

## Activities of Nigerian Chewing Stick Extracts against *Bacteroides gingivalis* and *Bacteroides melaninogenicus*

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**The in vitro activities of extracts of Nigerian chewing sticks against *Bacteroides gingivalis* and *B. melaninogenicus* are presented. The greatest inhibitory action was produced by *Serindeia werneckei*, whereas *Fagara zanthoxyloides* produced no appreciable inhibitory effect. A generally good correlation was found between the killing curves and MICs. Only extracts of *Anogeissus leiocarpus* showed acute toxicity in mice.**

Almost the entire rural population of Nigeria uses chewing sticks for orodental hygiene. Previous studies have demonstrated the antiplaque and antibacterial actions of extracts of these Nigerian chewing sticks (NCS) against oral bacteria, such as *Streptococcus mutans* (15), *Streptococcus mitis* (10), and oral anaerobes (9), which are the organisms commonly implicated in dental caries and orodental infections. Most of the studies on orodental infections stress the importance of oral anaerobes, particularly black-pigmented bacteroides, in the etiology of periodontal diseases (5, 11, 14). One of the most frequently isolated oral pathogens in these diseases is *Bacteroides gingivalis* (6, 12), although *Bacteroides melaninogenicus* has also been implicated (12).

The only reported clinical survey comparing the oral hygiene of chewing stick users with that of toothbrush users showed that dental caries and gingivitis were less common among the chewing stick users (7). It is believed that this may be due to the antimicrobial substances present in NCS. Alkaloid extracts of some other plants, e.g., *Sanguinaria canadensis*, incorporated into various dentifrices and oral rinses have been shown to possess broad-spectrum in vitro activity against a wide variety of microorganisms (1). Similarly, fagaronine, a compound extracted from an NCS, *Fagara zanthoxyloides*, has been shown to provide beneficial effects for the oral hygiene of some rural natives (8). The purpose of this study was to determine the antibacterial activity, kinetics of killing, and animal toxicity of the extracts of commonly used NCS.

The bacterial strains used for these experiments were standard reference strains of *B. gingivalis* (ATCC 33277) and *B. melaninogenicus* (ATCC 33184) maintained in an anaerobic chamber on brain heart infusion agar (BBL Microbiology Systems, Cockeysville, Md.) with supplements.

Nine different plants and plant parts (root and stem) indigenous to West Africa and used for oral hygiene were investigated. These include *F. zanthoxyloides*, *Vernonia amygdalina*, *Serindeia werneckei*, *Anogeissus leiocarpus*, *Bytyrospermum paradoxum*, *Terminalia glaucescens*, *Naucllea latifolia*, *Massularia accuminata*, and *Distemonanthus benthamianus*.

Extracts were prepared by removing the bark, mincing it into small pieces, and grinding it in a mill (Rival, Philadelphia, Pa.) into coarse powder. Twenty grams of each powder was added to 100 ml of phosphate-buffered saline (pH 7.2) in a wide-mouthed flask and then extracted by stirring for 4

days at 4°C. The resultant suspension was passed through a gauze filter to remove large debris and then centrifuged at 3,000 × g for 10 min. The supernatant fraction was carefully decanted and passed through a 0.22-μm (pore size) membrane filter (Nalgene Co., Rochester, N.Y.) under vacuum to remove insoluble materials. The clarified filtrate was dialyzed in two Spectrapor membranes with molecular weight limits of 3,500 and 6,000 to 8,000 (Spectrum Medical Ind., Inc.) against deionized distilled water. The dialysates and the predialysates were lyophilized, and the materials were kept at -70°C until tested.

Both the predialysates and the two dialysates were tested for inhibitory activity against reference strains of *B. gingivalis* (ATCC 33277) and *B. melaninogenicus* (ATCC 33184). Twenty grams each of the pre- and postdialyzed extracts was dissolved in 10 ml of phosphate-buffered saline and filter sterilized by passage through a 0.22-μm membrane filter. The resulting stock solution was serially diluted in prereduced brain heart infusion broth to give final concentrations of 0.1 to 512 μg/ml. These were then seeded with 0.1 ml of the bacterial inoculum (48-h culture in prereduced brain heart infusion broth adjusted to the turbidity of a 0.5 McFarland standard) and incubated in an anaerobic chamber at 37°C for 48 h. A medium without extract was similarly inoculated and served as a control. The MIC was taken as the lowest concentration that permitted no visible growth (13). All tubes showing no visible growth at 48 h were subcultured onto prereduced brain heart infusion agar by using an inoculum of 0.01 ml and incubated at 37°C for 5 days. The MBC was recorded as the lowest concentration that did not yield growth on solid medium. Killing curves were determined over 24 h for each of the bacterial strains in 5-ml glass test tubes with samplings at 0, 2, 4, 6, 12, and 24 h. Extracts were tested at twice the MIC. The tubes were inoculated with 0.1 ml of a 48-h broth culture diluted to give a final concentration of about 10<sup>6</sup> CFU/ml. Extract-free broths inoculated with the same organisms were used as controls. At the end of the appropriate incubation periods, viable counts were done on each sample with a Spiral Plater (Spiral Systems, Inc.). The colonies were counted after 5 days of incubation under anaerobic conditions.

