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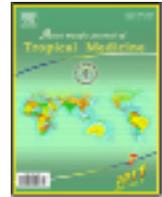
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Anticariogenic potentials of clove, tobacco and bitter kola

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ABSTRACT

Objective: To investigate three tropical plant materials – clove seeds [*Syzygium aromaticum* (*S. aromaticum*)], bitter kola fruits [*Garcinia kola* (*G. kola*)] and tobacco leaves (*Nicotiana* species) as potential targeted killers of *Streptococcus mutans* (*S. mutans*), a cavity-causing bacterium (gram-positive, facultative anaerobe) that resides in a multispecies microbial community (dental plaque) for the treatment of dental caries (tooth decay). **Methods:** Thirty one (31) teeth samples were collected from patients with obvious signs of tooth decay (swollen gum, weak or fallen tooth, etc.) using sterile swab sticks. These samples were collected from two major dental clinics in Nsukka, Enugu State, Nigeria and investigated by spread inoculation onto sterile blood agar and Mueller Hinton agar (MHA) respectively and incubated at 37 °C for 24 h. The discrete colonies obtained were further re-inoculated onto sterile Mitis salivarius agar (MSA) plates and incubated as above. The isolates were characterized by gram staining and catalase test. Tobacco leaves, clove seeds and bitter kola fruits were ground into powder, extracted with three different solvents (*n*-hexane, hot water and ethanol), filtered, dried and stored in clean containers, corked and kept until used. The plant extracts were investigated for phytochemistry, minimum inhibitory concentration (MIC), minimum cidal concentration (MCC) and compared with some conventional antibiotics commonly used against tooth decay. Antibiotic sensitivity test was also carried out. The results were statistically analyzed. **Results:** The extracts showed varied phytochemical composition but most abundantly the flavonoids. Our result also shows that females (16) have more tooth decay than males (15) and that 16 samples were very bloody while 15 were slightly bloody. The microbial characterization showed that 18 samples were catalase-positive indicating the presence of *S. mutans* while 13 were catalase-negative suspected to be *Staphylococcus* spp. The Gram reaction confirmed 13 Gram-negative and 18 Gram-positive organisms. The *n*-hexane extract had the best antimicrobial activity followed by the ethanol and lastly hot water. MIC showed that *n*-hexane clove extract had the largest inhibition zone diameter, followed by bitter kola extract and lastly tobacco extract. The antibiotic sensitivity test credited ciprofloxacin the best because it exhibited broad spectrum of action. **Conclusions:** Since the *n*-hexane extract of clove seeds demonstrated preferential growth-inhibitory activity against the causal cariogenic pathogens (*S. mutans*) in dental caries, we therefore, report here that clove extract be henceforth considered as a potential ingredient in toothpaste preparation.

1. Introduction

The human oral cavity contains complex and multispecies microbial communities^[1–6]. This means that the community residents should display extensive interactions while forming biofilm structures, carrying out physiological

functions, and inducing pathogenesis^[7]. Pathogenesis of dental caries suggests that *Streptococcus mutans* (*S. mutans*) metabolize sucrose more efficiently than other common oral bacteria making it an important resident in caries-associated biofilms^[8]. Unfortunately, many human diets are composed of high proportion of sucrose which makes *S. mutans* have a competitive advantage relative to other oral bacteria by converting sucrose to glucan (an adherent molecule) which promotes the attachment of the organism to the teeth as well as its rapid conversion of sucrose to lactic acid (an aciduric property) which gives it additional mechanism for successful competition^[9–11].

