroform and then we slowly added a few drops of concentrated sulfuric acid. The appearance of a reddish-brown stain at the interface of two phases indicates the presence of triterpenes.

2.5 Determination of Total Polyphenols

The method used was the Folin-Ciocalteu, described by Singleton and Rossi [10]. It is validated by checking the repeatability and linearity. Repeatability was performed by preparing the calibration range three (03) times under the same conditions and we determined the optical densities for each of the concentrations. For Linearity, tannic acid calibration line was obtained by linear regression of optical densities made on three measurements for concentrations between 0.025-0.125 g / L. According to Singleton and Rossi [10], 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 color reduction whose intensity is proportional to the amount of polyphenols present in the sample. These have an absorption maximum at 760 nm.

The Procedure is as follows. A stock solution of tannic acid was prepared by dissolving 125mg of acid in distilled water in sufficient quantity for 10 ml. This stock solution was then diluted with distilled water to obtained 5 daugther solutions of concentrations ranging from 0.025 to 0.125

mg/ml. From the daugther's solution, 100 µl of solution was taken separately and introduced in equivalent test tubes. After which 500 µl of Folin-Ciocalteu reagent (10 times diluted in distilled water) was added. It was mixed and left to stand for 2 minutes then 20% sodium carbonate NaCO3 was added, in order to trigger the oxidation-reduction reaction. The mixture was kept in the dark for 30 minutes at room temperature. The density of each solution was obtained by reading on a spectrophotometer at 760nm against a blank (Table 1).

These tests were performed thrice. From the results of the 3 (three) essays the graph of the calibration curve was plotted. The optical density (OD) values were considered to be compliant if a correlation coefficient R2 was as close as possible to 1 [11].

Determination of total polyohenols in the various extract. The solutions of the different samples to be assayed and the standard were prepared in the same way and under the same condition. Dosages were made thrice, and the concentrations were expressed as mean and were obtained from the calibration straight line obtained after validation of the analysis technique. Concentrations were therefore expressed in milligrams of tannic acid equivalent per gram of raw extracts.

2.6 *In Vitro* Evaluation of the Antibacterial Activity of *Clausena anisata* Plant

2.6.1 Sample collection of periodontal bacteria

This was carried out on patients with clinical evidence of active periodontitis, assessed and confirmed by 2 clinicians for consistency in diagnoses. The choice of the sampling site was made after analysis of the usual signs of the disease (inflammation, bleeding of the surrounding, loss of attachment, pocket depth). After considering these different clinical parameters, the sampling site was chosen from a quadrant having at least a pocket depth ≥4mm.

The supragingival plaque was first removed with curettes, the sulcus dried with gauze and air jet and normal saline on light pressure. After cleaning, the selected site was isolated from saliva by salivary cotton rolls and air- dried. Using a sterile college tweezer, a sterile absorbent paper point was introduced into the pocket or gingival sulcus. After 10 to 20 seconds the tip was carefully removed from the sulcus and the oral cavity making sure it doesn't come in contact with saliva, pus or oral mucosa. The sample was inserted into a tube containing the medium of transportation in an anaerobic chamber.

2.6.2 Sampling collection of cariogenic bacteria

After identification of the diseased tooth, it was cleaned, dried and isolated using salivary cottons rolls. A sterile dental excavator was used to collect carious dentine and immediately discharged into a tube containing the medium of transportation. The transport medium used was the brain heart broth to avoid a large oxygenation and the samples were treated within 24 hours.

2.7 Bacterial Culture

Once in the laboratory, the samples were first shaken by a voltex after which anaerobiosis was created near a bunsen burner by introducing few drops of mineral oil in the tubes containing the samples, then the samples were seeded on culture medium (chocolate agar) and bacterial growth was carried out at 37°C in anaerobic condition for 24hours.

Table 1. Determination steps of polyphenols according to Singleton and Rossi

	T0	T1	T2	T3	T4	T5	Sample 1	Sample 2
Tannic acid vol(ml)	0	0.4	0.8	1.2	1.6	2	-	-
Volume of distilled water(ml)	2	1.6	1.2	0.8	0.4	0		
Conc of tannic (mg/l)	0	25	50	75	100	125	-	-
Conc of sample (mg/ml)	-	-	-	-	-	-	1.4	3.2
Take 100µl of each solution								
Taking (µl)	100	100	100	100	100	100	100	100
Folin-Ciocaiteu diluted 10 times (µl)	500	500	500	500	500	500	500	500
Incubation for 2 min								
Sodium carbonate (20%) (µl)	2	2	2	2	2	2	2	2
Incubation 30 min in the dark absorbance 760 nm								

Since most of the pathogens responsible for periodontal diseases are anaerobic, it was necessary to grow them in an enclosure creating their living environment and for cariogenic pathogens. We also grew them in the same conditions since most of them are facultative aerobes that grow well in anaerobic condition. Anaerobiosis was obtained under the jar for a culture time of 24 hours at 37°C.

