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CRUDE AND ETHANOL EXTRACTS OF *ANACARDIUM OCCIDENTALE* AND *DENNETTIA TRIPETALA*: EFFECTS ON ORAL ORGANISMS AND POSSIBLE INCLUSION IN TOOTHPASTES.

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ABSTRACT

Oral infections continue to pose health threats despite improvements in oral hygiene in recent times. This work was aimed at studying the antimicrobial effects of *A. occidentale* and *D. tripetla* which are used in the treatment of oral infections locally. Crude and ethanol extracts of *Anacardium occidentale* and *Dennettia tripetala* were tested *in vitro* using Kirby-Bauer disc diffusion method on *Streptococcus mutans, Staphylococcus aureus, Pseudomonas aeruginosa, Escherichia coli and Lactobacillus acidophilus* isolated from patients attending Anambra State Teaching Hospital, Amaku, Awka, Dental Clinic. A new toothpaste (NT) was prepared according to formulations by Colgate-Palmolive® Thailand with commercial toothpaste ingredients and tested alone and in combination with extracts on the organisms. All extracts showed antimicrobial activity on isolates with mean zones of inhibition ranging from 7±1.00 mm of ethanol extract of *D. tripetala* in *S. aureus* and *P. aeruginosa* to 26±0.71mm of ethanol extract of *A. occidental* in *S. mutans*. *S. aureus* showed the highest sensitivity of 28.0% to all extracts as compared to *E. coli* of 13.0%. Activity of *D. tripetla* was generally low. Minimum Inhibitory Concentrations (MICs) ranged from 31.25mg/ml ethanol extract of *A. occidentale* in *S. mutans* to 500mg/ml of aqueous extract of *D. tripetala* on *E. coli* while the Minimum Bactericidal Concentration (MBC) ranged from 500 and above for most of the extracts. The newly formulated toothpaste exhibited little activity on the isolates and there was a significant difference ($p< 0.05$) in its activity when the extracts were

added to it. These extracts have potentials for use by industries to minimize reliance on synthetic antibiotics.

KEYWORDS: Oral infections, *A. occidental, D. tripetala*, Antimicrobial activity, Toothpaste.

INTRODUCTION

Dental caries is a microbial disease that result in the destruction of mineralized tissue of the teeth and is the most prevalent and costly oral infectious disease worldwide.[1,2] Dental plaque deposit on teeth is a concern for both cosmetic and its pathogenic nature. Presence of plaque may be the culprit for dental caries, gingivitis, periodontal problems, and halitosis.[3] Mechanical plaque control measures such as toothbrushes, dental floss, toothpicks and interdental brushes are very popular, most accepted methods of controlling plaque, and are mostly used in conjunction with chemical plaque control aids like mouth rinses and medicated toothpastes.[4] Several chemical preventive agents have beneficial effects in the control of plaque and to reduce or prevent oral disease. Chemicals, mainly triclosan and chlorhexidine, have been added in mouth rinses and dentifrices to prevent plaque and gingivitis. But some of these substances show undesirable side effects such as vomiting, diarrhoea, tooth staining and altered taste.[3,5] Not minding the effectiveness of many toothpastes formulations with antibacterial properties, there is an increasing societal desire to rely on naturally occurring compounds for health care which has also found its way into dentistry.[6,7] This had led to paying increased attention on using natural novel anti-infective ingredients in herbal dentifrices. Herbal ingredients have several benefits; chamomile has anti-inflammatory effect, echinacea has immune stimulatory property, sage and rhatany have anti-hemorrhagic properties, myrrh is a natural antiseptic, and peppermint oil has analgesic, antiseptic, and anti-inflammatory properties.[3,8]

The mouth harbours a diverse microbial community which inhabits the various surfaces of the normal mouth. The flora of normal healthy dentate mouth has 85% *Streptococci*, *Veillonella*, Gram positive diptheroids, Gram negative anaerobic rods, 5-7% *Neissaeria*, 2% *Lactobacilli*, 2% filamentous bacteria, 1% *Staphylococci* & *Micrococci*. The remainder are other bacteria, fungi, protozoa & viruses.[9]

