

Comparative Study of Ethanolic Wild African Nutmeg (*Pycnanthus angolensis* (Welw.) Stem Bark Extract Potentials and Selected Conventional Toothpaste against Hidden Resident Mouth Cavity Microfora

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Authors' contributions

This work was carried out in collaboration between both authors. Author OTO designed the materials and methods used in the course of the research work. Authors OTO and TTM designed the antimicrobial assay procedure. Author OTO wrote the first and the final draft of the manuscript. Both authors read and approved the final manuscript.

Article Information

DOI: 10.9734/AJBGMB/2020/v6i230147

Editor(s):

(1) Dr. Mangala Kohli, Vardhman Mahavir Medical College, India.

Reviewers:

(1) Pooja Kabra, Sharda School of Dental Science University, India.

(2) Gayatri Devi Gautam Varma, Mumbai University, India.

(3) Roger Antonio Rengifo Penadillos, National University of Trujillo, Perú.

Complete Peer review History: <http://www.sdiarticle4.com/review-history/62381>

Original Research Article

Received 27 August 2020
Accepted 02 November 2020
Published 26 November 2020

ABSTRACT

The aim of the study is to evaluate and compare the antibacterial activity of ethanolic stem extract of (Wild African nutmeg) *Pycnanthus angolensis* (Welw.) and some commercially available toothpaste against bacteria isolated from the hidden resident mouth cavity microfora. Bacteria were isolated from swabs of apparently healthy individuals and were identified using Staining procedure biochemical tests and the use of Bergey's manual of bacteria identification. The assay for antibacterial activity of *Pycnanthus angolensis* stem bark extract and the four toothpastes were determined using agar well diffusion method. The Gram positive bacteria isolated were *Streptococcus sanguis*, *Streptococcus ratti*, *Stomatococcus mucilaginous.*, *Peptostreptococcus sp.*, and *Streptococcus mutans* and the Gram negative bacteria were *Veillonella atypical*, *Veillonella parvula*, *Veillonella dispar* and *Acidiaminococcus sp.* Oral B toothpaste showed maximum efficacy of inhibition with inhibition zone diameter as wide as 20 mm at 100 mg/ml. Percentage frequency

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distribution of antibacterial activity of conventional toothpaste (Close-up) against hidden resident mouth cavity microfora depicts *Acidaminococcus* sp.13%, *Veillonella parvula* (10%), *Veillonella dispar* (12%), *Peptostreptococcus* sp.(12%), *Stomatococcus mucilaginous*.(9%), *Streptococcus ratti* (13%), *Veillonella atypical* (11%), *Streptococcus sangus* (9%) and *Streptococcus mutans* (11%), Percentage frequency distribution of antibacterial activity of conventional toothpaste (Oral B toothpaste) against hidden resident mouth cavity microfora reveals *Acidaminococcus* sp.(11%), *Veillonella dispar* (11%), *Veillonella parvula* (10%), *Peptostreptococcus* sp. (12%), *Stomatococcus mucilaginous*.(15%), *Streptococcus ratti* (11%), *Veillonella atypical* (8%), *Streptococcus sangus* (10%), and *Streptococcus mutans* (12%), Percentage frequency distribution of antibacterial activity of conventional toothpaste (MyMy toothpaste) against hidden resident mouth cavity microfora depicts *Acidaminococcus* sp.(12%), *Veillonella dispar* (9%), *Veillonella parvula* (8%), *Peptostreptococcus* sp.(10%), *Stomatococcus mucilaginous*.(16%), *Streptococcus ratti* (9%), *Veillonella atypical* (15%), *Streptococcus sangus* (9%) and *Streptococcus mutans* (12%), Percentage frequency distribution of antibacterial activity of conventional toothpaste (Olive toothpaste) against hidden resident mouth cavity microfora shows *Acidaminococcus* sp.(9%), *Veillonella dispar* (10%), *Veillonella parvula* (10%), *Peptostreptococcus* sp.(12%), *Stomatococcus mucilaginous*.(13%), *Streptococcus ratti* (10%), *Veillonella atypical* (17%), *Streptococcus sangus* (7%), and *Streptococcus mutans* (12%). *Pycnanthus Angolensis* stem bark extract inhibited the growth of the oral bacterial isolates with of zones of inhibition diameter ranging from 6 mm to 17 mm at a concentration of 100mg/ml. Secondary metabolite (Phytochemical) screening shows the presence of flavonoids, tannins, saponins, alkaloids, reducing sugars, steroid, phenol, terpenoid, pyrrolozidine alkaloid, glycoside and cardiac glycoside with glycoside and terpenoid most present. However, anthraquinones and volatile oil were absent. With menial antibacterial activity, *P. angolensis* can be use in the formulation of herbal toothpaste. It should be advocated that *Pycnanthus angolensis* should be added to our convention toothpaste to improve the functional ingredient of the toothpaste and Plant-based traditional knowledge has become a recognized tool in search for new sources of drugs. It is clear that the use of these herbal plants can offer a platform for further research.

Keywords: *Pycnanthus angolensis*; conventional toothpaste; mouth cavity microfora.

1. INTRODUCTION

Human oral cavity present an environment that allows the growth of characteristic microorganism found there. It provides a source of water and nutrients, as well as a moderate temperature [1]. It is one of the most dynamic habitats for numerous bacterial species where they undergo intense interspecies competition to for multispecies biofilm structure. Most of the resident mouth cavity organisms are nature commensals, but they may not be pathogenic until when their habitat become more favorable, this trigger up their pathogenic nature, this is a factor dependant on individual personal hygiene [2]. Several chemical formulations with anti bacterial agents have been tried in toothpastes but with less potent activity [3]. Chemicals, mainly triclosan and chlorhexidine, have been added in mouth washers and conventional tooth pastes, to prevent tooth decay, plaque and gingivitis. But some of these substances show undesirable side effects such as tooth staining and altered taste [4]. This has led to paying increased attention on using natural ingredients in herbal oral paste. Many medicinal plants with

known antimicrobial property have used in pharmaceutical formu lations for therapeutic purposes. *Pycnanthus angolensis* is one of the medicinal plants used in herbal medicine with various medicinal properties. The plant is known as wild African nutmeg, it is a lowland tree forest, native to West and East Africa. *Pycnanthus angolensis* has various English names which include African nutmeg, In Africa it is widely known Asilomba, In Nigeria, it is known as Akwa-mili and Oje in Igbo and Akomu in Yoruba [5].

Pycnanthus angolensis (welw.)warb belongs to family Myristicaceae, also known as numerous fruit trees, fragrant spicy plants whose dried fruits are used as condiment. it has a reputed medicinal activity for its analgesic, stomachic, aperative, carminative, anti-inflammatory, haemostatic and antimicrobial actions [6]. It has also been reported to be useful for treatment of various ailment like female sterility, gonorrhoeal infertility, rhinopharyngeal and bronchial pneumonia and as a poison antidote [7].



Plate 1. Some common toothpastes available in Nigeria

Pycnanthus angolensis is an evergreen tree grows up to 40 meters tall and sometimes up to 1.5 meters or more. The trunk is straight and cylindrical in shape with fissures and flaking bark, the sap is honey-colored and the branches are in whorls. The leathery leaves are up to 31cm (centimeters) long by 9 wide. The blades have pointed tips, heart-shaped bases, and thick midribs. They are hairless on top and coated with rusty, feltlike hairs on the undersides. The leaves usually bear signs of insect damage, a feature so common it is considered characteristic of the species. The flowers are arranged in dense,

rusty panicles up to 15 centimeters long. The individual flowers are difficult to see in the tight panicle until the stamens develop, being only about a millimeter long. The flowers are hairy and fragrant. The fruit is a rounded drupe reaching over 3 centimeters long and wide, borne in clusters. It is hairy brown when new, turning yellow-orange, and has cartilaginous flesh that dries woody. It contains a black seed with a redaril which resembles that of nutmeg. The fruit ripens over a long period continuing into the next flowering season, which begins around October [8].

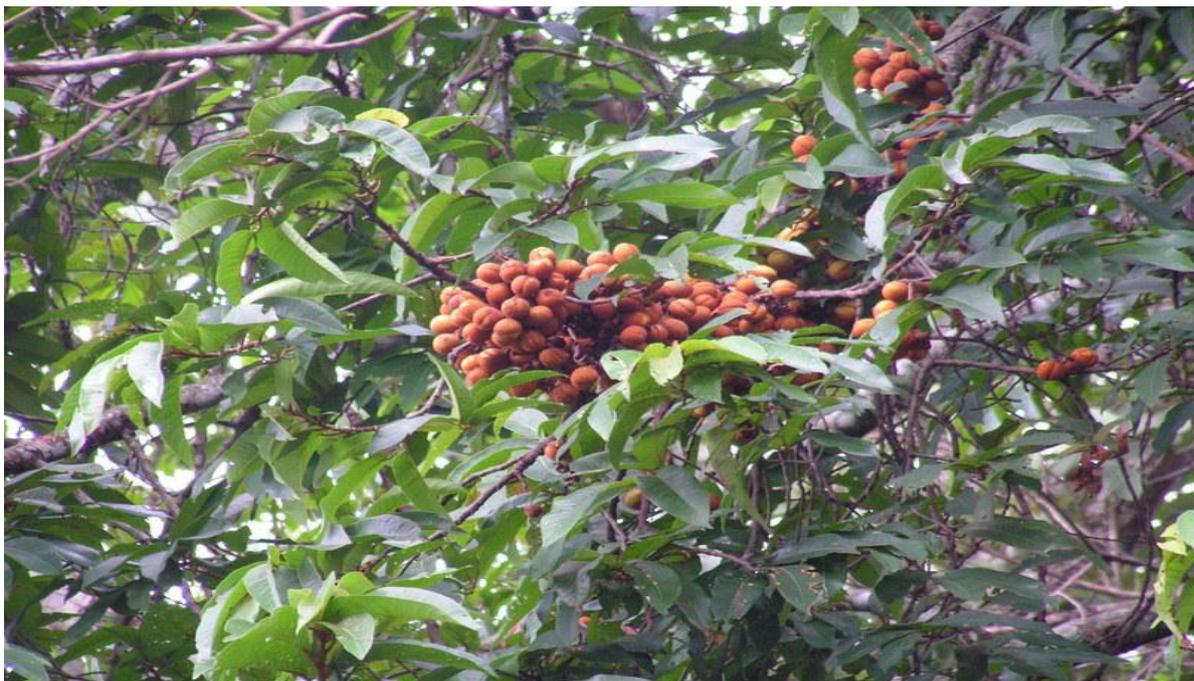


Plate 2. *Pycnanthus angolensis* plant

Most parts of the tree have been used in traditional African medicine. The sap has been used to control bleeding. It is made into an eyewash to treat cataracts and filariasis of the eye [8]. The bark has been used as a poison antidote and a treatment for leprosy, anemia, infertility, gonorrhoea, and malaria. Leaf extracts are consumed or used in an enema to treat edema. Root extracts are used to treat parasitic infections, such as schistosomiasis. The seed oil is used to treat thrush [8,9]. It should be known that Stem, Young Twigs, Leaves, Bark, Fruit, Spines, Seeds and latex of *Pycnanthus angolensis* are the parts of trees being exploited for oral health care [10] in West and Central Africa

In the context of this research work, there is need to find a potent medicinal plant that can serve as a suitable replacement for conventional chemical used in the formulation of toothpaste which will have a deleterious effects on hidden mouth cavity microflora, it may also be used in addition to conventional chemical used in preparation of toothpaste or mouth washers with various side effects. Plant-based traditional knowledge has become a recognized tool in search for new sources of drugs.

2. MATERIALS AND METHODS

2.1 Collection of Hidden Resident Mouth Cavity Microflora Isolates

Four (4) toothpastes samples were bought from a supermarket in Akungba community. The four toothpastes collected were Close-up, My-My, Oral-B, and Olive. The toothpastes were stored at appropriate temperature as recommended by the producers.

2.2 Collection of *Pycnanthus angolensis* Sample

Pycnanthus angolensis Stem bark part were collected from Isale-Akungba swamp in Akungba community of Ondo State. The plant part was authenticated at the herbarium of the Department of Plant Science and Biotechnology, Adekunle Ajasin University, Akungba-Akoko, Ondo State, Nigeria. A voucher number was assigned for future reference (AA105).

2. Preparation of *Pyc. angolensis* Extract

Pycnanthus angolensis stem bark was transported to the Microbiology laboratory of Adekunle Ajasin University, washed with sterile

water and was air dried for 10 days. The dried stems were then chopped into small pieces to increase surface area. About 500 g of the stem bark of *Pycnanthus angolensis* was soaked in one (1) liter of ethanol for 7 days. After which it was filtered with Whatman No. 1 filter paper. The solvent was recovered and the crude extract obtained using rotary evaporator. The crude extract was thus kept in refrigerator at 4°C for further screening.