A first culture of isolation of the bacteria of the different samples was performed. Then a transplantation on chocolate agar was carried out with isolated bacteria to get only pure cultures. These cloned bacteria were used for identification tests.

2.7.1 Identification of bacteria

A simple microscopic examination of the unfixed preparation (mobile or immobile, aspect of colonies) where the morphologic characteristic of the bacterial present on the agar was identified. The preparation was then stained with standard stains such as Gram stain. Microscopically, Gram negative bacteria appeared in pink whereas those with Gram positive showed a violet color.

The information collected on the size and morphology of colonies and their pigmentation was used to confirm the type of microorganisms. The diagnosis was confirmed by the use of standardized test for each family of bacteria. Catalase test and oxidase test were used to characterise the microorganisms.

2.7.2 Antibacterial tests

The antibacterial activity was evaluated by looking for three parameters that are the MIC, MBC and the inhibition diameter of the different extracts and an antibiotic (Amoxicillin) as a control. The choice of Amoxicillin was made for it usual use in dental practice.

2.7.3 Preparation of the plant extracts

The stock solutions of the different extracts of our plant were prepared at concentrations of 800mg/ml for the leaves and stem bark, as dilution solvent we used distilled water.

2.7.4 Preparation of bacteria inoculums

Near the flame of a bunsen burner, for each bacterial strain, some colonies of 18 to 24 hours of life were collected using a platinum loop. They were then introduced into a certain volume of sterile physiological saline (Nacl 9/1000) up to obtaining a turbidity similar to that of 0.5 Mc Farland scale, corresponding to the concentration of 1.5 × 108 Colony Forming Units / ml (CFU /mL) [35]. Sensitivity test.

2.8 Determination of the Minimum Inhibitory Concentration of C anisata

Minimum Inhibitory Concentration (MIC) was determined as the lowest concentration of *C. anisata* extract which inhibited the growth of the tested microorganisms. The minimum inhibitory concentration (MIC) of *C. anisata* was determined using the broth dilution method. A stock solution was prepared at 800 mg/ml of each crude extract. Next, 2 ml of each extract at 800mg/ml was introduced into the first tube of the dilution range. For this a series of tubes were prepared with broth to which various concentrations of the *C. anisata* extracts were added viz., 0 mg/ml (negative control) ,0.78 mg/ml , 1.56 mg/ml, 6.25 mg/ml, 12.5 mg/ml, 25 mg/ml, 100 mg/ml, 200 mg/ml, 400 mg/ml. The antibiotic gentamycin was taken as positive control (.08 µg/ml). Then, 15 µL of bacterial inoculum was added to each test tube and incubated at 37°C. the tests tubes were examined and MIC was determined. All sets were read visually and MIC values were recorded as the lowest concentration of the *C anisata* that had no visible turbidity (Fig. 2).

2.9 Determination of Minimum Bactericidal Concentrations (MBC)

The minimum bactericidal concentration (MBC) is the minimum concentration corresponding to the lowest concentration of substance capable of killing more than 99.9% of bacterial inoculum (i.e less than 0.1% of survivors) after 18 to 24 hours of incubation at a temperature of 37°C. Thus, their determination is based on the subculture from the MIC on an agar medium.

In each of the tubes lacking a bacterial pellet (unobserved visible growth) and the controls for the determination of the MIC were streaked on Mueller Hinton agar on Petri dishes. The petri dishes were incubated for 18 to 24 hours at 37 ° C. The MBC of each extract was deduced from the lowest concentration at which no culture was observed on Mueller Hinton agar [15]. The operation was repeated three times (Fig. 3).

2.10 Evaluation of the Ratios MBC / MIC

These ratios make it possible to confirm the bacteriostatic or bactericidal character of a substance. When these ratios are greater than or equal to 4, the substance is said bacteriostatic; if these ratios are less than 4, the substance is considered bactericidal. If they are equal to 1, then it is called "absolute bactericidal".