Dentifrices generally have a similar basic formulation and most toothpastes are available in paste form. A few powder dentifrices are available containing abrasives, surfactants,

flavoring, coloring agents and sweeteners. Toothpastes contain all these agents as well as binding agents, humectants, preservatives and water.[10] In addition, some herbal toothpastes also exist which has other components like natural lemon extract, flavor containing natural blend of mint, eucalyptus, rosemary, chamomile, sage, myrrh & other natural oils depending on the manufacturer.[11]

Dennettia tripetala is a plant which belongs to the Annonaceae family. It has edible fruits and is rich in vitamin C. The leaves have found importance in folk medicine in the treatment of fever, cough, asthma catarrh, diarrhea and rheumatism. It has been used for a long time in the treatment of toothaches.[12,13] *Anacardium occidentale* is a medicinal plant. Many parts of the plants are used in the traditional medicine of the Patamona of Guyana. They grind the seeds into poultice for treating snakebites, apply nut oil to cracked heels or as an antifungal agent, and use the fruits, bark, and leaves for many other purposes including anti-fungal activity, for sores and rashes, or as an antipyretic, and for antidiarrheal applications.[14] *Anarcardium occidentale* and *Dennettia tripetala* are rich in many phytochemicals as has been reported by many authors and are used in the treatment of oral infections in the Southern and Eastern part of Nigeria.[12,13] The aim of this research therefore is to scientifically determine their antimicrobial activities against selected oral micro-organisms as no such work has been carried out. The establishment of such will be of immense importance because the plants are readily available and have some pleasant flavours which can substitute some synthetic flavours presently used in toothpastes.

MATERIALS AND METHOD

Plant material

D. tripetala seeds were purchased from a local market in Awka and immature leaves of *A. occidentale were procured from Agu –Ukwu Nri all in* Anambra State Nigeria. These samples were identified in Botany department of Nnamdi Azikiwe University, Awka, Anambra State.

Test Organisms

Clinical oral organisms: *Streptococcus mutans, Staphylococcus aureus, Pseudomonas aeruginosa, Escherichia coli and Lactobacillus acidophilus* obtained from Anambra State University Teaching Hospital, Amaku, Awka, Dental Clinic were used for the study. Confirmation tests were carried out on the organisms according to the methods of.[15]

Preparation of plant extract

Immature freshly plucked *A. occidentale* leaves and fresh mature fruits of *D. tripetala* were dried in the oven at 40°C for 5days. The dried fruits of *D. tripetala* were dehulled mechanically. These plant materials were ground into powder using electric blender. 50grms each of the powder were soaked separately in 500mls of distilled water and absolute ethanol for 6 h and 24h respectively with intermittent shaking and filtered with a mess sieve followed by further filtration with Whatman No 1 filter paper. The supernatants were collected and evaporated on an evaporator at 40^0C.The extracts were carefully scraped off unto sterile pre-weighed sterile universal bottles and then re-weighed. The difference in the weights for each is the weight of the crude extracts. They were stored in air tight containers until when needed.[16]

Sterility Testing

The sterility of the extracts was checked by streaking on Nutrient agar plates and incubating for 24-48 hours. Uninnoculated sterile Nutrient agar plates were kept for media sterility control.

Preparation of Turbidity Standard

A 0.5 McFarland Standard was prepared by adding 0.5ml of 0.048M $Bacl_2$ (1.17% w/v $Bacl_2$2H2O) to 99.5 ml of 0.18 M H_2SO4 (1% v/v) with constant stirring. A barium sulphate precipitate was checked for optical density using matches curvettes with 1 cm path and distilled water as a blank standard. A UV-Vis spectrophotometer was used to measure the absorbance at 625nm. An absorbance of 0.1 was obtained which was in the accepted range of 0.08-0.13. The approximate cell density corresponding to 0.5 McFarland is 1×10^6 cells/ml.