In order to eliminate or reduce dental caries, numerous

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studies have been focused, through various means, on the prevention of *S. mutans* colonization. Vaccination is yet to yield effective result as well as outright sterilization of the oral cavity which rather result in secondary infections and resistance evolutions^[12–14]. Current dental therapy is primarily focused on removing the whole dental plaque which rather removes both commensal bacteria together with oral pathogens thereby creating niches for pathogens to repopulate the oral cavity. The other approach of inhibiting adherence with antagonists such as use of sucrose analogues to reduce caries susceptibility^[15], as well as the enzyme dextranase, to reduce the synthesis of glucan molecule (adherent) from sucrose by *S. mutans* has been successfully incorporated in toothpastes to reduce dental caries in children^[16]. Also replacement of cariogenic *S. mutans* by competitor noncariogenic strains which produce a bacteriocin active against other *S. mutans* strains naturally occurring cariogenic strains has been assessed with effective result but awaiting evaluation of efficacy in humans^[17]. Other probiotic approaches abound and are prospective^[18]. Interference with signaling mechanisms have also been investigated as prospective means of reducing the colonization of the oral cavity by *S. mutans*^[14,19]. Within the repertoire of antibiotics available to a prescribing clinician, the majority affect a broad range of microorganisms, including the normal flora. The ecological disruptions therefore results in secondary infections or other negative clinical consequences. To address this problem in the realm of achieving a targeted killing of *S. mutans*, we reasoned that certain natural herbs such as cloves [*Syzygium aromatic* (*S. aromatic*)], bitter kola [*Garcinia kola* (*G. kola*)] and tobacco leaves (*Nicotiana* Spp.) which have some folkloric claims of activity against dental caries^[20], be substantiated in this study, from where another lead molecule may probably be discovered for cure or treatment of dental caries. Therefore, the potential effectiveness of the different plant extracts on *S. mutans* and other organisms incriminated in this investigation would be compared with the activity of some conventional antibiotics commonly used against tooth decay.

2. Materials and methods

2.1. Materials

All materials used for this study were of analytical grade as obtained from their local suppliers and as stock in Microbiology Laboratory, University of Nigeria, Nsukka. They included: Mitis salivarius agar, potassium tellurite, nutrient agar, Mueller Hilton agar, barium chloride, peptonecrystal violet, Lugol's iodine, safranin, alcohol, hydrogen peroxide (H₂O₂).

2.2. Media preparation

Media used were: Mitis salivarius agar (MSA) which is selective for *S. mutans*, Blood agar (BA) and Mueller Hinton agar (MHA) as well as peptone water. The procedures employed in their preparations were according to the manufacturer's instructions.

2.2.1. Preparation of 0.5 McFarland standards

One (1) mL of concentrated H₂SO₄ was added to 99 mL

of distilled water and mixed properly. Then a 1% w/v of barium chloride solution was made by mixing 1.0 g in 100 mL of distilled water properly. Concentrated H₂SO₄ solution was mixed with the barium chloride solution to give a turbid solution of barium sulphate. After that, a small volume of the turbid solution was transferred into a capped tube, of the same type as that used for test and control inocula. Then, it was stored in a well sealed container at temperatures between 20–28 °C.

2.3. Collection of teeth samples

Thirty one (31) tooth samples were collected from two dental clinics: University of Nigeria, Nsukka (UNN) Dental Clinic at CEC and Supreme Laboratory, University Market Road, Nsukka. Samples were collected from patients with obvious signs of tooth decay (swollen gum, weak or fallen tooth, etc.) with sterile swab sticks. However, in cases where the decay was severe, and needed pulling, samples were collected directly from the decaying cavity in the teeth by the dentist using a pair of extraction forceps and elevator.

2.4. Inoculation/subculturing

Collected samples were spread-inoculated onto sterile blood agar and MHA respectively and incubated at 37 °C for 24 h. Discrete colonies were further re-inoculated onto sterile MSA plates and incubated at 37 °C for 24 h.

2.5. Gram Staining

Gram staining was carried out on isolates. A drop of saline was used to solubilise an aliquot of discrete colonies on a clean glass slide to make a thin, uniform smear. This was then allowed to air-dry, after which the smear was heat-fixed over Bunsen flame and allowed to cool. Crystal violet was added to cover the smear for 1 min, after which it was washed with tap water. To this was added Lugol's iodine and left for 1 min; then washed off. Decolourization was done with acid alcohol, which was added quickly and uniformly to the slide and left for about 5 s, after which it was quickly washed off with water. Counter-staining was done for 1 min by addition of safranin and then washed with water and air-dried.