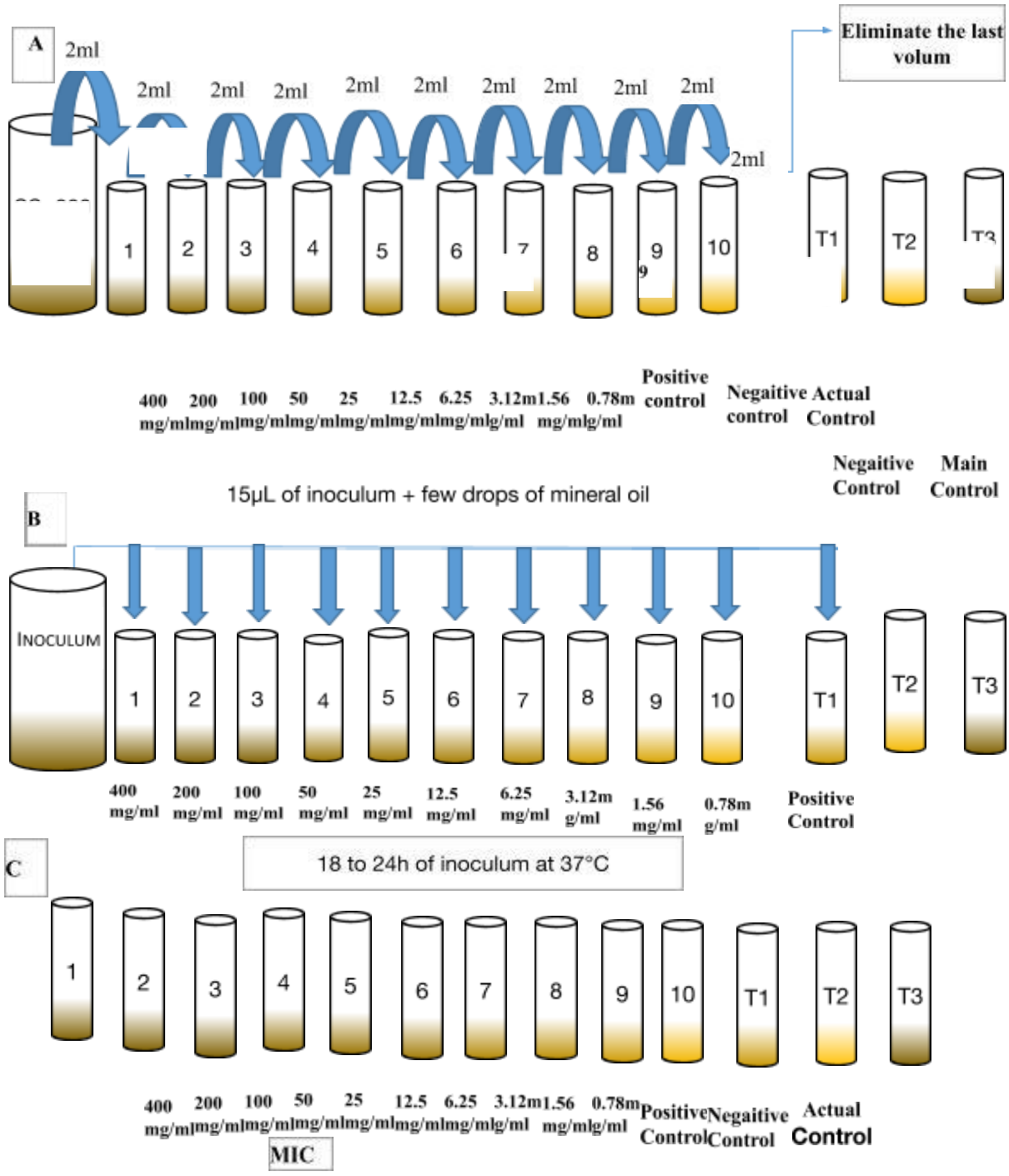

Fig. 2. Method of determining the MIC

SS = stock solution, Positive Control: broth + inoculum, Negative Control: simple broth, Actual Control: broth + extract