Standardization of the Test Organisms

The organisms were inoculated and incubated in nutrient broth for 24 hours. The turbidity resulting from this was adjusted to 0.5 McFarland turbidity standard using the same Nutrient agar broth medium. The broth culture was diluted 1:200 by mixing 0.1ml of the inoculums and 19.9ml of the broth. This gives working inoculums that should contain 10^5-10^6 cells/ml within the 30 minutes it was used.

Sensitivity Screening

Sensitivity screening was carried out by the method of.[18] Briefly antimicrobial activities of the extracts were tested using Mueller-Hinton Agar (MHA). Sterile discs (6mm in diameter)

were made from Whatman No 1 filter paper impregnated with 0.2ml of 100mg/ml prepared by homogenizing 0.5g of extract in 5mls of dimethyl sulphoxide (DSMO). The discs were allowed to dry in the oven at 40^0C aseptically. Sterile swab sticks were used to inoculate 0.2ml of the standardized test organisms evenly on solidified Mueller-Hinton Agar plates. The inoculated plates were allowed to dry for ten minutes. Then sterile forceps was used to place the impregnated discs on the surface of the solidified agar. This was done in duplicate.[19,20] Discs impregnated with ciprofloxacin (5µg/ml) served as positive control whereas discs saturated with sterile water served as negative control. This was incubated for 24hrs at 37^0C and the zones of clearance were measured in (mm) using a ruler and recorded.

Determination of Minimum Inhibitory Concentration (MIC)

The MIC values were determined by broth dilution assay. Sterile homogenized extracts were serially diluted (two-fold) in sterile Nutrient broth in test tubes for the organisms to obtain a concentration range of 500mg/ml to 31.25mg/ml. Then 0.1ml of each standardized test organism was added to each of the test tubes and the preparation was incubated at 37^0C for 24 hours. Negative controls were equally set up using broth cultures of test organisms without extracts. Tubes with medium only were set as controls for sterility of the medium. Test tubes were evaluated for the presence or absence of visible turbidity in the broth after the incubation period. The lowest concentration (highest dilution) of the mixture preventing appearance of turbidity (growth) was considered and recorded as the MIC.[21]

Determination of Minimum Bactericidal Concentrations (MBC)

From the tubes showing no visible growth or turbidity in MIC, 0.1 ml of the suspension was inoculated onto sterile Nutrient agar. The plates were incubated at 37^0C for 24hours. The least concentration that did not show any visible growth of the test microorganism was considered as the MBC for the organisms. A plate with media only was set as negative control to check the sterility of the media.[22]

Preparation of Toothpaste

The toothpaste was prepared according to formulations of.[23] The ingredients and their quantities are as stated in the table below;

Table 1: Toothpaste Ingredients and their Quantities

Ingredients	Functions	%(w/w)
Phase A		
Xanthan/ Cellulose gum	Thickner	0.8
Sorbitol	Humectant	23.38
Glycerin	Humectant	25.0
Phase B		
Deionized water		17
Sodium fluoride	Anticaries agent	0.22
Sodim benzoate	Preservative	0.3
Phase C		
Silica	Abrasive	21
Titanium dioxide	Whitening agent	0.5
Pepper Mint	Flavouring agent/antiseptic	0.8

All reagents were of analytical and edible grade

The procedure used in preparing the toothpaste according to[23] is as follows:

Phase A: Disperse Xanthan gum/Cellulose gum in sorbitol under intensive stirring. Phase B: Dissolve all additive in water, whilst stirring during 10 minutes. Add B to A and keep stirring during 45minutes until a homogenous gel is formed. Then gradually add C to A and B. Keep stirring the mixture slowly during 30 minutes. At last introduce the surfactant while continuing stirring during 5 minutes. The process was aseptically carried out. The newly prepared toothpaste was sterilized and kept till when needed.

Sterility Testing

The sterility of the new toothpaste was checked by streaking on Nutrient agar plates and incubating for 24-48 hours. Uninnoculated sterile Nutrient agar plates were kept for media sterility control.