For the acute toxicity test, adult CD1 mice (Charles River Breeding Laboratories, Inc., Wilmington, Mass.) weighing about 25 g were used. Sets of four mice were allocated to three groups. Predetermined 50% lethal doses of *F. zanthoxyloides* (4) were administered to the mice by the intravenous, intraperitoneal, and per os routes at doses of 8, 20,

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TABLE 1. MICs and MBCs of extracts

Chewing stick extract	Concn (µg/ml) for:			
	<i>B. gingivalis</i>		<i>B. melaninogenicus</i>	
	MIC	MBC	MIC	MBC
<i>F. zanthoxyloides</i>	256	>512	>512	>512
<i>S. werneckei</i>	0.25	0.25	0.5	0.5
<i>V. amygdalina</i>	2	2	2	2
<i>M. accuminata</i>	0.5	0.5	4	4
<i>A. leiocarpus</i>	4	16	8	16
<i>B. paradoxum</i>	2	2	4	4
<i>D. benthamianus</i>	2	2	2	2
<i>T. glaucescens</i>	2	4	4	8
<i>N. latifolia</i>	1	4	2	4

and 50 g/kg (body weight), respectively. The mice were maintained in separate cages and observed for 72 h. Post-mortem examination was performed on those that died.

Identical results were obtained for the predialysates and the two dialysates with molecular weight limits of 3,500 and 6,000 to 8,000, indicating that antibacterial activity may be ascribed to plant components with molecular weights larger than 8,000.

Table 1 shows the MICs and MBCs of individual NCS extracts for the two test strains. *B. gingivalis* was more susceptible than *B. melaninogenicus* to the chewing stick extracts. The MICs ranged from 0.25 to 256 µg/ml for *B. gingivalis* and 0.5 to >512 µg/ml for *B. melaninogenicus*. *S. werneckei* extract produced the greatest inhibitory activity; the MICs were 0.25 and 0.5 µg/ml for *B. gingivalis* and *B. melaninogenicus*, respectively. All the NCS except *F. zanthoxyloides* produced significant inhibitory effects on the two bacterial strains.

Figures 1 and 2 show the killing curves of the two organisms produced by six of the eight active extracts. Almost identical curves were obtained for both test bacterial

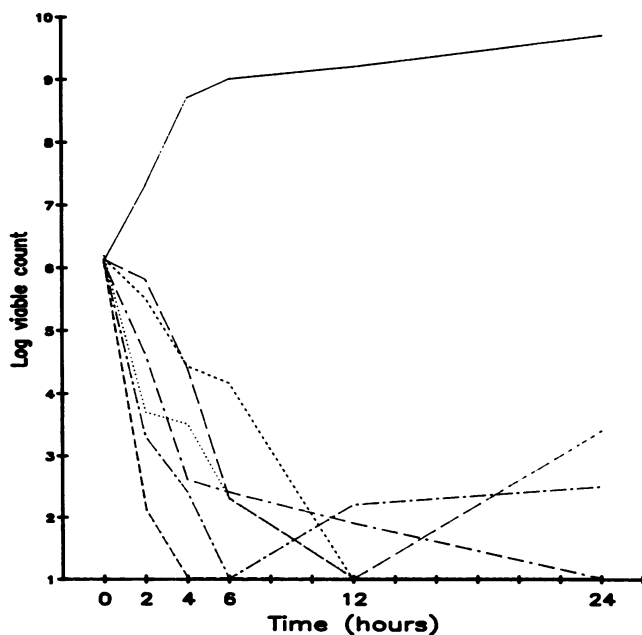


FIG. 1. *B. gingivalis* killing curves. —, Broth control; — —, *A. leiocarpus*; - - - -, *D. benthamianus*; — — —, *V. amygdalina*; ..... , *B. paradoxum*; — — — —, *N. latifolia*; - - - - - , *S. werneckei*.