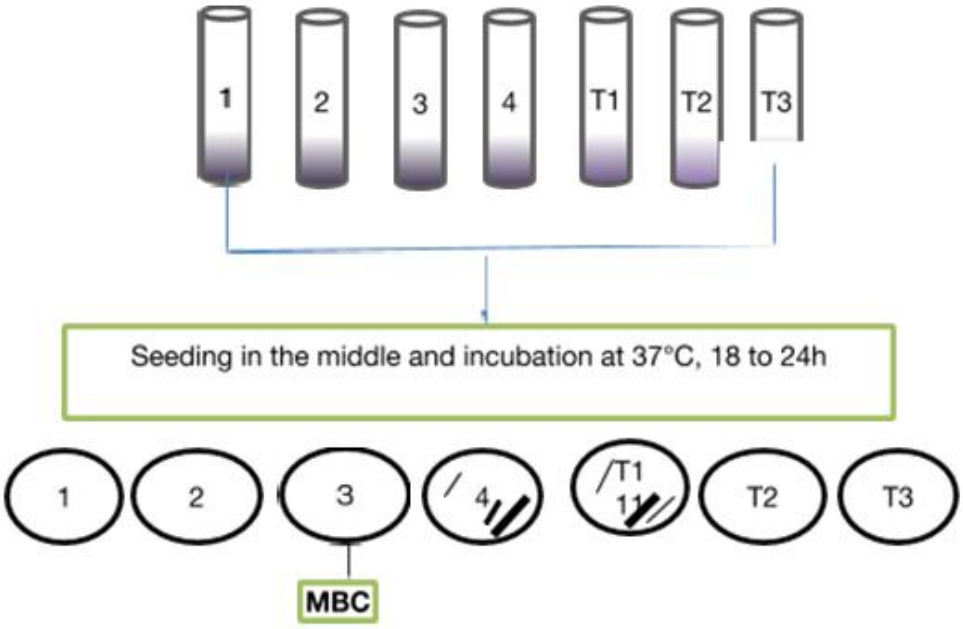

Fig. 3. Illustrates the technique described above.
T_1: broth + inoculum, T_2: simple broth, T_3: broth + extract

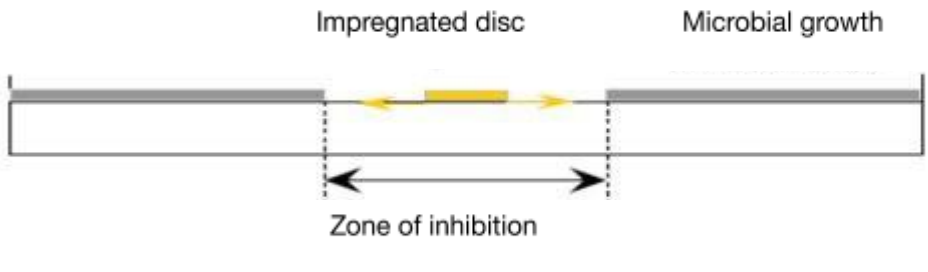

Fig. 4. Illustration of sensitivity test

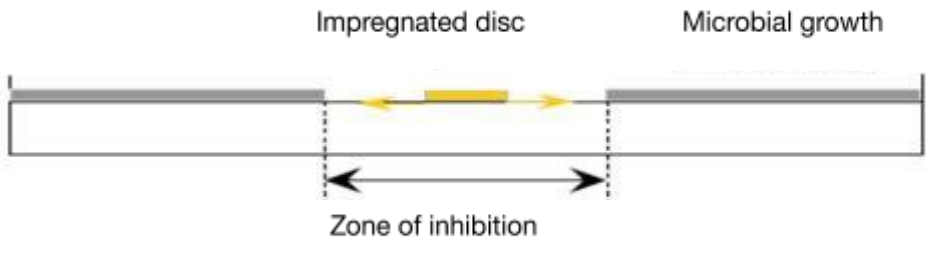

Fig. 5. Illustration of sensitivity test

2.11 Measurement of Inhibition Diameters

Antimicrobial Assay Antibacterial activity was determined by the well diffusion method according to the NCCLS [11]. The test organisms were inoculated on Mueller Hinton agar plates and spread uniformly to form a lawn. Wells (5 mm diameter) were cut into the agar. The measurement of the inhibition diameters by the solid medium diffusion method was performed from the MIC and MBC concentrations for each strain. This test was performed on each strain

thrice. Sterile disc of 6mm in diameter cut from Whatman paper n°1, impregnated with 15 µl of the different extracts of known concentration solutions (MICs and MBCs) were gently deposited on the surface of Mueller Hinton agar previously seeded by swabbing with bacterial inoculum. An Amoxicillin antibiotic disc (30 µg) was deposited. Upon application, disc of 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 (Fig. 4).

After incubation, the inhibition diameter was measured in millimetres using a graduated ruler, the diameter of each disk included. This test was repeated three times for each strain. According to Moreira et al. [36], sensitivity to different extracts was classified according to diameter of the inhibition zones as follows:

- Not sensitive (-) or resistant: diameter <8 mm,
- Sensitive (+): diameter between 8 – 13.9mm,
- Very sensitive (++): diameter between 14 to19mm.
- Extremely sensitive (+++): diameter > 19 mm (Fig. 5).

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3. RESULTS

3.1 Extraction Yield

The yield of the leaves were 1.4 times higher than that of the stem bark extract (Table 2).

Table 2. Extraction yield of hydro-alcoholic extracts

Plant part	Mass of powder(g)	Mass of dry extract obtained(g)	Yield (%)
Leaves	300g	22.6g	7.5
Stem bark	500g	26.18g	5.3

Table 3. Phytochemical constituents of *Clausena anisata* hydroethanolic extracts

Secondrymetaboites	CALE	CASE
Phenols	+++	+++
Tannins	+++	+
Triterpenes	+++	+++
Flavenoids	++	+
Coumarines	+++	++
Alkaloids	++	+++
Saponosides	+++	++

Legend: CALE: Clausena anisata leaves extrac, CASE: Clausena anisata stem bark extract, +++: High concentration,++: Moderate concentration, +: Low concentration.