Sensitivity Screening of the New Toothpaste (NT).

The sensitivity screening of NT was carried out as described by.[2] Briefly, with the NT regarded as solid, 0.5grams of NT was weighed aseptically and dissolved in 5mls of distilled water to obtain 100mg/ml of the toothpaste. The sensitivity of toothpaste was tested against the test organisms also using the Kirby-Baeur disc diffusion assay described above. 100mg/ml each of the extracts was mixed with the NT and sensitivity also tested. Medium, culture method, incubation time and temperature were as employed in the sensitivity test for the extracts.

Statistical Analysis

The tests were carried out in duplicates and values for the diameter of the zone of inhibition reported as mean ± standard deviation. Also, the data obtained were subjected to one-way ANOVA using Statistical package for Social Science (SPSS) 15.0 for Windows Evaluation, Version 2006. *p*-values < 0.05 were considered statistically significant.

RESULT AND DISCUSSION

The disc diffusion method was used to determine the antimicrobial potency of *A. occidentale* and *D. tripetala* against clinical isolates; *Streptococcus mutans, Staphylococcus aureus, Pseudomonas aeruginosa, Escherichia coli and Lactobacillus acidophilus* isolated from patients attending Anambra State Teaching Hospital, Amaku, Awka, Dental Clinic. The result of the antimicrobial activity of the extracts on test microorganisms is presented in table 2. The extracts had varying degrees of activities against the test organisms. *Streptococcus mutans* showed the highest sensitivity with an inhibition zone diameter of 26.50±0.71mm for ethanol extract of *A. occidentale*, followed by *L. acidophilus of* 24.0±1.0mm for ethanol extract of *A. occidentale and Streptococcus mutans of* 22.0±1.14 for aqueous extract of *A. occidentale*. These observations indicate the presence of antibacterial activity, which confirms their medicinal potential and more interestingly the fact that ethanol extract of *A. occidentale* could compare with the antimicrobial activity of a standardized drug ciprofloxacin.[16] The ability of aqueous and ethanol extract of plants to exhibit antimicrobial activity has been previously reported.[24] *D. tripetala's* antimicrobial activities were generally low as compared to *A. occidentale*. The finding in this study is in agreement with that of[13] which evaluated the activities of the leaves on the same plant and reported it as high antimicrobial activity but the inhibition zone is not very different from that of the present study. The ethanol extracts in this study recorded more activity that the aqueous extracts and this agrees with the work of[25] that though carried out his work with methanol also recorded higher activity of the methanol extracts than the aqueous extract. The ability of the extracts to inhibit the growth of the test microorganisms might be as a result of the presence of bioactive substances (alkaloids, flavonoids, phenols saponins, steroids and tannins) in their leaves.[13,26] The MIC and MBC of the extracts on the test organisms is shown in table 3. The MIC ranges from 31.25mg/ml ethanol extract of *A .occidentale* on *S. mutans* to 500mg/ml of aqueous extract of *D. tripetala* on *E. coli* while the Minimum Bactericidal Concentration *(*MBC) ranged from 500 and above for most of the extracts. The MIC and the MBC are results are in partial agreement with the works of.[16]