2.4 Specimen Collection and Isolation of Hidden Resident Mouth Cavity Microflora

Bacterial isolates to be used as test organisms were isolated from the Mouth Cavity. Sterile swab was used to swab the mouth of volunteers and bacteria were isolated using serial dilution and pour plate method. The media used were nutrient agar, Mannitol salt agar and blood agar. The swab was dipped into sterile distilled water and serial dilution was carried out. Aliquot of 1ml from 10^3 and 10^5 were dispensed into sterile petri dishes and molten agar was poured onto the plate, swirled, and allowed to solidify. The plate was then incubated at 37°C for 24 hours after which the media were further examined for bacterial growth. Colonies were subcultured on freshly prepared agar. Distinct colonies were picked into nutrient agar slant and stored at 4°C until further use [11].

2.5 Standardization of Hidden Resident Mouth Cavity Microflora Isolates

Pure culture of the test organisms was transferred into 5 ml of nutrient broth and incubated for 24 hours. 0.1 ml of the overnight culture was transferred into 9.9 ml of distilled water in a test tube using a sterile needle and syringe and then mixed by shaking it. The liquid contains approximately 106cfu/ml of bacterial suspension [12].

2.6 Standardization of *Pycnanthus angolensis* stem Bark Extracts

The extracts were standardized by adding 1 g of each extract to 7.5 ml of distilled water and 2.5 ml of Dimethyl sulfoxide(DMSO) making it 100 mg/ml. The concentration was reduced by adding 5 ml of distilled water into three sterile bijou bottles labeled A, B and C. 5 ml from the 100 mg/ml bijou bottle was taken and dispensed into the bijou bottle A making it 50 mg/ml. Same

process was repeated to get a concentration of 25 mg/ml and 12.5 mg/ml [12]

2.7 Identification of Hidden Resident Mouth Cavity Isolates

The bacterial colonies isolated from mouth cavity were identified by studying cultural morphology and biochemical tests. Growth characteristics were studied on different media such as MacConkey agar, Mannitol salt agar, Blood agar. Morphology was studied with Gram's staining. The biochemical tests were done to characteristics bacterial isolates includes Catalase, Oxidase and Coagulase, interpreted according to Bergey's Manual of Systematic Bacteriology.

2.8 Gram Staining of Hidden Resident Mouth Cavity Microfora Isolates

A loopful of sterile distilled water was dropped on a clean grease free slide by using a sterile inoculating loop after which an inoculum from the culture was mixed with the water on the slide. The smear was allowed to air dried and then heat fixed gently by passing it quickly over a Bunsen flame. The smear was flooded with crystal violet solution for 60 seconds (one minutes) and rinsed with water. The smear was again flooded with Lugol's iodine for 30 seconds and rinsed with water, 70% alcohol was poured on the slides for 15 seconds until the crystal violet had been completely washed off. It was then counterstained with Safranin for 60 seconds and allowed to dry. The slides were then observed under oil immersion objective. Gram positive cells remained purple while Gram negative cells appeared red or pink [13].

2.9 Biochemical Test of Hidden Resident Mouth Cavity Isolates

2.9.1 Catalase test

A drop of hydrogen peroxide solution was placed on a clean grease free slide. A flamed inoculating loop was used to place a loopful of an inoculum on the slide and gently mixed after which it was observed for bubbles or effervescence which is an indication of catalase positive organism [13].

2.9.2 Motility test

A little immersion oil was placed round the edge of the depression of a cavity slide and then a loopful of the bacterial colony was transferred to

the centre of a clean dry cover slip with a sterile inoculating loop. The cavity slide was inverted over the cover slip such that the culture drop was in the centre of the slide depression. The culture drop appeared hanging. This was examined immediately for motility under the oil immersion microscope [13]

2.9.3 Indole test

Three millimeters of 1% Tryptone broth was placed into different tubes after which a loopful of the bacterial isolates were inoculated into different test tubes leaving one of the tubes uninoculated to serve as the control. The test tubes were then incubated at 37°C for 48 hours. After incubation, 0.5 ml of Kovacs's reagent was added and shaken gently after which it was allowed to stand for 20 minutes to permit the reagent to rise to the top. A red colour at the surface of the tubes indicated a positive result while a yellow colouration of the surface layer indicated a negative result [13].

2.9.4 Coagulase test

A loopful of normal saline solution was placed on each glass slide and was emulsified. Human plasma was added to one of the suspension and was stored properly for 15 minutes while the other was left as control. Coagulase positive was indicated by clumping which did not re-emulsify [13].

2.9.5 Sugar fermentation test

Nine milliliters of nutrient broth was placed into different test tubes and 1 ml of the sugars was added into the different test tubes after which Durham tubes were placed inside the test tubes. The test tubes were then covered with cotton wool, sterilized and then allowed to cool. The organisms were then inoculated into the test tubes and they were incubated at 37°C alongside an uninoculated test tube which serves as a control. It was checked at 24 hrs and 48 hrs for colour change and gas production [13].

2.10 Antibacterial Activity of Collected Toothpastes against Hidden Resident Mouth Cavity Isolates

An assessment of tooth pastes for antibacterial activity was tested by agar well diffusion method. A stock solution was prepared by mixing 1g of toothpaste in 10 ml of distilled water. The bacterial strains were grown in Nutrient broth

medium at 37°C for 24 hrs and were diluted to 0.5M MacFarland turbidity standard. The tooth pastes were also constituted into concentrations of 100 mg, 50 mg, 25 mg, and 12.5 mg. About 0.1 ml of the standardized inoculums was spread on Mueller Hinton agar plates. Wells of 6 mm in diameter were punched off into medium with a sterile cork borer and filled with 50 µl of toothpaste stock solutions in aseptic condition. All the plates were kept in a refrigerator to allow pre-diffusion of extract for 30 minutes. Further, the plates were incubated at 37°C for 24 hrs and antibacterial activities were evaluated by measuring the diameters of zones of inhibition [14].

2.11 Antimicrobial Screening of Ethanol Stem bark Extract of *Pycnanthus angolensis* against Hidden Resident Mouth Cavity Isolates

The ethanol extract of *Pycnanthus angolensis* stem bark was screened for antibacterial activity against the oral bacterial isolates. This was carried out using the agar well diffusion method. A stock concentration of 100 mg/ml was constituted by dissolving 1g each, of the extracts in 10 ml of Dimethyl sulfoxide (DMSO) diluted with sterile distilled water in ratio 1:3. 50 mg/ml, 25 mg/ml and 12.5 mg/ml concentrations of the extracts were prepared using dilution formula ($C_1V_1=C_2V_2$). 1 ml of 10^{-4} normal saline dilution of a 24 hours broth culture was mixed with 19 ml of the agar in a sterile universal bottle and poured into sterile petri dish. The agar plate was left to solidify and wells were bored on them using 6 mm cork borer. 50 µl of each concentration of the extracts was poured into each well and incubated at 37°C for 24 hours. The diameter zones of inhibition were measured and recorded in millimeter and the results interpreted according to the Clinical Laboratory Standard Institute [15] guidelines. Piperacillin/tazobactam (0.125 mg/ml) and Demethyl sulfoxide (DMSO) were used as positive and negative controls respectively [16].

2.12 Minimal Inhibitory Concentration and Minimal Bacteriocidal Concentration

The minimal inhibitory concentration (MIC) was determined using the tube dilution method. Graded concentrations of the extract was prepared using Mueller Hinton broth medium into different test tubes. The concentrations were 100 mg/ml, 50 mg/ml, 25 mg/ml, 12.5 mg/ml, 6.25 mg/ml and 3.125 mg/ml. standardized inoculum

of 24 hours broth culture was inoculated into the test tubes was incubated at 37°C for 24 hours. After incubation, the test tubes were examined for sign of growth (turbidity) and the minimal concentration with no growth was recorded as the MIC.

The minimal bacteriocidal concentration (MBC) was determined by streaking out samples from the test tubes with no growth on the surface of freshly prepared nutrient agar. The plates were then incubated at 37°C for 24 hours, after which plates were observed for any bacterial growth. Again, the minimal concentration with no growth was taken as the MBC.

2.13 Qualitative Secondary Metabolite Screening of Ethanolic Stem bark *Pycnanthus Angolensis* Extract

Plant filtrate was prepared by boiling 20 g of the fresh plant in distilled 5ml of water. The solution was filtered through a vacuum pump. The filtrate was used for the Secondary metabolite (Phytochemical) screening for Flavonoids, Tannins, Saponins, Alkaloids, Reducing Sugars, Anthraquinones and Anthocyanosides.

2.13.1 Test for alkaloids

About 0.2gram of plant extract was warmed with 2 ml of H_2SO_4 for two minutes, it was filtered and few drops of Dragendoff's reagent were added. Orange red precipitate indicates the present of Alkaloids [17]

2.13.2 Test for tannins

One milliliter of the filtrate were mixed with 2ml of $FeCl_3$, A dark green colour indicated a positive test for the tannins [17].

2.13.3 Test for saponins

One milliliter of the plant filtrate were diluted with 2 ml of distilled water; the mixture were vigorously shaken and left to stand for 10min during which time, the development of foam on the surface of the mixture lasting for more than 10 mm, indicates the presence of saponins [18].

2.13.4 Test for anthraquinones

One milliliter of the plant filtrate was shaken with 10 ml of benzene; the mixture was filtered and 5 ml of 10% (v/v) ammonia were added, then shaken and observed. A pinkish solution indicates a positive test [19].

2.13.5 Test for anthocyanosides

One milliliter of the plant filtrate was mixed with 5 ml of dilute HCl; a pale pink colour indicates the positive test.

2.13.6 Test for flavonoids

One milliliter of plant filtrate was mixed with 2 ml of 10% lead acetate; a brownish precipitate indicated a positive test for the phenolic flavonoids. While for flavonoids, 1 ml of the plant filtrate were mixed with 2ml of dilute NaOH; a golden yellow colour indicated the presence of flavonoids [20].

2.13.7 Test for reducing sugars

One milliliter of the plant filtrate was mixed with Fehling A and Fehling B separately; a brown colour with Fehling B and a green colour with Fehling A indicate the presence of reducing sugars [21].

2.13.8 Test for cyanogenic glucosides

This was carried out subjecting 0.5 g of the extract 10 ml sterile water filtering and adding sodium picrate to the filtrate and heated to boil.

2.13.9 Test for cardiac glucosides

Legal test and the killer-kiliani was adopted, 0.5 g of the extract were added to 2ml of acetic anhydride plus H₂SO₄ [18].

2.14 Quantitative Secondary Metabolic Screening of Ethanolic Stem Bark *Pycnanthus angolensis* Extract

2.14.1 Saponins

About 20 grams each of dried plant samples were ground and, put into a conical flask after which 100 ml of 20 % aqueous ethanol were added. The mixture was heated using a hot water bath. At about 55°C, for 4 hour with continuous stirring, after which the mixture were filtered and the residue re-extracted with a further 200 ml of 20% ethanol. The combined extracts were reduced to 40 ml over a water bath at about 90°C. The concentrate was transferred into a 250 ml separatory funnel and 20 rnl of diethyl ether were added and then shaken vigorously. The aqueous layer was recovered while the ether layer was discarded. The purification process was repeated three times. 60 rnl of n-butanol

were added. The combined n-butanol extracts were washed twice with 10 ml of 5% aqueous sodium chloride. The remaining solution was heated in a water bath. After evaporation, the samples were dried in the oven to a constant weight; the saponin content was calculated as percentage of the starting material [22]

2.14.2 Flavonoids

The concentration of flavonoids in the extract was estimated spectrophotometrically according to the procedure of Sun et al. The extract (0.1 g) was dissolved in 20 ml of 70% (v/v) ethanol to give a final concentration of 0.5 mg/ml. To clean dry test tubes (in triplicate) were pipetted 0.5 ml of working solution of sample and diluted with 4.5 ml distilled water. To each test tube was added 0.3 ml of 5% (w/v) NaNO₂, 0.3 ml of 10% Aid3 and 4 ml of 4% (w/v) NaOH. The reaction mixtures were incubated at room temperature for 15 minutes. The absorbance was read at 500 nm against reagent blank. The standard calibration curve was prepared by pipetting 0.2, 0.4, 0.6, 0.8, 1.0 ml of 1 mg/ml rutin into clean dry test tubes. The volumes were made up to 5 ml with distilled water. To each of the tubes were added 0.3 ml of 5% (w/v) NaNO₂, 0.3 ml of 5% (w/v) AlCl₃ and 4 ml of 4% (w/v) NaOH. At room temperature for 15minutes, the reaction mixtures were incubated. Absorbance was taken at 500nm and was plotted against the concentration to give the standard calibration curve. The concentrations of the flavonoids in the extract was extrapolated from standard calibration curve and expressed as milligram rutin equivalent per g of extract (mg RE/g extract) [12].