3.2 Phytochemical Screening

Clausena anisata leaves extract (CALE) was found to contain moderate amounts of Flavonoids and Alkaloids, but large amounts of Saponosides, Coumarins, Tannins, Triterpenes and Phenols, whereas *Clausena anisata* stem bark extract (CASE) possessed trace amount of Flavonoids and Tannins, moderate amounts of Coumarins and Saponosides but large amounts of Triterpenes, Alkaloids and Phenols (Table 3).

3.3 Total Polyphenols

The repeatability and linearity of the extracts showed that the mean optical density was most elevated (0.233) at a concentration of 0.025 g/l (Table 4).

3.4 Linearity

From the results of the 3 (three) essays the graph of the calibration curve was plotted. The quantitative evaluation of tannic acid shows a positive correlation between the change in tannic acid concentration (0.025-0.125 g / L). The equation of the line is $Y = 0.9653X + 0.0001$; $R^2 = 0.9994$ and the optical density with a correlation coefficient $R^2 = 0.9994$ (Fig. 6).

Table 4. Repeatability of the method of Folin –Ciocalteu

Concentration (g/l)	Optical Dencity 1	Optical Dencity 2	Optical Dencity 3	Average optical dencity	Standard deviation
0.025	0.024	0.021	0.025	0.233	± 0.002
0.05	0.05	0.049	0.05	0.049	± 0.0005
0.075	0.067	0.075	0.077	0.0730	± 0.0052
0.1	0.097	0.1	0.09	0.095	± 0.0051
0.125	0.121	0.12	0.122	0.121	± 0.001