Despite various reports on the potent in vitro antimicrobial activity of some of these plants, the findings of this study only indicates mild to moderate in vitro antimicrobial activity since any compounds/extracts with potent in vitro pharmacological activity (that is, antimicrobial) should exert their activity at EC50/IC50 value of less than or equal to 30µg/ml (≤ 30µg/ml).[27] This discrepancy could be due to the type of extract used in the antimicrobial screening phase.[16] However these agents could still be useful as the oral flora of the mouth need not be eliminated but maintained in a healthy state. So it is expected that agents used in the oral products should be bacteriostatic and not bactericidal. The newly prepared toothpaste was of fine form and typical of conventional toothpastes save for colour. The ingredients used in the preparation of the toothpaste are shown in table 1. The antimicrobial activity of the neat toothpaste and different combinations of the new toothpaste and extracts is presented in table 4. The toothpaste had some degree of activity probably due to the presence of sodium fluoride which has dual functions in toothpaste; as remineralizing and anticaries agent and pepper mint.[3,8] The addition of the extracts as indicated in the table increased the activity of the toothpaste but it should be noted that the combined effects is not arithmetic sum of the effects of the neat toothpaste and the extracts. This suggests that some components of the toothpaste may be militating against the effect of the extracts. It is interesting to record the effect of the extracts especially *A. occidentale* on *S. mutans* which is a major culprit in dental caries. Natural products offer a rich source of structurally diverse substances with a wide range of biological activities, which could be useful for the development of alternative or adjunctive anticaries therapies. However, it is a challenging approach owing to complex chemistry and isolation procedures to derive active compounds from natural products.[1]

CONCLUSION

Conclusively, the present finding confirms scientifically the antimicrobial activity of the extracts on clinical oral organisms and that they are useful resource in the production of natural dentifrice. Though the extracts of *D. tripetala* were not as effective, they could still be harnessed for theirs pleasant aroma in the preparation of dentifrice. However, further studies are needed to isolate the active components of these extracts. Animal studies are also recommended to further authenticate the use of the extracts on living organisms.

Table 2: In-vitro Antimicrobial Activity of Extracts against the Test Microorganisms (size of disc 6mm)

	AEAO	EEAO	AODT	EEDT	CPX
S. aureus	22.0±1.14	20.0±1.41	8.3±1.52	20.3±1.15	26.5±1.29
S. mutans	12.5±2.12	26.5±0.71	8.3±1.52	20.5±1.15	27.5±1.29
P. aeruginosa	11.5±0.71	12.3±0.71	8.3±1.52	9.00±1.15	20.25±1.71
E. coli	8.0±1.41	12.3±0.57	6.67±1.15	8.00±1.15	24.0±1.82
L. acidophilus	11.0±1.41	24.0±1.00	7.00±1.00	8.00±1.15	23.0±1.29

Key:

AEAO = Aqueous extract of *A. occidentale*, EEAO = Ethanol extract of *A. occidentale*

AODT = Aqueous extract of *D. tripetala*, EEDT = Ethanol extract of *D.tripetala*

CPX = Ciprofloxacin

Table 3: In-vitro respective Minimum Inhibitory Conc. (MIC) and Minimum Bactericidal Conc. (MBC) of extracts on the Test Microorganisms (mg/ml).

	AEAO	EEAO	AODT	EEDT
S. aureus	125, 500	62.5, 125.0	250,>500	250,500
S. mutans	125, 250	31.25,125	125,>500	62.5,250
P. aeruginosa	250,500	125.0,500	250,>500	250,500
E. coli	125, >500	250,>500	500,>500	125,500
L. acidophilus	125,500	62.5,500	125,500	62.5,500

Key:

AEAO = Aqueous extract of *A. occidentale*, EEAO = Ethanol extract of *A. occidentale*

AODT = Aqueous extract of *D. tripetala*, EEDT = Ethanol extract of *D. tripetala*

Table 4: In-vitro Antimicrobial effects of New Toothpaste only and different Combinations of Extracts (size of disc 6mm)

	NT	NT+EEDT	NT+AEAO	NT+EEAO
S. aureus	16.0±1.14	26.0±2.12	26.5±0.707	25.5±1.06
S. mutans	18.5±0.70	27.75±1.06	25.5±0.707	31.5±1.06
P. aeruginosa	13.5±2.12	17.25±1.06	19.5±0.707	20.5±1.06
E. coli	10.5±0.70	16.75±1.06	17.0±1.141	19.0±0.70

Key: NT = New toothpaste, NT+EEDT = NT and ethanol extract of *D. tripetala*

NT+ AEAO = NT and aqueous extract of *A. occidentale* and

NT+ EEAO = NT and ethanol extract of *A. occidentale*

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