2.14.3 Cardiac glucosides

To 2ml of filtrate hydrolysate, 3 ml of ethyl acetate was added and shaken, ethyl acetate layer was separated and 10% ammonia solution was added to it. Formation of pink color indicated the presence of cardiac glycosides.

2.14.4 Tannins

About 500 mg of the plant sample were weighed into a 50 ml plastic bottle. 50 ml of distilled water was added and shaken for 1 hour on a mechanical shaker. This was filtered into a 50 ml volumetric flask and made up to the marked level. Then, 5 ml of the filtrate was transferred into a test tube and mixed with 2 ml of 0.1 M FeCl₃ in 0.1 M HCl and 0.008 M potassium ferro cyanide. The absorbance was measured at 120

nm within 10 minutes. The tannins content was calculated using a standard curve of extract [22].

2.14.5 Alkaloids

Five grams of the plant sample were weighed into a 250 ml beaker and 200 ml of 10% acetic acid in ethanol was then be added, the reaction mixture were covered and allowed to stand for 4 hour. This was filtered and the extract will be concentrated on a water bath to one-quarter of the original volume. Concentrated ammonium hydroxide was added drop-wise to the extract until the precipitation is complete. The whole solution were allowed to settle and the precipitate was collected, washed with dilute ammonium hydroxide and then filtered; the residue being the alkaloid, which was dried and weighed to a constant mass [22]

2.14.6 Phlobatannins

About 0.5 grams of each plant extracts were dissolved in distilled water and filtered. The filtrate was boiled in 2ml of HCl, red precipitate show the present of phlobatannins.

3. RESULTS

Table 1 this table describe the result of biochemical and Gram staining result obtained on the hidden resident mouth cavity microfora.. Ten (10) isolates were isolated and identified, it was observed that Gram stain revealed that five (5) were Gram positive and five (5) were Gram negative. The Gram positive organisms include *Streptococcus sanguis*, *Streptococcus ratti*, *Stomatococcus mucilaginous* *Peptostreptococcus* sp., and *Streptococcus mutans* while the Gram negative organisms are *Veillonella atypical*, *Veillonella parvula*, *Veillonella dispar* and *Acidiaminococcus* sp.

Table 2 qualitative Secondary metabolite screening of ethanol extracts of *Pycnanthus angolensis* stem extract. Flavonoid, tannins, saponins, alkaloids, reducing sugars, steroid, phenol, terpenoid, pyrrolozidine alkaloid, glycoside and cardiac glycoside were present while anthraquinones and volatile oil were not detected

Fig. 1 antibacterial activities of different conventional toothpaste against hidden resident mouth cavity microfora were described in this section of Fig. 1. All the toothpastes showed inhibitory activity against the hidden resident mouth cavity microfora, with zones ranging from

6 mm to 18 mm in diameter. Close-up toothpaste has the highest antibacterial activity against *Stomatococcus mucilaginous* at 20 mm and lower activity on MyMy toothpaste at 7.0 mm against *Streptococcus sanguis*.

Fig. 2 antibacterial activity of ethanolic (Wild African Nutmeg) *Pycnanthus angolensis* (Welw.) extract against hidden resident mouth cavity microfora. It was observed that *Acidaminococcus* sp. has the highest zones of inhibition against hidden resident mouth cavity microfora with 20.0 mm at 100 mg/ml while *Streptococcus ratti* has the lowest zones of inhibition of 6.0 mm at 12.5 mg/ml concentration.

Figs. 3, 4, 5, 6, shows the percentage frequency of antibacterial activity of conventional toothpaste against hidden resident mouth cavity microfora., *Streptococcus ratti* and *Acidaminococcus* sp. (13%) respectively has the highest percentage frequency on close up toothpaste, *Stomatococcus mucilaginous*.(15%) (Oral B and MyMy toothpaste) and *Veillonella atypical* (17%) (Olive toothpaste) respectively.

Fig. 3 percentage frequency distribution of antibacterial activity of conventional toothpaste (Close-up toothpaste) against hidden resident mouth cavity microfora depicts *Acidaminococcus* sp.(13%), *Veillonella parvula* (10%), *Veillonella dispar* (12%), *Peptostreptococcus* sp.(12%), *Stomatococcus mucilaginous*9%), *Streptococcus ratti* (13%), *Veillonella atypical* (11%), *Streptococcus sanguis* (9%), and *Streptococcus mutans* (11%).

Fig. 4 percentage frequency distribution of antibacterial activity of conventional toothpaste (Oral B toothpaste) against hidden resident mouth cavity microfora reveals *Acidaminococcus* sp. (11%), *Veillonella dispar* (11%), *Veillonella parvula* (10%), *Peptostreptococcus* sp. (12%), *Stomatococcus mucilaginous*15%), *Streptococcus ratti* (11%), *Veillonella atypical* (8%), *Streptococcus sanguis* (10%), and *Streptococcus mutans* (12%).

Fig. 5 percentage Frequency Distribution of Antibacterial Activity of conventional toothpaste (MyMy toothpaste) against hidden resident mouth cavity microfora depicts *Acidaminococcus* sp. (12%), *Veillonella dispar* (9%), *Veillonella parvula* (8%), *Peptostreptococcus* sp. (10%), *Stomatococcus mucilaginous* (16%), *Streptococcus ratti* (9%), *Veillonella atypical* (15%), *Streptococcus sanguis* (9%), and *Streptococcus mutans* (12%).

Table 1. Biochemical and Gram staining of hidden resident mouth cavity microflora

Isolate	Gram stain	Shape	Coagulase	Catalase	Motility	Nitrate	Glucose	Mannitol	Xylose	Indole	citrate	Urease	V.P	TDA	Probable organism
Iso 1	+	Diplococcus	+	+	-	-	+	+	+	-	-	-	-	+	<i>Streptococcus sanguis</i>
Iso 2	-	Diplicoccus	-	-	-	+	+	+	+	-	-	-	+	-	<i>Veillonella atypica</i>
Iso 3	+	coccus	+	-	-	+	+	+	+	-	-	-	+	-	<i>Streptococcus ratti</i>
Iso 4	+	coccus	-	+	-	+	+	+	+	-	-	-	-	-	<i>Stomatococcus mucilaginous.</i>
Iso 5	+	Diplococcus	-	-	+	-	+	+	+	-	-	+	+	-	<i>Peptostreptococcus sp.</i>
Iso 6	-	Diplococcus	+	-	-	+	+	+	+	-	-	-	+	-	<i>Veillonella parvula</i>
Iso 7	-	coccus	+	-	-	+	+	+	+	-	-	+	+	-	<i>Veillonella dispar</i>
Iso 8	-	Diplococcus	+	+	-	+	+	+	+	-	-	-	-	+	<i>Acidaminococcus sp.</i>
Iso 9	+	coccus	+	+	-	+	+	+	+	-	-	-	-	-	<i>Streptococcus mutans</i>
Iso 10	-	Diplococcus	+	+	-	+	+	+	+	-	-	-	-	+	<i>Acidaminococcus sp.</i>

KEY: Negative (-), Positive (+)

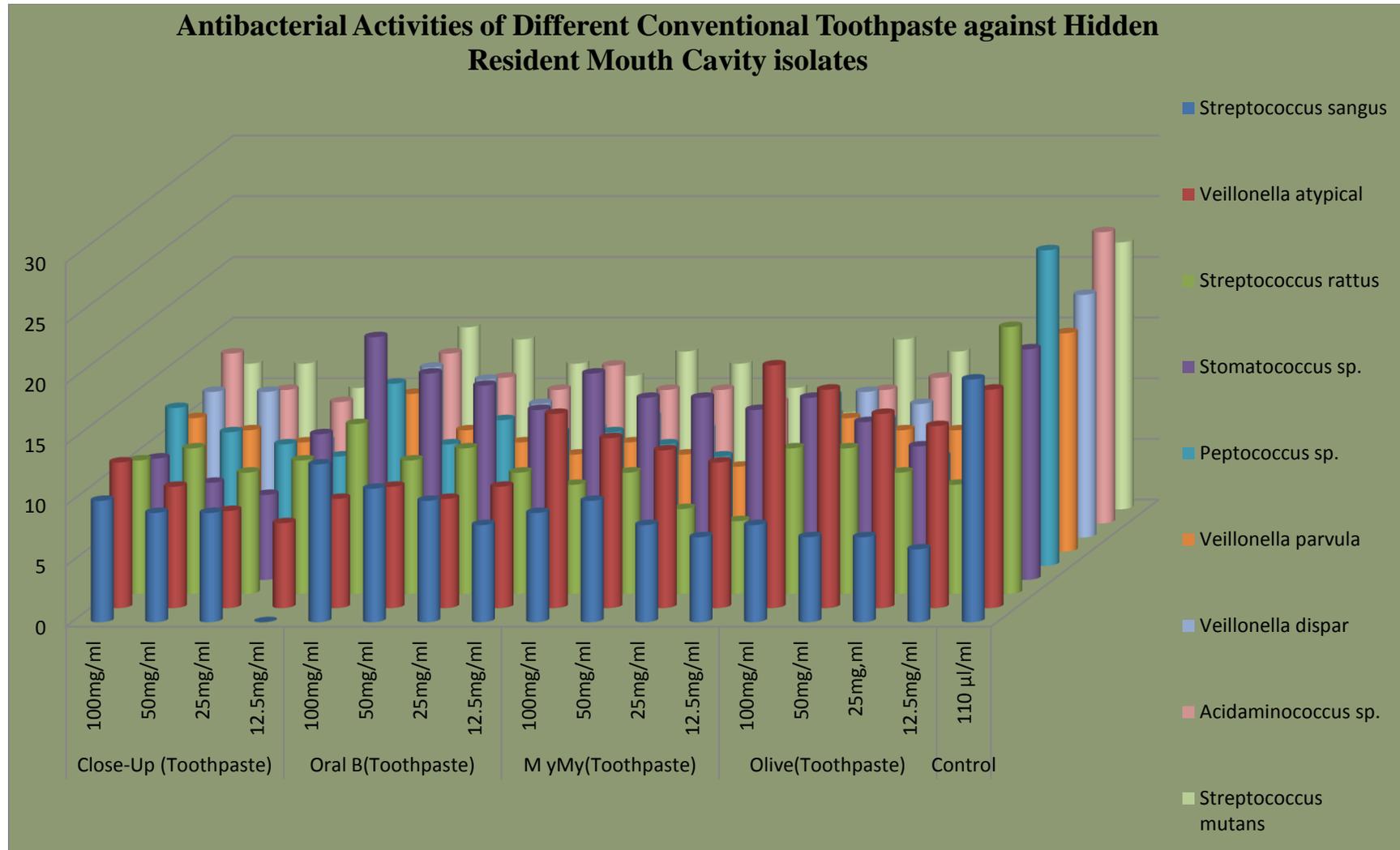


Fig. 1. Antibacterial activities of different conventional toothpaste against hidden resident mouth cavity microfora

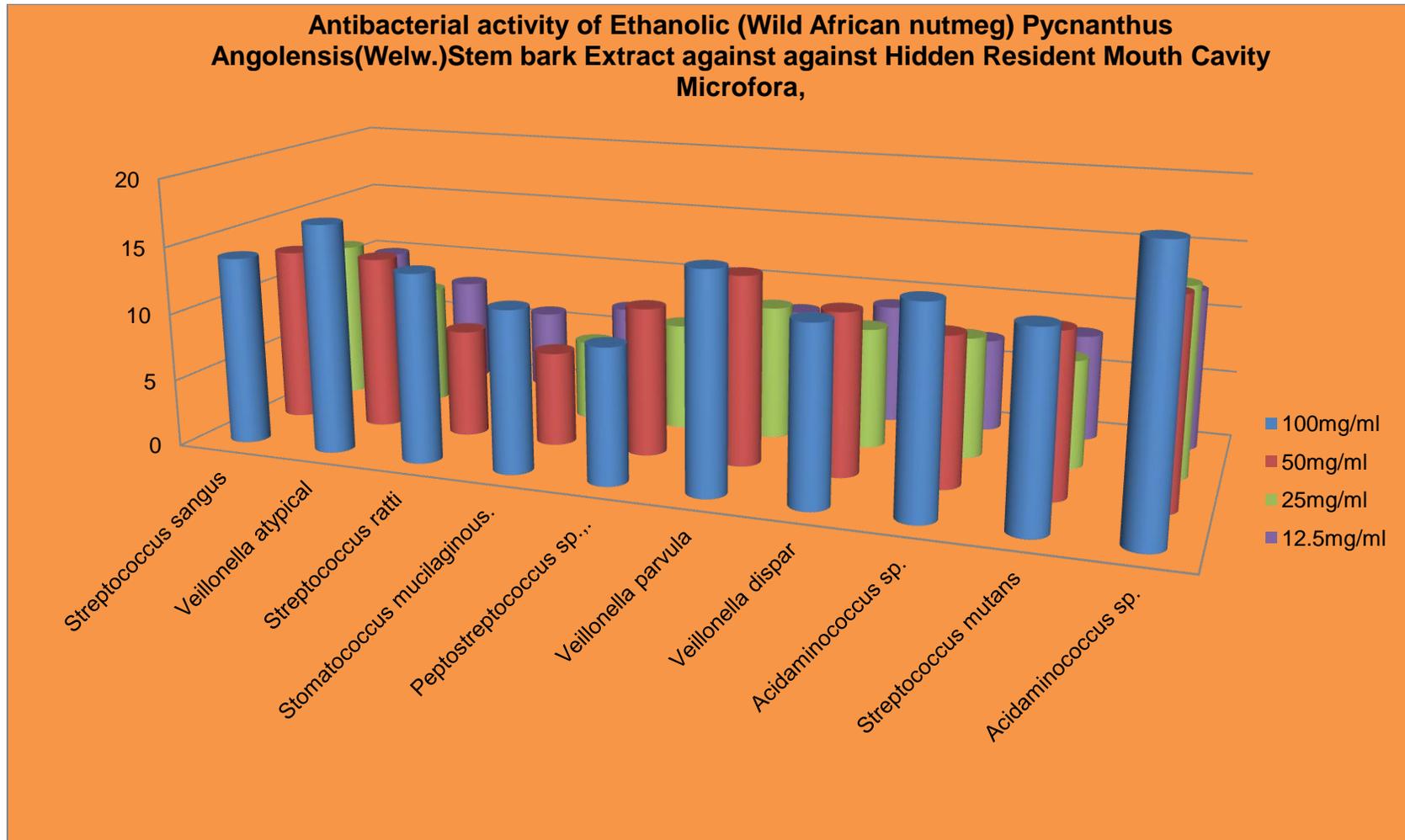


Fig. 2. Antibacterial activity of stem bark ethanolic (Wild African nutmeg) *Pycnanthus angolensis* (Welw.) extract against against hidden resident mouth cavity microfora