2.6. Catalase test (Biochemical test)

To a smear of each isolate on a clean glass slide was added a drop of 3% hydrogen peroxide (H₂O₂) and observed for effervescence which denoted positive reactions and negative if it did not show any effervescence.

2.7. Collection and extraction of plant samples

Plants used were tobacco (snuff), bitter kola and cloves. The plants were all purchased from Ogige market in Nsukka, Enugu State.

Tobacco leaves, clove seeds and bitter kola fruits were ground into powder using a local grinding machine. Crude extraction was done with *n*-hexane, hot water and ethanol. Twenty-five (25 g) of each was mixed with 250 mL of *n*-hexane, ethanol, and hot water. In the case of *n*-hexane and ethanol, they were left overnight for proper extraction, after which they were filtered with a filter paper. The filtrate

was placed in a flat clean tray and allowed to dry properly. The dried extract was then scraped into a clean container, corked and kept for further use.

The same process was followed for the hot water extract but they were never soaked overnight, instead they were left till they cooled to about 45 °C before being filtered.

2.8. Phytochemistry of the plants

The plant extracts were investigated by standard methods for the presence of total phenol, alkaloids, saponins, flavonoids, tannins and hemagglutinins.

2.9. Antibioqram

One (1) g of clove extract earlier extracted with *n*-hexane was weighed into a test tube containing 2 mL of *n*-hexane to get 500 mg/mL concentration of the clove extract. Aseptically, 1 mL of this stock solution was put into the next test tube and mixed properly to make 250 mg/mL solutions of the plant extracts. This serial dilution was continued until a dilution of 15.625 mg/mL was reached for *n*-hexane in the sixth test tube. This procedure was repeated for all the plant extracts using their respective extracting solvents.

2.10. Minimum inhibitory concentration (MIC)

This is the smallest concentration of an antibacterial agent capable of reducing or stopping the growth of an organism. A 0.1 mL of *S. mutans* in peptone water broth was standardized to 0.5 McFarlands standard. An aliquot of the organism equals 0.5 McFarlands was evenly spread on the already prepared MHA agar aseptically with a glass spreader and the plates were allowed to solidify for a while before boring seven holes on the plates to contain the six dilutions of the extracts and the control. After appropriate labeling, the plates were incubated at 37 °C for 24 h.

2.11. Minimum cidal concentration (MCC)

This is the smallest concentration of an antibacterial agent that could kill the population of microorganisms present. To 9 mL of the prepared molten MHA was added 1 mL of the different concentrations [500, 250, 125, 62.5, 31.25 and 15.625 (mg/mL)] of the plant extracts. This was thoroughly mixed and poured into sterile petri dishes, and allowed to set. Then 0.1 mL of the *S. mutans* equal to 0.5 McFarland was spread-inoculated onto the plates, allowed to air dry and incubated at 37 °C for 18 h.

2.12. Antibiotic sensitivity test

The following antibiotics and their various concentrations were used:

Ciprofloxacin (CPX)	50 μg
Streptomycin (S)	30 μg
Rocephin (R)	25 μg
Pefloxacin (PEF)	30 μg
Gentamycin (GN)	10 μg
Ampiclox (APU)	30 μg
Zinnacef (Z)	20 μg
Erythromycin (E)	10 μg
Septtrin (SXT)	25 μg
Amoxacillin (AM)	30 μg

Approximately 0.1 mL of test organism was spread-inoculated on sterile MHA plates. To these were carefully added Gram-positive antibiotic disc and pressed gently down the agar before incubating at 37 °C for 24 h.

2.13. Statistical analysis

All experiments were performed in replicates for validity of statistical analysis. Results were expressed as mean ± SD. ANOVA and student's *t*-test was performed on the data sets generated using Predictive Analytics Software. Differences were considered significant for *P* values < 0.05–0.01.

3. Results

3.1. Efficiency of solvent extraction

Table 1 shows that *n*-hexane extracted more (nearly double-fold) than ethanol and hot water from clove, *G. kola* and tobacco leaves respectively.

3.2. Phytochemical analysis of the herbs

Table 2 shows the quantitative and qualitative result of phytochemical determination of components (phenol, flavonoid, saponin, alkaloids, hemagglutinin and tannins) of the various herbs (*S. aromaticum*, *G. kola* and *Nicotiana* Spp.). The degree of the presence of the components is expressed as % composition.