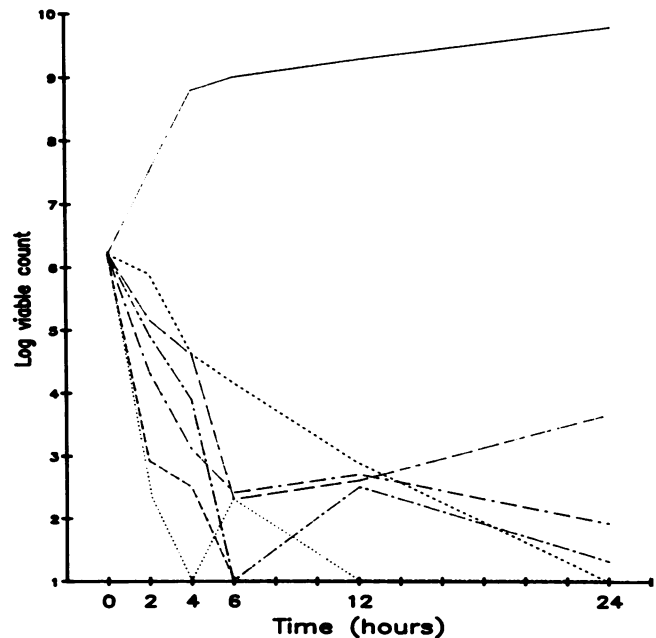


FIG. 2. *B. melaninogenicus* killing curves. —, Broth control; — —, *A. leiocarpus*; - - - -, *D. benthamianus*; — — —, *V. amygdalina*; ..... , *B. paradoxum*; — — — —, *N. latifolia*; - - - - - , *S. werneckei*.

strains. There was remarkable reduction in the growth of both organisms in the first 6 h of incubation by all the extracts, although some extracts were more potent than others. All the NCS extracts, except extracts of *A. leiocarpus* and *N. latifolia*, were effective in killing the organisms after 24 h of exposure.

All the extracts, except that of *A. leiocarpus*, showed no evidence of acute toxicity to adult mice at the doses given by the three routes. Extracts of *A. leiocarpus* were lethal to mice within 5 s after intravenous injection of 8 g/kg and within 60 s after intraperitoneal injection of 20 g/kg. Doses of 50 g/kg given per os produced no detectable toxicity. The postmortem results of the dead mice showed no gross pathological changes in the organs and viscera.

The data presented in this report represent preliminary information on the in vitro activities of NCS with respect to their antimicrobial activities and acute toxicities. Our results demonstrate that eight of the nine NCS possess significant antimicrobial action against *B. gingivalis* and *B. melaninogenicus*, two representative oral anaerobes often associated with periodontal diseases. This observation suggests wider implications and may explain, in part, the relatively lower incidence of dental caries and gingivitis among chewing stick users compared with toothbrush users in Nigeria (3, 7).

The killing curves of the two organisms after exposure to supraconcentrations of six NCS extracts showed good correlation with the MICs and the MBCs. The curves also demonstrated that *B. gingivalis* was more susceptible than *B. melaninogenicus* to the extracts. The implication of the results of these killing curves in a clinical setting can only be speculative until a controlled clinical trial is done.

Results of acute toxicity testing showed that most of the NCS were nontoxic. The *A. leiocarpus* extract was very toxic parenterally, but this may not be relevant since it was not acutely toxic by oral administration. The clonic convulsions before spontaneous death and the lack of postmortem gross pathology suggest that the extract is a neurotoxin. We

are investigating the toxic effect of NCS with chronic administration.

We can only hypothesize what the ingredients in these chewing sticks are that produce the antimicrobial action and the toxic effect of the *A. leiocarpus* extract. Studies by previous investigators of the active moiety are inconclusive, although our preliminary experience shows that the active substances are heat stable (9), unlikely to be proteins, and nondialyzable through membranes with molecular weight limits of 8,000.

It is conceivable that the NCS serve as an important factor in dental disease control, based on the readily demonstrated antimicrobial effects. Data on the oral health of rural Nigerians who depend on this method for hygiene suggest that NCS are efficacious (3, 7). We are investigating the chemical identity and the mode of action of the active moiety of these chewing sticks.

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