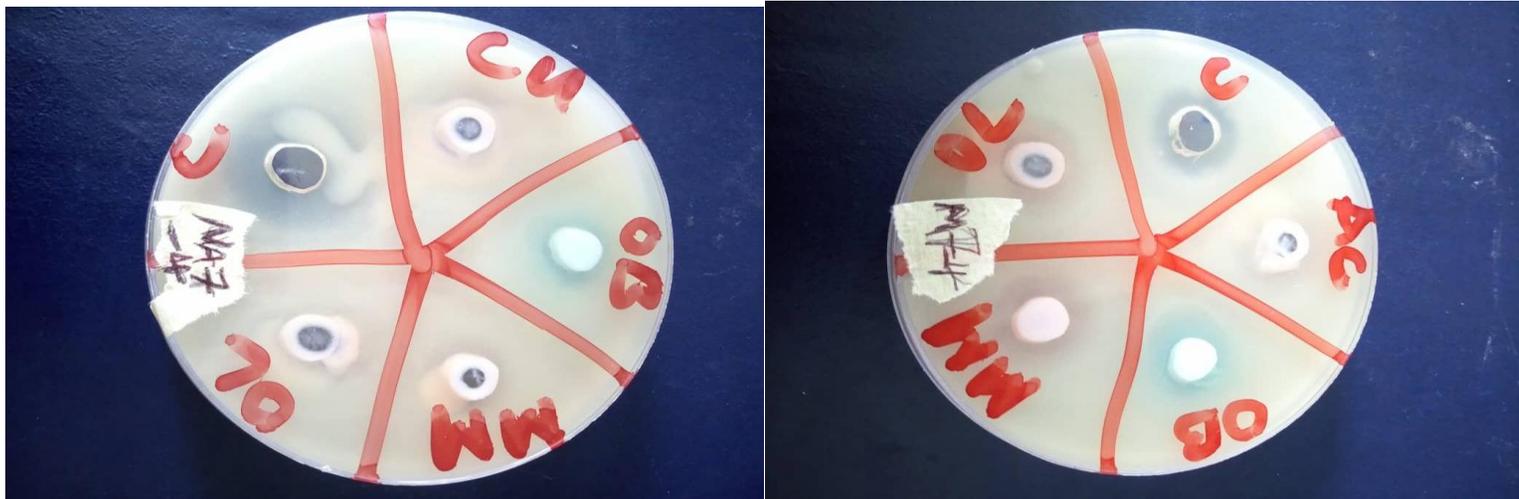


Plate 3. Antibacterial activity of the four toothpastes against hidden resident mouth cavity microflora

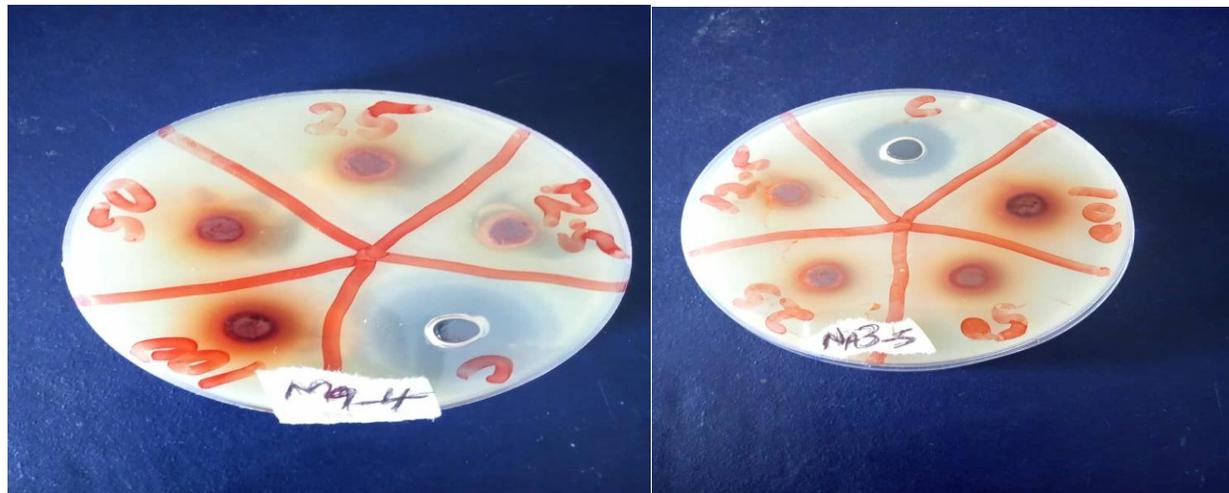


Plate 4. Antimicrobial screening of stem bark *Pycnanthus angolensis* extract against hidden resident mouth cavity microflora

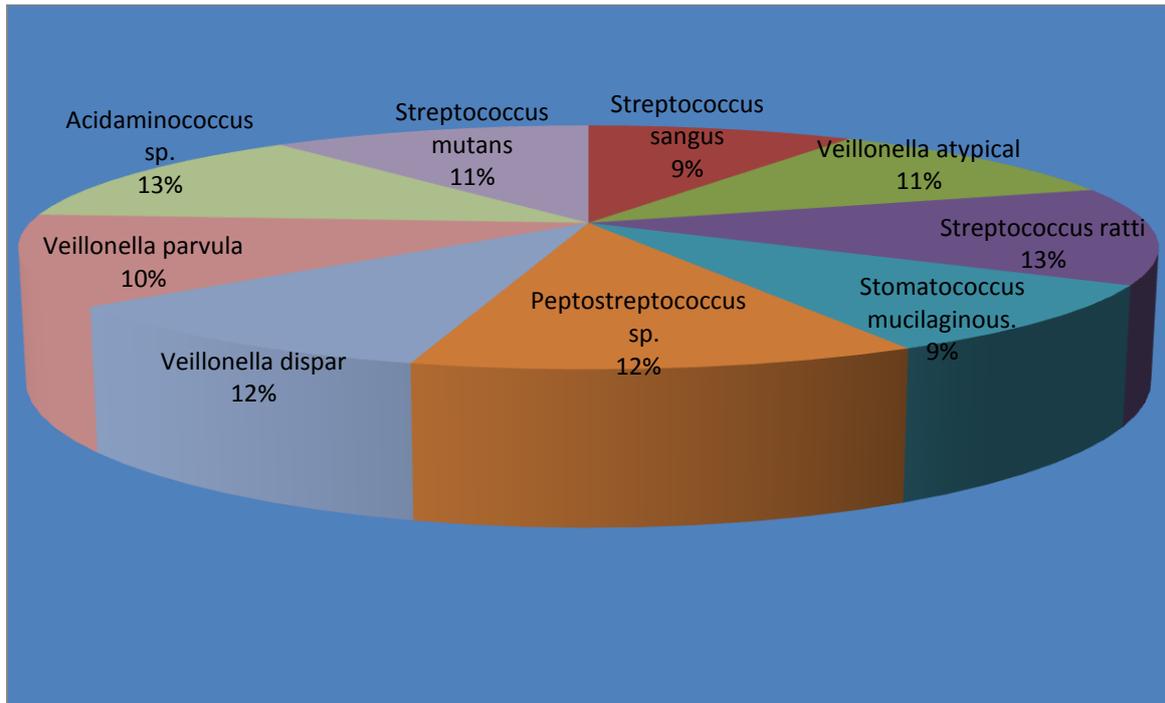


Fig. 3. Percentage frequency distribution of antibacterial activity of conventional toothpaste (Close-up) against hidden resident mouth cavity microflora

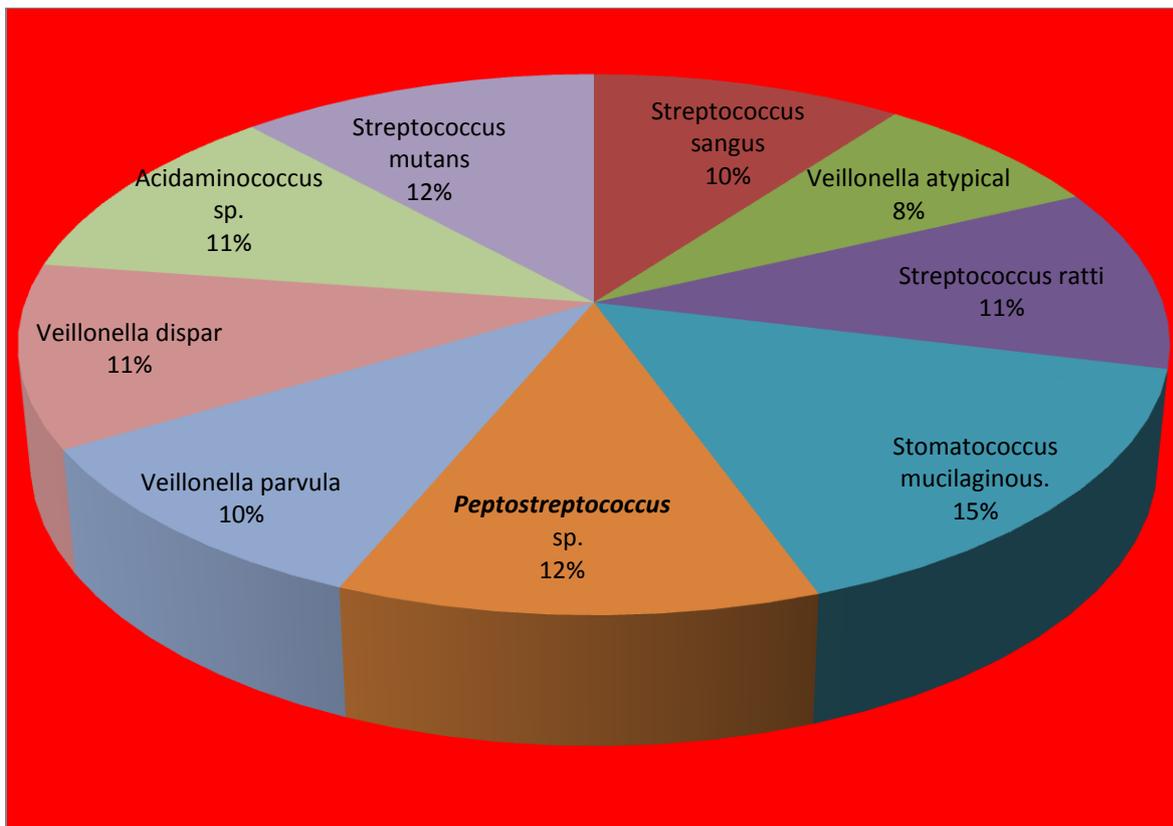


Fig. 4. Percentage frequency distribution of antibacterial activity of conventional toothpaste (Oral B) against hidden resident mouth cavity microflora