Table 1

Extractive power of the solvents.

Solvents	<i>G. kola</i>	<i>S. aromaticum</i>	<i>Nicotiana</i> Spp.
<i>n</i> -hexane	1.9	4.2	0.0
Ethanol	2.5	2.9	0.0
Hot water	0.5	0.3	0.3

α = 0.05.

3.3. Gram reaction and catalase test

Table 3 shows the various biodata of the patients whom samples were collected from, their tooth profile and the morphological characteristics of the isolates of the 31 samples. 15 samples were collected from males, while 16 were from females. In the tooth profile, 20 samples collected were highly decayed while 10 samples were slightly decayed and among them were 16 very-bloody samples and 15 were slightly bloody. It was observed that tooth decay occurred more in females than in males. Eighteen (18) samples were catalase-positive indicating the presence of *S. mutans*, which are Gram-positive organisms while 13 others were catalase-negative which were also Gram-negative organisms suspected to be *Staphylococcus* Spp. UDC code, represents samples collected from University Dental Clinic, Nsukka, while SL indicates samples collected from Supreme Laboratory, Nsukka.

3.4. Antimicrobial evaluation

Susceptibility tests were carried out to determine the minimum inhibitory concentration (MIC) and minimum cidal concentration (MCC) of both the plant extract and control

Table 2

Phytochemical analysis.

Plant materials	Compositions (%)					
	Flavonoids	Saponin	Phenol	Alkaloids	Hemagglutinin	Tannins
<i>S. aromaticum</i>	17.40	6.89	22.01	5.22	1.5	5.68
<i>G. kola</i>	8.01	8.11	4.20	4.10	8.1	–
<i>Nicotiana</i> Spp.	6.01	–	5.90	–	–	–

+++ : Highly present; ++: moderately present; +: present, –: absent.

antibiotics. The MIC showed that *n*-hexane extracted more than ethanol and hot water since the organism was shown to be more inhibited with this solvent (*n*-hexane) extract especially, the clove extract.

3.5. Antibiotic sensitivity test

Table 4 shows the various antibiotics, their concentrations as well as their sensitivities. It was shown that ciprofloxacin exhibited the largest zone of growth inhibition because generally ciprofloxacin is known to be a broad spectrum antibiotic and it is the preferred antibiotic for a broad range of pathogenic organisms.

Table 3

Gram staining and biochemical test result.

Specimen code	Gram reaction	Catalase reaction	Suspected organism
UDC 1	–	–	–
SL 1	–	–	<i>Staphylococcus</i> spp.
UDC 2	–	–	<i>Staphylococcus</i> spp.
SL 2	+	+	<i>Streptococcus</i> spp.
UDC 3	–	–	<i>Staphylococcus</i> spp.
SL 3	+	+	<i>Streptococcus</i> spp.
UDC 4	–	–	<i>Staphylococcus</i> spp.
SL 4	–	–	<i>Staphylococcus</i> spp.
UDC 5	–	–	<i>Staphylococcus</i> spp.
SL 5	+	+	<i>Streptococcus</i> spp.
UDC 6	+	+	<i>Streptococcus</i> spp.
SL 6	+	+	<i>Streptococcus</i> spp.
UDC 7	+	+	<i>Streptococcus</i> spp.
SL 7	+	+	<i>Streptococcus</i> spp.
UDC 8	+	+	<i>Streptococcus</i> spp.
SL 8	+	+	<i>Streptococcus</i> spp.
UDC 9	+	+	<i>Streptococcus</i> spp.
SL 9	+	+	<i>Streptococcus</i> spp.
UDC 10	+	+	<i>Streptococcus</i> spp.
SL 10	+	+	<i>Streptococcus</i> spp.
UDC 11	+	+	<i>Streptococcus</i> spp.
SL 11	+	+	<i>Streptococcus</i> spp.
UDC 12	+	+	<i>Streptococcus</i> spp.
SL 12	–	–	<i>Staphylococcus</i> spp.
SL 13	–	–	<i>Staphylococcus</i> spp.
SL 14	+	–	<i>Streptococcus</i> spp.
SL 15	+	+	<i>Streptococcus</i> spp.
SL 16	+	+	<i>Streptococcus</i> spp.
SL 17	–	–	<i>Staphylococcus</i> spp.
SL 18	–	–	<i>Staphylococcus</i> spp.
UDC 13	–	–	<i>Staphylococcus</i> spp.