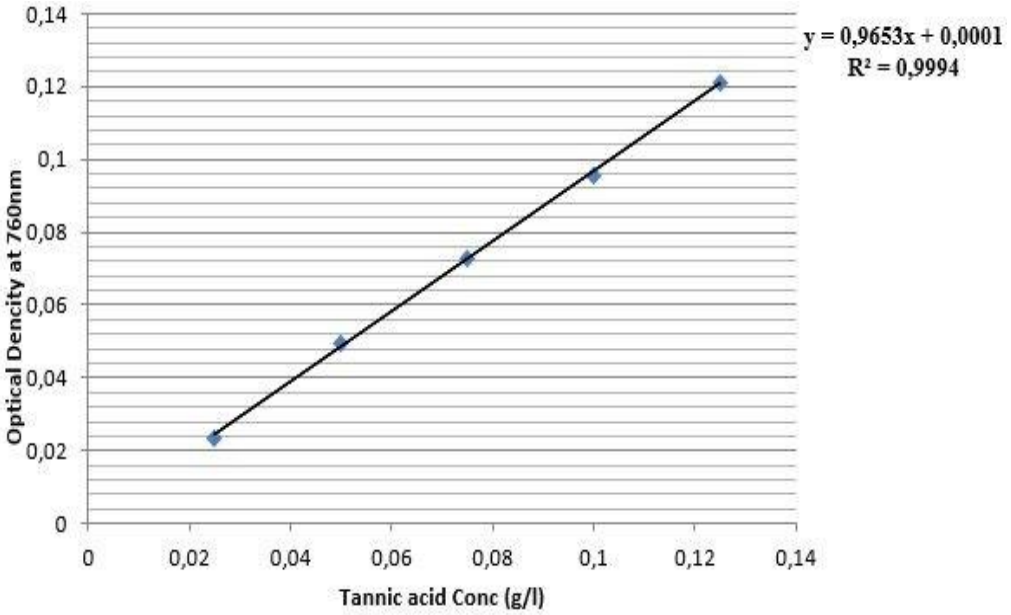

Fig. 6. Calibration right for the determination of total polyphenols

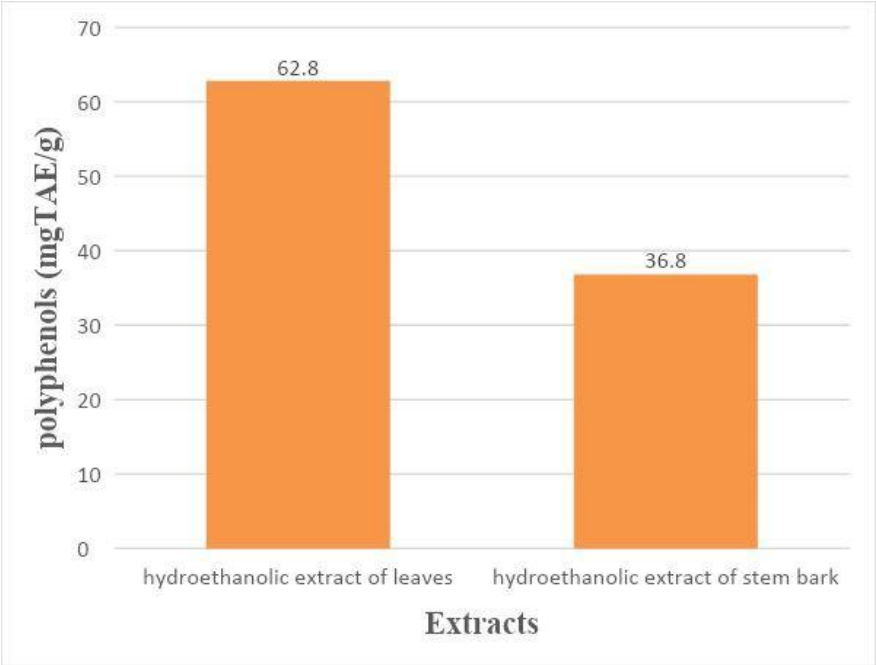

Fig. 7. Comparative diagram of total polyphenols in crude extracts

3.5 Total Polyphenol Content of the Various Extracts

The polyphenol contents of the leaves extracts was 62.8% while that of the stem extract was 36.8% (Fig. 7).

3.6 Antibacterial Activities of *Clausena anisata* Plant

*Streptococcus mutans, Lactobacillus ssp, Aggregatibacter actinomycetemcomitans, Fusobacteriumnucleatum and Prevotellaintermidia*were the micro-organisms isolated.

We obtained MICs between 50 mg / mL and 100 mg / mL with the two extracts; the lowest was 50 mg / mL for *Aggregatibacterium actinomycetemcomitan* and *Fusobacterium nucleatum* isolate of the leaves extract and *Aggregatibacterium actinomycetemcomitan* isolate with the stem bark extract. The largest was 100 mg / ml for the isolate of *Fusobacterium nucleatum* with the stem bark, *Prevotella intermedia, Streptococcus mutans, Lactobacillus spp* isolate with both leaves and stem bark extracts.

By comparing the MICs of the hydro-ethanolic extracts with each other, it was found that the leaves extract had more antibacterial properties against *Aggregatibacterium actinomycetemcomitan* and *Fusobacterium nucleatum* isolate meanwhile that of the stem bark extract against *Aggregatibacterium actinomycetemcomitan.*

The MBC varied from 100 mg/ml to 400 mg/mL with the leaves extract, *Aggregatibacterium actinomycetemcomitan* being the lowest, *Prevotella intermedia* and *Lactobacillus spp* the highest.

For the stem bark, it varied from 200 mg/mL to 400 mg/mL with *Aggregatibacterium actinomycetemcomitan* being the lowest, *fusobacterium nucleatum, Prevotella inermedia, Streptococcus mutans* and *Lactobacillus spp*the highest.

The MBC / MIC ratios were 2 for *Aggregatibacterium actinomycetemcomitan* and *Streptococcus mutans* then 4 for *Fusobacterium nucleatum, Prevotella intermedia* and *Lactobacillus spp* isolate with the hydroethanolic extract of the leaves, and 4 for all the bacterial isolate with the stem bark extract.

Table 5. Determination of MIC, MBC and the ratios MBC/MIC of the different hydroethanolic extracts on isolated strains

	Leaves extract			Stem bark extract		
Strains	**MIC**	**MBC**	**MBC/ MIC**	**MIC**	**MBC**	**MBC/ MIC**
Aggregatibacterium Actinomycetem comitan	50	100	2	50	200	4
Fusobacterium nucleatum	50	200	4	100	400	4
Prevotella intermedia	100	400	4	100	400	4
Streptococcus mutans	100	200	2	100	400	4
Lactobacillus spp	100	400	4	100	400	4

Legent: MIC: Minimum inhibitory concentration, MBC: Minimum bactericidal concentration, MBC/MIC: ratio Minimum bactericidal concentration/Minimum inhibitory concentration

Table 6. Inhibition diameters of the different hydroethanolic extracts of *Clausena anisata* plant and Amoxicillin on isolated strains

	Leaves extract		Stem bark extract		Amoxicillin
Strains	**MIC (mm±s.d)**	**MBC (mm± s.d)**	**MIC (mm±s.d)**	**MBC (mm±s.d)**	**Ø (mm±s.d)**
Aggregatibacterium actinomycetemcomitan	12.00±1.41	15.00±1.4	9.50±0.71	11.00±1.41	27.50±0.71
Fusobacterium nucleatum	9.0±1.41	9.50±0.71	8.25±0.35	8.50±0.71	14.50±0.71
Prevotellainermedia	10.00±1.41	11.50±0.71	8.50±2.12	9.50±0.71	16.50±0.71
Streptococcus mutans	10.50±2.12	13.50±2.12	8.00±1.41	10.00±1.41	18.00±1.41
Lactobacillus spp	8.50±0.71	11.00±1.41	8.50±0.71	10.00±1.41	13.50±0.71

Ø = Diameter

The action of the hydro-ethanolic extract of the leaves was bactericidal on *Aggregatibacterium actinomycetemcomitan* and *Streptococcus mutans*, bacteriostatic on *Fusobacterium nucleatum, Prevotella intermedia* and *Lactobacillus spp* meanwhile that of the stem bark was bacteriostatic on all the isolated bacteria (Table 5).