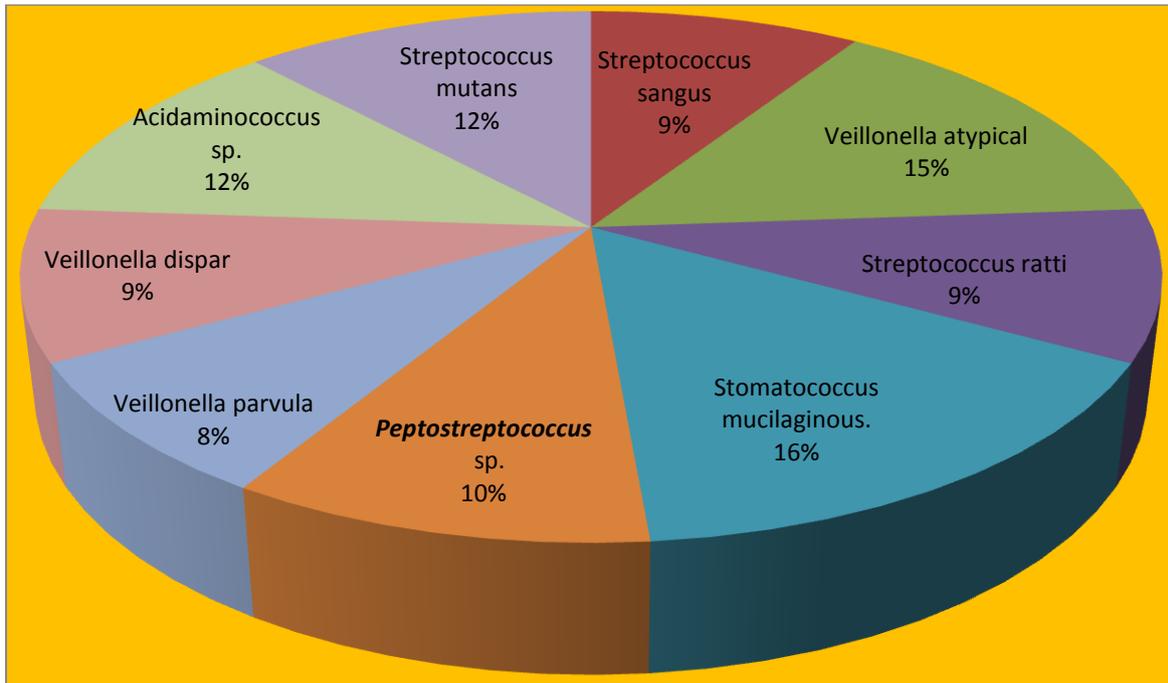


Fig. 5. Percentage frequency distribution of antibacterial activity of conventional toothpaste (MyMy) against hidden resident mouth cavity microflora

Fig. 6 percentage frequency distribution of antibacterial activity of conventional toothpaste (Olive toothpaste) against hidden resident mouth cavity microflora shows *Acidaminococcus* sp.(9%), *Veillonella dispar* (10%), *Veillonella parvula* (10%), *Peptostreptococcus* sp.(12%), *Stomatococcus mucilaginous*.(13%), *Streptococcus ratti* (10%), *Veillonella atypical* (17%), *Streptococcus sangus* (7%), and *Streptococcus mutans* (12%).

Fig. 7 percentage frequency distribution of antibacterial activity of ethanolic *Pycnanthus angolensis* (Welw.) stem extract against hidden resident mouth cavity microflora. From the figure, it was observed that *Acidaminococcus* sp. (14 %) has the highest while *Peptostreptococcus* sp., (7%) has the lowest percentage frequency of Antibacterial activity of ethanolic *Pycnanthus angolensis* (Welw.) stem extract against hidden resident mouth cavity microflora. The other percentage frequency are *Streptococcus mutans* (10%), *Veillonella dispar* (9%), *Veillonella parvula* (11%), *Stomatococcus mucilaginous*.(8%), *Streptococcus ratti* (10%), *Veillonella atypical* (12%) and *Streptococcus sangus* (9%).

Fig. 8 minimal inhibitory concentration (MIC) and the minimal bacteriocidal concentration (MBC) of ethanolic stem extract of *Pycnanthus angolensis* against hidden resident mouth cavity microflora.

The extract showed maximum efficacy against *Streptococcus sangus* and *Acidaminococcus* with the lowest MIC of 12.5 mg/ml and MBC of 50 mg/ml.

Fig. 9 quantitative Secondary metabolite screening of the ethanol stem extract of *Pycnanthus angolensis* stem extract. Glycoside and terpenoid were most present with a concentration of 14.01 while saponins were least present with a concentration of 3.21%.

Fig. 10 synergistic antibacterial activities of different conventional toothpaste and *Pycnanthus angolensis* against Hidden Resident Mouth Cavity isolates. Fortification of Close-up toothpaste with *Pycnanthus angolensis*, It can be depicted that *Acidaminococcus* sp. has the highest zone of inhibition of 24.0 mm at 100 mg/ml while *Veillonella atypical* has the lowest 7.0 mm at 12.5 mm. Addition of Oral B toothpaste and *Pycnanthus angolensis*, it can be deduced that *Peptostreptococcus* sp and *Streptococcus mutans* has the highest zones of inhibition of 25 mm at 100 mg/ml and *Streptococcus sangus*, *Veillonella atypical* and *Veillonella parvula* has the lowest zones of inhibition of 8mm at 12,5 mg/ml. fortification of MyMy toothpaste with *Pycnanthus angolensis* shows that *Veillonella atypical*, *Streptococcus sangus* and *Peptostreptococcus* sp has the

highest zones of inhibition of 26.0 mm at 100 mg/ml while *Veillonella parvula* has the lowest zones of inhibition of 7.0 mm at 12.5 mg/ml. Addition of Olive toothpaste and *Pycnanthus angolensis* depicts that *Peptostreptococcus* sp

has the highest zones of inhibition of 24.0 mm at 100 mg/ml while *Acidaminococcus* sp and *Peptostreptococcus* sp has the lowest zones of inhibition of 7.0mm at 12.5 mg/ml.

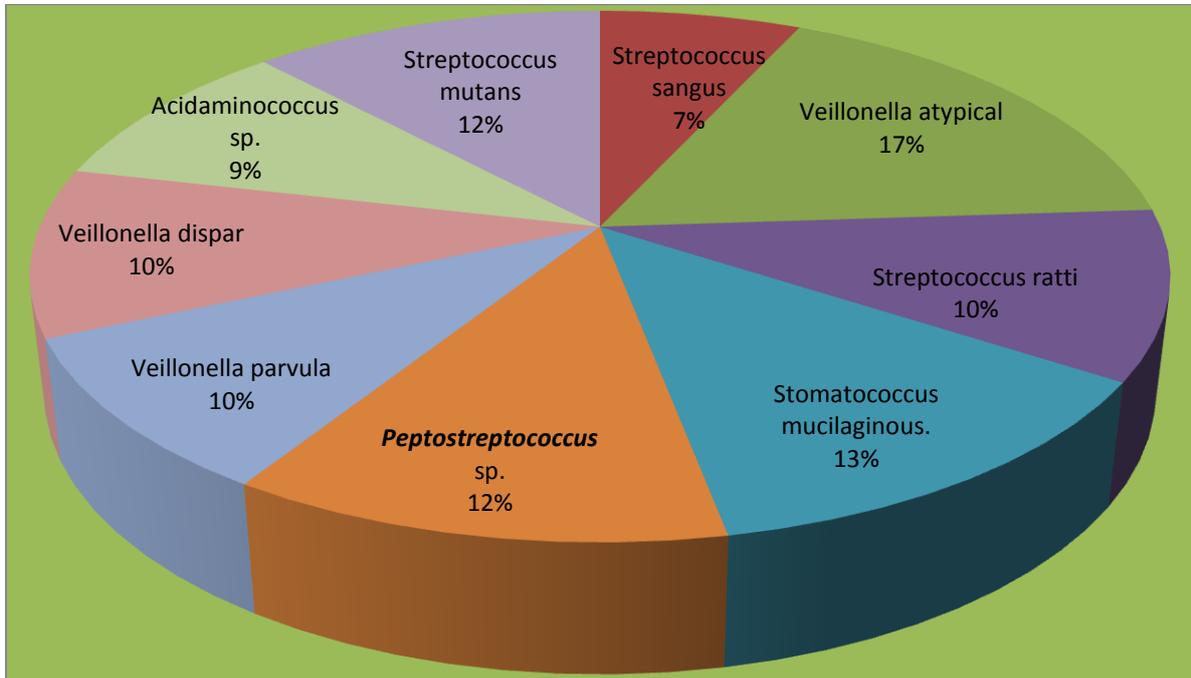


Fig. 6. Percentage frequency distribution of antibacterial activity of conventional toothpaste (Olive) against hidden resident mouth cavity microflora

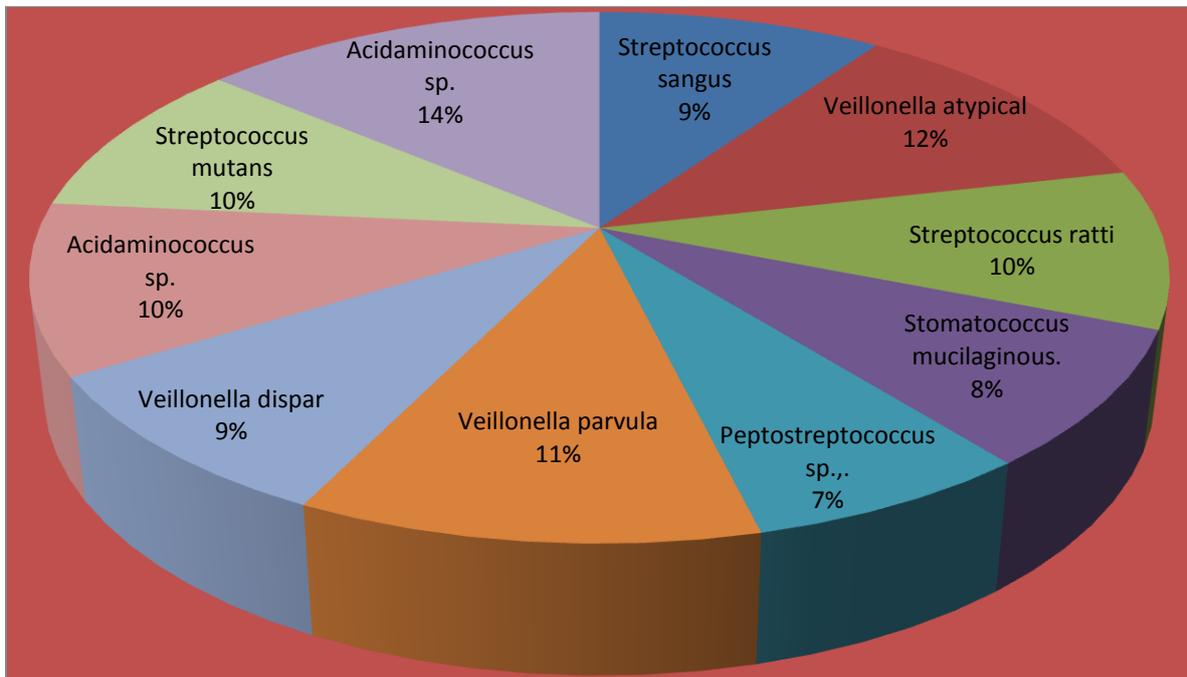


Fig. 7. Percentage frequency distribution of antibacterial activity of ethanolic *Pycnanthus angolensis* (Welw.) stem bark extract against hidden resident mouth cavity microflora