(+) = Gram positive/catalase positive; (–) = Gram negative/catalase negative.

Table 4

Antibiotics sensitivity test.

Antibiotics	Concentration (μ g/L)	Extent of sensitivity, IZD (mm)
CPX	50	23
S	30	20
R	25	10
PEF	30	5
GN	10	2
APU	30	0
Z	20	0
E	10	0
SXT	25	0
AM	30	0

4. Discussion

We observed that *n*-hexane extracted more than ethanol and hot water. This agrees with an earlier work that aliphatic alcohols (with up to 3 carbon atoms) or mixtures of alcohol with water are the solvents with the greatest extractive power for almost all natural substances of low molecular weight^[21], even though van Vuuren and his colleague argued that aqueous extracts indicated better activities than organic solvent extraction in the isolation of *S. mutans* and other implicated bacteria on toothbrushes^[22]. This was further confirmed by the subsequent growth inhibitory activity of the *n*-hexane extract especially from the clove extract as against ethanol. Generally, clove extract showed the greatest activity even with hot water extraction as supported by evidence from the work of Cai and Wu, who proposed that crude extract of *S. aromaticum* (clove) exhibited preferential growth-inhibitory activity against periodontal oral pathogens including *S. mutans* and concluded that compounds from clove possess growth-inhibitory activity against oral pathogens^[23].

Comparatively, *G. kola* was next to clove in activity while tobacco showed no activity even with *n*-hexane, which normally extracted best and showed most inhibition with other plants. Our observation showed that hot water extract of clove plant inhibited the growth of *S. mutans* at two different concentrations of 31.25 and 15.625 mg/mL while hot water extract of *G. kola* showed a small zone of inhibition of *S. mutans* at the MIC of 15.625 mg/mL. This obviously means that clove extract had more activity than *G. kola*. However, the hot water extract of tobacco Spp. showed slim (minimum) growth inhibition at 62.25 mg/mL while the MCC study revealed no growth with the water extract at this concentration but showed more growth of *S. mutans* at the same concentration of the corresponding *n*-hexane and ethanol extracts of both clove and *G. kola*. This clearly shows

that tobacco has no activity on *S. mutans*^[24], implying that tobacco in any form, for instance snuff which is basically a consumption form of tobacco, has no effect against *S. mutans* and is not advisable to be taken. This is in consonance with Cerami and his colleagues who suggested that tobacco intake is also a source of toxic reactive glycation products as well as increases the risk of gum diseases^[25].

In antibiotic sensitivity test, ciprofloxacin showed the largest zone of inhibition followed by streptomycin. Statistically, it was discovered that at α -level of 0.05, the treatment was not significant with regards to the effect of the herbs in inhibiting the growth of *S. mutans*. However, the problems resulting from wide spectrum antibiotic use, combined with the emergence of drug-resistant strains, highlight the fundamental need for new “targeted” antibiotic therapies to combat mucosal pathogens with a minimum impact on normal microflora. So to improve the selectivity of ciprofloxacin activity on *S. mutans*, a co-formulation with pure clove extract could do well.

Conclusively, due to the outstanding antimicrobial activity observed in clove plant as against bitter kola and tobacco leaves in the growth inhibition of the causal organism in tooth decay (*S. mutans*), we therefore report that clove be recommended as an ingredient for tooth paste preparation or mouthwash formulations, example, gargles. We recommended further studies to address synergy when clove is combined with bitter kola in such preparations. Following the inactivity of tobacco leaves in this study in addition to its changing the dental colour of a consumer, we advise therefore, that people should not use tobacco especially in form of snuffs on the teeth.

Conflict of interest statement

The authors report no declaration of interest.

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