3.7 Antibacterial Activity of *Clausena anisata* Leaves and Stem Bark Extracts

Comparing the zones of inhibition diameters, the minimal inhibitory and bactericidal concentrations of C. *Anisata* and Amoxicillin. The susceptibility test of *Clausena anisata* extracts was performed on all isolated bacterial including three Gram-negative (*Aggregatibacter actinomycetemcomitans, Fusobacterium-nucleatum and Prevotella intermedia*) and two Gram-positive (*Streptococcus mutans* and *Lactobacillus spp*). All bacterial isolate tested were susceptible to *Clausena anisata* extracts. The leaves extract showed the best antibacterial activity with MIC and MBC having inhibition diameter of 12.00 mm (MIC) and 15 mm (MBC) with *Aggregatibacter actinomycetemcomitans* whereas the lowest was found to be Lactobacillus spp with 8.50 mm (MIC) as well as *Fusobacterium nucleatum* with 9.5 mm (MBC).

For the stem bark extract, *Aggregatibacter actinomycetemcomitans* was also found to have the best activity with inhibition diameters of 9.5 mm (MIC) and 11.00 mm (MBC) meanwhile *Streptococcus mutans* was the lest with 8 mm (MIC) as well as *Fusobacterium nucleatum* 8.5 mm (MBC).

The hydro-ethanolic extract of the leaves generates larger inhibition diameters than those of the stem bark extract on all isolated bacteria. The different diameters obtained with Amoxicillin were high in comparison with those of the plant extracts. The negative control (MHB) did not produce any zone of inhibition (Table 6).

4. DISCUSSION

This study revealed that the leaves and stem bark collected in Wabane South West Region of Cameroon of *Clausena anisata* have potent antibacterial properties against isolated oral microorganisms responsible for periodontitis and tooth decay.

In the current study, polyphenols (substances responsible for the antioxidant, antibiotic and antiinflammatory properties of plants) was higher in the leave than the stem bark extracts. Kenechukwu et al. [13] in Nigeria who obtained yields 10 times higher than the yield in our study while Adamu et al. [14] reported that the extraction of *Clausena anisata* leaves with acetone gave the same yield as in our study. These discrepancies in yields might be due to the different methods and solvent used. Also if one takes into account these results one would be tempted to prefer the ethanolic extract and the Soxhlet method used by kenechukwu that gives a greater yeild. A quantifiable yield will be beneficial in the extraction of the extracts during drug manufacturing.

In the current study, the leaves extract was more concentrated in secondary metabolites than the stem bark extract. The most metabolites abundant in the leaves extract were phenols, triterpenes, coumarins, tannins and saponosides meanwhile that in the stem bark extract were phenols, alkaloids and triterpenes. Kenechukwe et al. [13] also reported that leaves possess trace amount of alkaloids and large amount of saponoside but no flavonoids and tannins whereas the stem bark contains large amounts of saponosides, flavonoids, alkaloids and tannins. The differences of the chemical compounds of any plant can vary greatly depending upon goegraphical region, the age of the plant, local climatic condition, seasonal conditions, experimental conditions and the nature of solvent used for extraction [13,14]. Indeed, the presence of these high concentration of secondary metabolites can explain much of the biological activity in the leaves than the stembark.

The current study also showed that the quantification of polyphenols was higher in the leaves (62.8 mgTAE/g) and low in the stem bark (36.8 mgTAE/g). This polyphenols content corresponds to the high antimicrobial activity seen from the leave extracts.

The hydro-alcoholic extracts of *Clausena anisata* showed that these extracts have antimicrobial activity. The extracts showed activity at different concentrations on all isolated strains. The MICs obtained were between 50 mg/mL to 100 mg/mL with the two extracts. Aligiannis et al. [14] proposed a classification of crude extract activity based on the results of MIC; there is a strong inhibition when the MIC <0.500 mg/ml, moderate inhibition when the MIC is 0.600 mg/ml – 1.500

mg/ml, low inhibition when the MIC > 1.600 mg/ml. If we stick to it we would admit that the extracts used have a low inhibition capacity on the bacterial Strains used. The ratio MBC/MIC of 2 showed bactericidal activity on *Aggregatibacter actinomycetem comitans* and *Streptococcus mutans*. The MBC/MIC of 4 on *Fusobacterium nucleatum, Prevotella intermedia* and *Lactobacillus spp* with the leaves and bark extracts is bacteriostatic. This is confirmed by the high concentration of secondary metabolites present in the leaves than the stem bark.