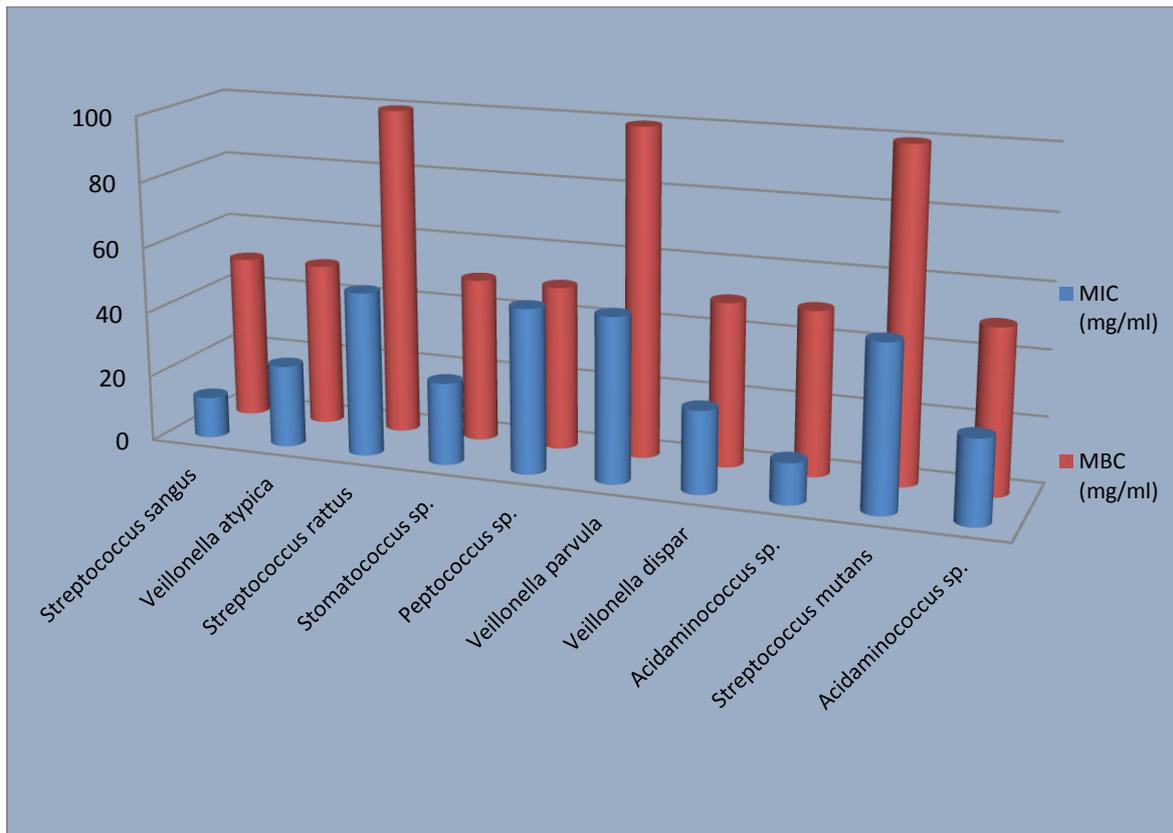


Fig. 8. Minimal inhibitory concentration (MIC) and minimal bacteriocidal concentration (MBC) of ethanolic stem bark extract of *Pycnanthus angolensis* against hidden resident mouth cavity microflora

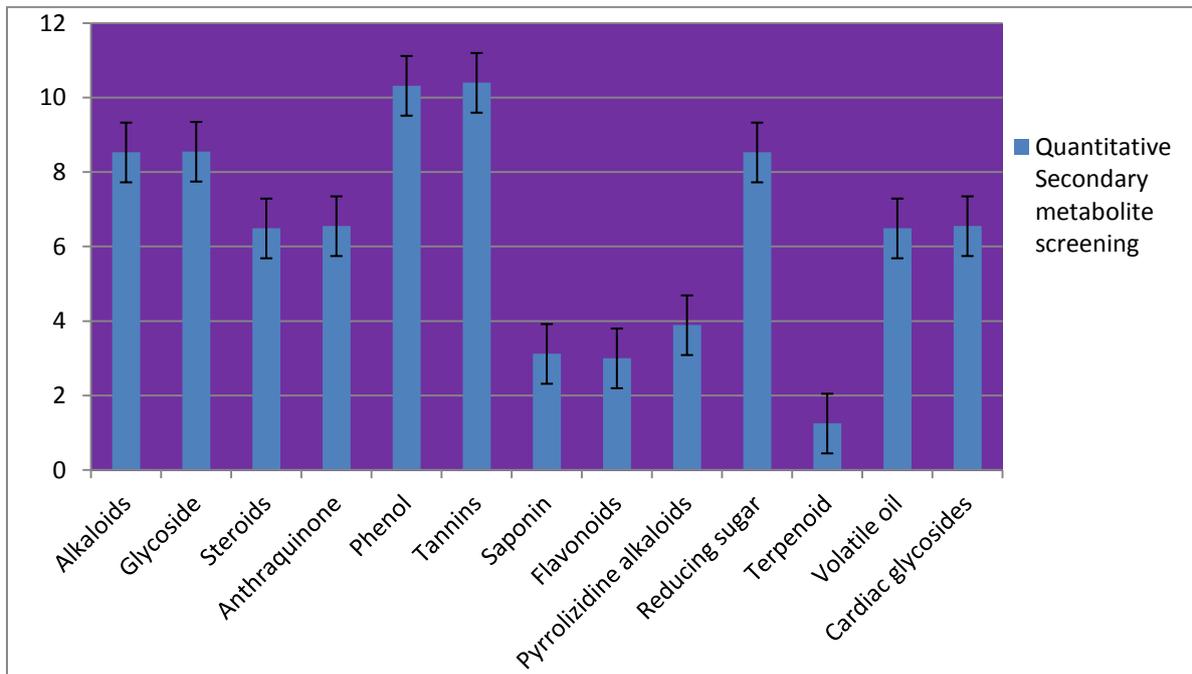


Fig. 9. Quantitative secondary metabolite screening of the ethanol stem bark extract of *Pycnanthus angolensis* stem extract

Table 2. Qualitative secondary metabolite screening of ethanol stem bark extracts of *Pycnanthus angolensis*

Constituent	Presence
Alkaloids	+
Glycoside	+
Steroids	+
Anthraquinone	-
Phenol	+
Tannins	+
Saponin	+
Flavonoids	+
Pyrrolizidine alkaloids	+
Reducing sugar	+
Terpenoid	+
Volatile oil	-
Cardiac glycosides	+

KEY: + = Present, - = Absent and ND = Not Detected

Fig. 11 synergistic percentage frequency distribution of antibacterial activity of conventional toothpaste (Close-up tooth paste) and *Pycnanthus angolensis* against hidden resident mouth cavity microflora isolates. It was observed that *Acidaminococcus* sp.(13%), *Veillonella dispar*(11%), *Veillonella parvula*(11%), *Pepto streptococcus* sp. (12%), *Streptococcus mutans* (13%), *Streptococcus ratti*(11%), *Veillonella atypical* (11%), *Streptococcus sangus* (10%) and *Stomatococcus mucilaginous*.(11%).

Fig. 12 synergistic percentage frequency distribution of antibacterial activity of conventional toothpaste (Oral B toothpaste) and *Pycnanthus angolensis* against hidden resident mouth cavity microflora isolates. It was observed that *Acidaminococcus* sp(12%), *Veillonella dispar* (12%), *Veillonella parvula*(11%), *Peptostreptococcus* sp. (12%), *Streptococcus mutans* (10%), *Streptococcus ratti*(11%), *Veillonella atypical* (9%), *Streptococcus sangus* (11%) and *Stomatococcus mucilaginous*.(12%).

Fig. 13 Synergistic percentage frequency distribution of antibacterial activity of conventional toothpaste (MyMy toothpaste) and *Pycnanthus angolensis* against hidden resident mouth cavity microflora isolates. It was observed that *Acidaminococcus* sp(11%), *Veillonella dispar* (10%), *Veillonella parvula* (10%), *Peptostreptococcus* sp.(11%), *Streptococcus mutans*(14%), *Streptococcus ratti* (10%), *Veillonella atypical* (13%), *Streptococcus sangus*(9%) and *Stomatococcus mucilaginous* (12%).

Fig. 14 Synergistic Percentage frequency distribution of antibacterial activity of

conventional toothpaste (Olive toothpaste) and *Pycnanthus angolensis* against hidden resident mouth cavity microflora isolates. It was observed that *Acidaminococcus* sp. (13%), *Veillonella dispar* (11%), *Veillonella parvula*(11%), *Peptostreptococcus* sp.(13%), *Streptococcus mutans*(9%), *Streptococcus ratti*(11%), *Veillonella atypical* (13%), *Streptococcus sangus*(9%) and *Stomatococcus mucilaginous*.(12%).

4. DISCUSSION

The aim of the study is to evaluate and compare the antibacterial activity of ethanolic stem extract of *Pycnanthus angolensis* and some commercially available toothpaste against bacteria isolated from the hidden resident mouth cavity microflora the resident microflora plays an important in health of human. Microflora acts against the pathogens and protects the body from entering of several microbes. Resident Microflora helps in the metabolism of the body. However, most of these commensals can become pathogenic in responses to changes in the environment or other triggers in the oral cavity, including the quality of an individual's personal hygiene.

In this study, the commensals/ hidden resident mouth cavity microflora the isolated are *Streptococcus sangus*, *Streptococcus ratti*, *Stomatococcus* sp., *Peptostreptococcus* sp., *Streptococcus mutans*, *Veillonella atypical*, *Veillonella parvula*, *Veillonella dispar* and *Acidaminococcus* sp.. This is similar to the report of Rahman et al. [23] and Subramonian et al. [24] who also reported isolation of *Streptococcus* spp. from the oral cavity of human.

Streptococcus sanguinis, also known as *Streptococcus sanguis*, is a Gram-positive facultative anaerobic coccus species of bacteria and a member of the Viridans *Streptococcus* group. *S. sanguinis* is a normal inhabitant of the healthy human mouth where it is particularly found in dental plaque, where it modifies the environment to make it very difficult for other strains of *Streptococcus* that cause cavities, such as *Streptococcus mutans* [25].

Veillonella atypical, a type of bacteria found in the guts of athletes, but not in sedentary people, may be transformed into a probiotic that can enhance health and physical performance in individuals that cannot exercise effectively. *Veillonella* are Gram-negative bacteria (Gram stain pink) anaerobic cocci. This bacterium is

well known for its lactate fermenting abilities. It is a normal bacterium in the intestines and oral mucosa of mammals. In humans they have been implicated in cases of osteomyelitis and endocarditis [26].

Streptococcus ratti is a species of Streptococcus. Streptococcus ratti can be viewed as a type of oral bacteria. It is a type of bacteria that may be found in any healthy individuals. One example may be oral cavities. Streptococcus Ratti is also a component of dental biofilms [27].

Stomatococcus mucilaginous is a Gram-positive, coagulase-negative, encapsulated, non-spore-forming and non-motile coccus, present in clusters, tetrads or pairs, that is a part of the normal oropharyngeal flora. Belonging to the family Micrococcaceae, it was first isolated from the mucous membrane of the cheek and gingiva. It is an oral commensal, that has been linked to causing severe bacteremia in immune-compromised patients. This bacterium has also been shown to form biofilms, similar to that of *Pseudomonas aeruginosa*. *S. mucilaginous* is a cohabitant in the lower airways of patient with bronchiectasis [27,28]

Peptostreptococcus is genus of anaerobic, Gram-positive, non-spore forming bacteria. The cells are small, spherical, and can occur in short chains, in pairs or individually. They typically move using cilia. *Pepto strepto coccus* are slow-growing bacteria with increasing resistance to antimicrobial drugs [29]. *Peptostrep tococcus* species are commensal organisms in humans, living predominantly in the mouth, skin, gastrointestinal, and urinary tracts. They are members of the gut microbiota. Under immune suppressed or traumatic conditions these organisms can become pathogenic, as well as septicemic, harming their host. *Pepto streptococcus* can cause brain, liver, breast, and lung abscesses, as well as generalized necrotizing soft tissue infections. They participate in mixed anaerobic infections, a term which is used to describe infections that are caused by multiple bacteria that do not require or may even be harmed by oxygen [30,31]

Veillonella parvula is a bacterium in the genus *Veillonella*. It is a normal part of the oral flora but can be associated with diseases such as periodontitis and dental caries as well as various systemic infections [32]. *Veillonella* is part of the normal flora of the mouth and gastrointestinal tract. *Veillonella* is often

mistaken for the more serious gonococcal infection. *Veillonella* spp. are often regarded as contaminants; they are often associated with oral infections; bite wounds; head, neck, and various soft tissue infections; and they have also been implicated as pathogens in infections of the sinuses, lungs, heart, bone, and CNS [33].

Streptococcus mutans is a facultative anaerobic, gram-positive coccus (round bacterium) commonly found in the human oral cavity and is a significant contributor to tooth decay. It is part of the "streptococci" (plural, non-italic lowercase), an informal general name for all species in the genus *Streptococcus*. *Streptococcus mutans* is a Gram-positive bacterium that lives in the mouth. It can thrive in temperature ranging from 18-40 degrees Celsius. The bacterium metabolizes different kinds of carbohydrates, creating an acidic environment in the mouth as a result of this process. This acidic environment in the mouth is what causes the tooth decay. It is the leading causation of dental caries (tooth decay) worldwide. *S. mutans* is considered to be the most cariogenic of all of the oral *Streptococcus* [34].

The Some microorganisms that colonize humans are commensal, meaning they co-exist without harming humans; others have a mutualistic relationship with their human hosts. The organisms has a living relationship with other organism, to obtain nutrient and benefit from the other organism without harm on the dependant organism and they are part of the normal flora of the mouth. it is an observable fact that the conventional toothpaste may not be able to remove completely all this resident organisms and commensals in the mouth cavity, this is one of the reasons, conventional toothpaste should be fortified with medicinal plants like *Pycnanthus angolensis*, to improve the oral cleanliness of the mouth cavity and remove both the commensal and hidden oral cavity bacteria [11].

In comparing the activity of medicinal plant '*Pycnanthus angolensis*' and conventional toothpaste, it can be observed that the zones of inhibition of *Pycnanthus angolensis* stem bark extract is more elevated than the conventional toothpaste. this is to say that the arrays of chemical present in *Pycnanthus angolensis* is much more than the convectional toothpaste. Conventional tooth paste contains Sodium fluoride(Close up toothpaste), Sodium phosphate, Trisodium phosphate, sodium

fluoride (Oral B toothpaste), Sodium mono fluoro phosphate, (MyMy toothpaste and Sodium carboxymethyl, sodium fluoride (Olive toothpaste), this active ingredient are derivative of plant extract and couple of this active ingredient can be found naturally in medicinal plant in large quantity, in order word, it is better to use medicinal plant like *Pycnanthus angolensis* with relatively high arrays of chemical than conventional tooth .paste with just one synthetic active ingredient [16].

Moreover, medicinal plant like *Pycnanthus angolensis* do not have any side effects like gum irritation, gum bleeding, gingivitis and etc, compared to convectional toothpaste, during use, many side effects may arises. With the observable features of this research work, it is better to combine both medicinal plants like *Pycnanthus angolensis* and conventional toothpaste for adequate oral hygiene and removal of hidden mouth cavity microflora to prevent tooth decay, dental plaques and tooth removal.

The result of the assay for antibacterial activity of *Pycnanthus angolensis* against the resident mouth cavity microfora showed that the plant has inhibitory effect on the growth of oral bacteria. This is because most of the bioactive secondary metabolites are present in the extract in appreciable quantity and quality. Stem-bark of *Pycnanthus angolensis* contains secondary metabolites i.e phytochemicals such as tannin, flavonoid, anthraquinone and phenols. Stem-bark of *Pycnanthus angolensis* has inhibitory effects on resident mouth cavity microfora, plant are traditionally reported to remedy to oral bacterial. Tannins, flavonoids and phenolic compounds have been the major phytochemicals associated with antibacterial activity in medicinal plants [35]. They protect against allergies, inflammations, microorganisms, ulcers, viruses and tumours (Okwu and Omodamiro,2005;41); [36].

Tannins are antiseptic, have astringent properties and hasten the healing of wounds in an inflamed membrane as the wounds are free from attack of parasitic fungi, insects and yeasts infections [11]. Flavonoids, a water soluble, super antioxidant and free radical scavenger as antioxidant, anticarcinogens, antimicrobial, anti tumor. Saponins were observe to have bitter taste, foaming property, haemolytic effect on red blood cells and emulsifying agent. It was also observed that saponin containing plants are good

expectorant, cough suppressant, hemolytic activity. Tannins were observable in medicinal plant under study.they have unpleasant taste, tans leather, used in the production of leather and ink, in treating wounds, varicose ulcers, hemorrhoids, frostbite and burns, and it has a soothing relief, regenerates skin, anti-inflammatory and diuretics in its activity [16].

Some strains such as *Streptococcus salivarius* produce hats bacteriocin called as salivaricin. It shows activity against Group A streptococci [37]. Production of bacteriocin by such strains in the pharynx will reduce bacterial colonies in the mouth. Similarly, many oral bacteria produce other inhibitors such as hydrogen peroxide, volatile fatty acids, they change local environmental conditions (e.g. redox potential or pH), which may exclude exogenous species and suppress opportunistic pathogens. For example, the production of hydrogen peroxide by the members of the *Streptococcus mitis* which can suppress the growth in the dental plaque of periodontal pathogens, such as *Acidaminococcus* spp. Several clinical studies have demonstrated the efficacy of toothpastes against oral and gingival bacteria [38].

The results of this present study revealed that commercially available oral toothpastes exhibited wide variation in their effectiveness against oral bacteria and the Oral B toothpaste brand establish superior inhibition activity against oral bacteria isolated from oral swab samples. Triclosan and Fluoride containing Toothpastes were found to be highly effective against the oral acidogenic bacteria [39] and were recommended by the WHO and FDI. Also, it should be mentioned that the inhibition effect of the toothpastes may not be unconnected with the different active ingredients which can diffuse at different rates [40]. Use of fluorides has been the foundation of caries counteractive action and the use of fluoridated toothpaste is the most widely recognized types of caries control being used today. Many commercial toothpastes claim to have abrasive, spreadability, foaming ability and have caries counteractive action. Since it is now known that dental plaque is made of large numbers of commensal bacteria together with a limited number of pathogens [41,28,42], such an approach may not be effective since the "remove all or kill all" approach creates open, non-competitive surfaces for pathogens to repopulate the oral cavity.

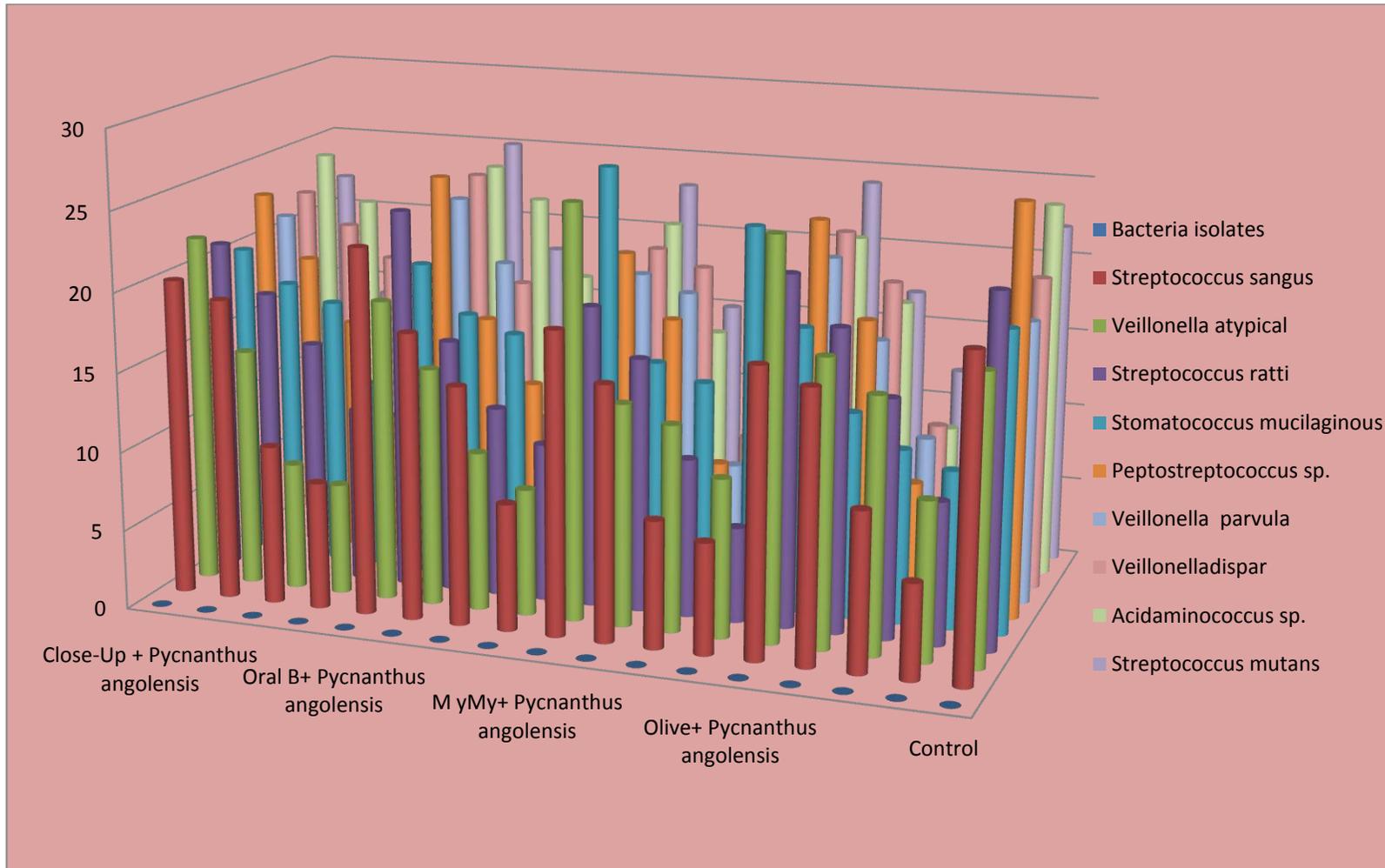


Fig. 10. Synergistic Antibacterial activities of different conventional toothpaste and *Pycnanthus angolensis* stem bark extract against hidden resident mouth cavity isolates

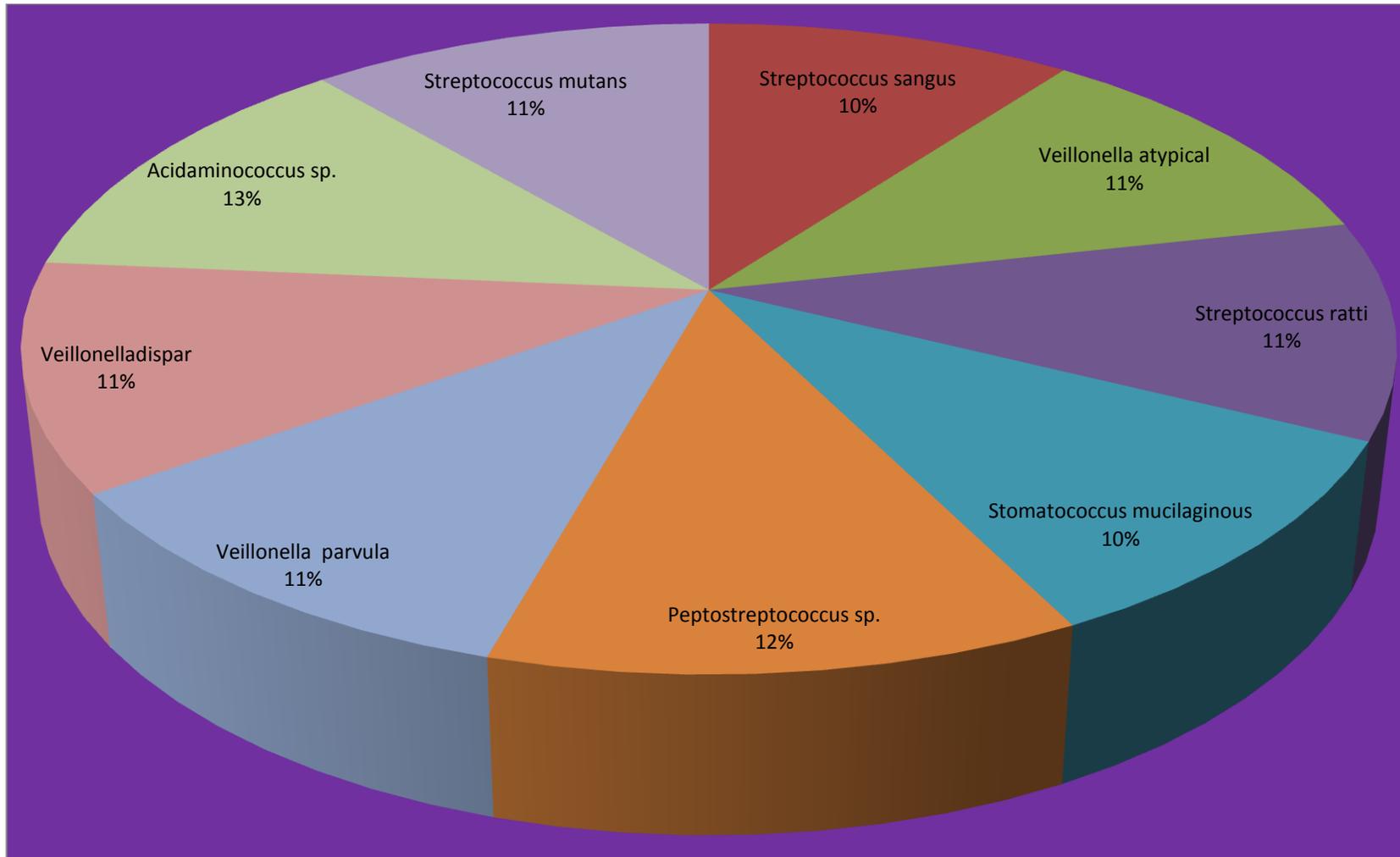


Fig. 11. Synergistic percentage frequency distribution of antibacterial activities of conventional toothpaste(Close up) and *Pycnanthus angolensis* stem bark extract against hidden resident mouth cavity isolates