The values of the inhibition diameters obtained vary from one bacterial type to another, probably in relation to the activity of secondary metabolites present in the plant. According to Moreira et al. [12]. 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, sensitive (+) 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. It reveals that the action of an extract can be non-susceptible or susceptible on bacteria. In the current study, the MIC and MBCs were susceptible at different concentrations with the two extracts. In general, by comparing the inhibition diameters of the different extracts with their corresponding MICs and MBCs, it was found that smaller concentrations exhibited larger diameters of inhibition. The *Clausena anisata* leaves extract showed the best antimicrobial activity with MIC and MBC having inhibition diameters ranging from 8.5 mm to 12 mm and 11 mm to 15 mm for MIC and MBC respectively on all strains tested. *Aggregatibacter actinomycetemcomitans* generated the highest inhibition diameter with MIC (12 mm) and MBC (15 mm) while Lactobacillus spp generated the least inhibition diameter with MIC (8.5 mm) as well as *Fusobacterium nucleatum* with MBC (9.5 mm). The stem bark of *Clausena anisata* also showed antibacterial activity with MIC and MBC having inhibition diameters ranging from 8.5 mm to 9.5 mm and 10 mm to 11 mm respectively. *Aggregatibacter actinomycetemcomitans* generated the highest inhibition diameter of 9.5 mm (MIC) and 11 mm (MBC) whereas Streptococcus mutans generate the least inhibition diameter of 8 mm (MIC) as well as *Fusobacterium nucleatum* with 8.5 mm (MBC). Amoxicillin produced zone of inhibition between 13.5 mm and 27.5 mm. Comparing the inhibition diameters of the different extracts with that of the Amoxicillin, it observed that the inhibition diameters of the leaves extract were closer to that of Amoxicillin, signifying the potency of the leaves extracts. On the contrary, the inhibitory diameters of the stem bark extract were small compare to that of the Amoxicillin. In view of these results, it appears that the hydroethanolic extract of the leaves generates larger inhibition diameters than those of the stem bark extract on all isolates. Our study showed that *Clausena anisata* leaves are more effective than the stem bark extract on all bacterial tested. The antimicrobial activity of essential oil from *Clausena anisata* has been reported to have antiactirial activity *against Staphylococcus aureus, Streptococcus pneumonia, Enterococcus* spp*, Salmonella typhimurium* and *Pseudomonas aeruginosa* [15,16,17]. The present study provides more evidence of the antimicrobial potential of this plant.

The variable composition of *C. anisata* makes it useful in the control of tooth decay and periodontal diseases because these germs are part of the commensal flora and should not be killed but rather be inhibited in their growth. As a result, extracts of *Clausena anisata* could be used in the fabrication of several products such as toothpastes and mouthwashes. It would therefore be interesting to pay special attention to this plant which could in the near future be a medicinal alternative more respectful of the oral flora.

5. CONCLUSION

Aggregatibacter actinomycetemcomitans, Fusobacterium nucleatum, Prevotella intermedia were isolated from patients with periodontal diseases while *Streptococcus mutans* and *Lactobacillus spp*) from patients with tooth decay. This study showed that *Clausena anisata* extracts contained secondary metabolites such as phenols, alkaloids, triterpenes, flavonoids, saponosides, coumarins and tannins these being more abundant in the leaves extract. The yields of the leaves and stem bark obtained were 7.5% and 5.3% respectively.

The evaluation of the antibacterial activity of our various extracts by determination of MIC and MBC has revealed that *Clausena anisata* antibacterial properties against oral microorganisms. The leaves extract exhibited bactericidal activities on *Aggregatibacter actinomycetemcomitans* and *Streptococcus mutan*s, and bacteriostatic on *Fusobacterium*

nucleatum, Prevotella intermedia and *Lactobacillus* spp whereas the stem bark extract exhibited bacteriostatic activity on all isolated strains tested.

RESEARCH GAPS

The isolation and characterization of the active compounds present in *Clausena anisata* plant in order to identify the different molecules responsible for the antimicrobial activity;

- Evaluate the in vivo antimicrobial activity of *C. anisata.*
- Evaluation of the toxicity of the extract, and exposure in vivo.
- To study the feasibility of a possible pre-formulation of a mouthwash using *C. anisata.*

CONSENT

As per international standard or university standard, patient's written consent has been collected and preserved by the authors.

ETHICAL APPROVAL

Approval to carry out this research was obtained from the institutional review board of the Université des Montagnes. Then, an authorization of study was obtained from the management of Cliniques Universitaires des Montagnes. The participation was voluntary in strict respect of the confidentiality of the patient's data.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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