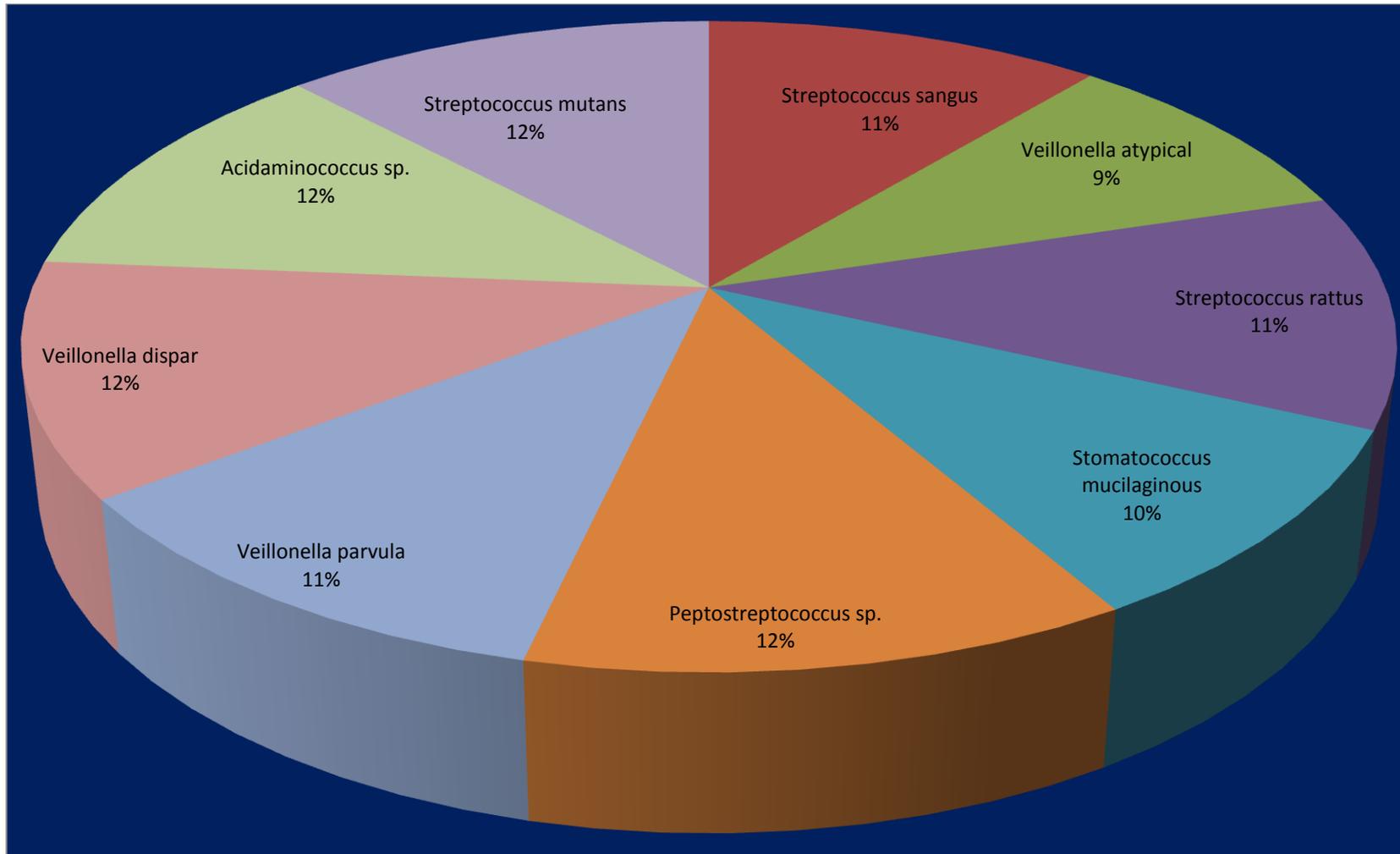


Fig. 12. Synergistic percentage frequency distribution of antibacterial activities of conventional toothpaste (Oral B) and *Pycnanthus angolensis* stem bark extract against hidden resident mouth cavity isolates

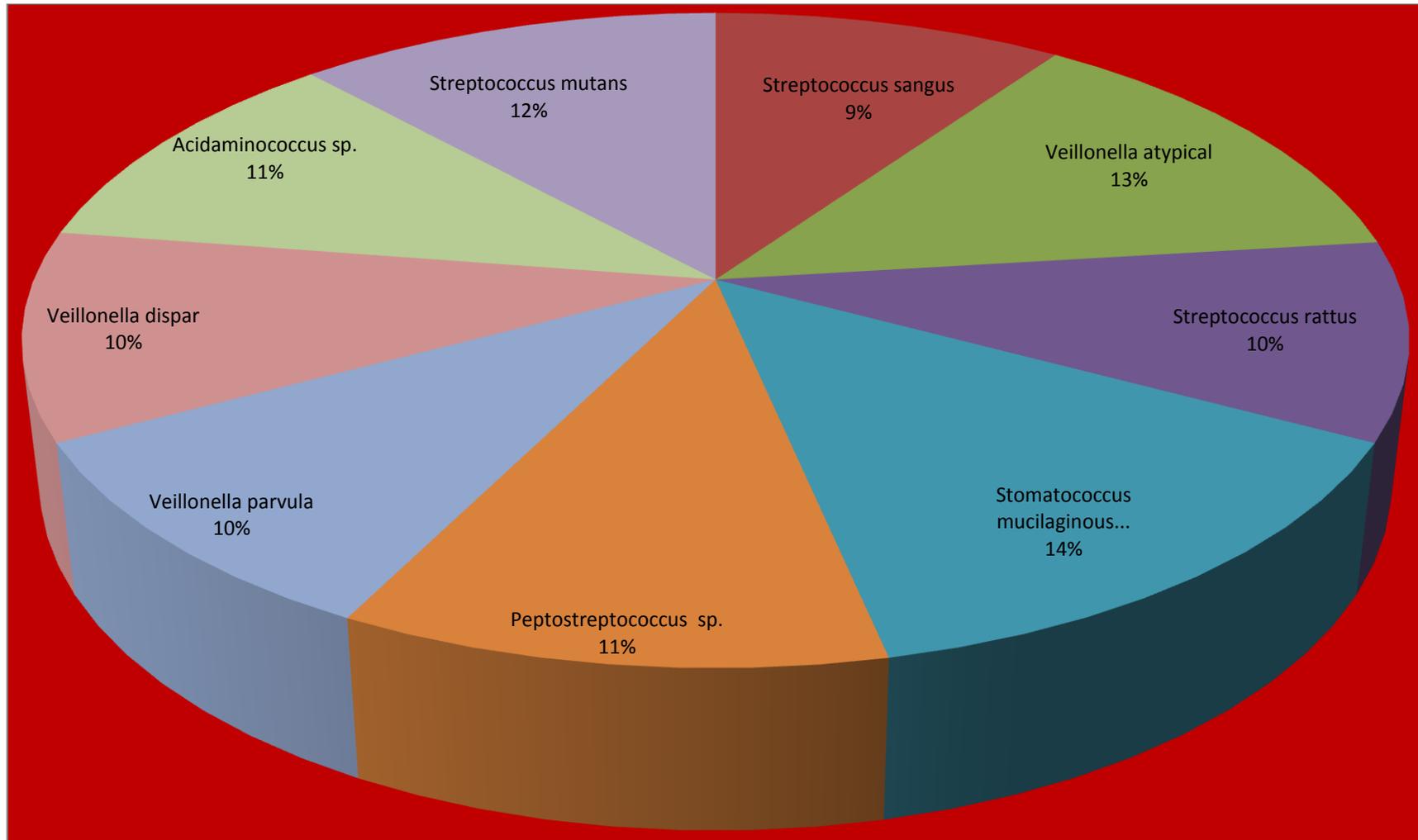


Fig. 13. Synergistic percentage frequency distribution of antibacterial activities of conventional toothpaste (MyMy) and *Pycnanthus Angolensis* stem bark extract against hidden resident mouth cavity isolates

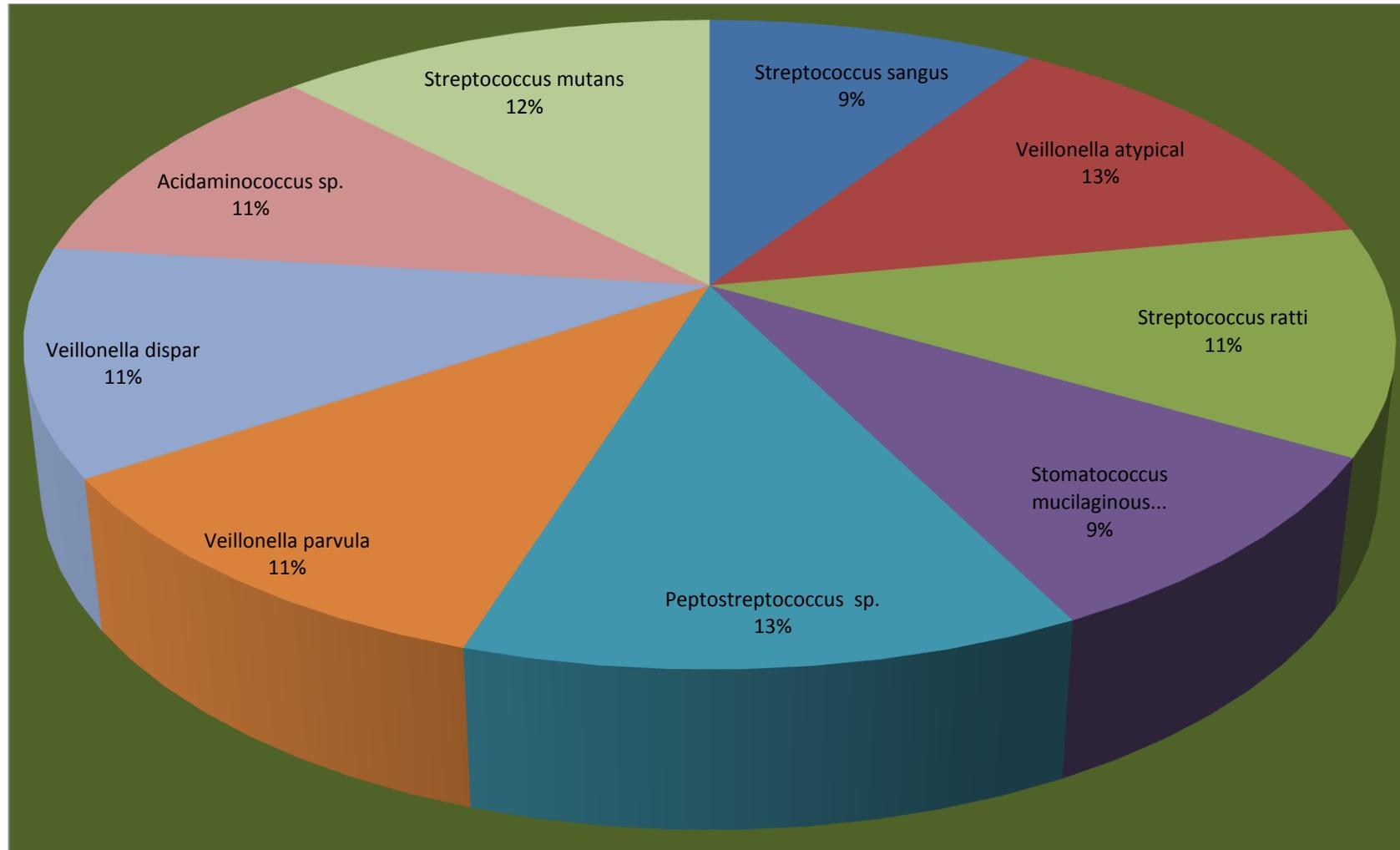


Fig. 14. Synergistic percentage frequency distribution of antibacterial activities of conventional toothpaste(Olive) and *Pycnanthus Angolensis stem bark extract* against hidden resident mouth cavity isolates

It should be mentioned here that the conventional toothpaste has a limited action on resident human oral cavity bacterial, because of the approach of 'remove all and kill all' which has a short coming, the oral bacterial finds a way to repopulate the mouth due to our diet and hidden resident mouth cavity microfora, there is need to reformulate the convention toothpaste with medicinal plants used as chewing sticks, this will help the efficacy of the conventional toothpaste with less side effect of bacterial re-population, therefore medicinal plants used as chewing stick become a veritable tools for conventional toothpaste reformulation [43]. Many plant species are used as chewing sticks and natural tooth brush. Certain plants are used for management of gum bleeding, toothache, sores in mouth and bad breath. Stem, young twigs, leaves, bark, fruit, spines, seeds and latex are the parts of plants being exploited for oral health care [11, 43]. It is clearly stated that secondary metabolite plays a tremendous role in our health and physical well being especially against this hidden resident mouth cavity microflora, this brings the knowledgeable fact that medicinal plant like *Pycnanthus angolensis* should be added to our convention toothpaste to improve the functional ingredient of the toothpaste.

5. CONCLUSION

In conclusion, Plant-based traditional knowledge has become a recognized tool in search for new sources of drugs. It is clear that the use of these herbal plants can offer a platform for further research.

DISCLAIMER

The products used for this research are commonly and predominantly use products in our area of research and country. There is absolutely no conflict of interest between the authors and producers of the products because we do not intend to use these products as an avenue for any litigation but for the advancement of knowledge. Also, the research was not funded by the producing company rather it was funded by personal efforts of the authors.

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ACKNOWLEDGEMENT

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

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