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Antimicrobial Screening of *Breynia nivosus* and *Ageratum conyzoides* Against Dental Caries Organisms

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Abstract: The antimicrobial effect of the aqueous and ethanolic extracts of *Breynia nivosus*, *Ageratum conyzoides* and the combination of herbal preparations on *Streptococcus mutans* isolated from dental caries patients attending the federal dental clinic Enugu, Nigeria was investigated. The hot water and ethanolic extracts of *Breynia nivosus* showed zones of inhibition ranging from 10-15 mm at concentrations of 25-400 mg mL⁻¹. The Minimum Inhibition Concentration (MIC) was 25 mg mL⁻¹. All the extracts of *Ageratum conyzoides* had no inhibitory effect on the test organism. The herbal combinations had 19 mm zone of inhibition at the 400 mg mL⁻¹ concentration of the ethanolic extracts. Phytochemical studies of *Breynia nivosus* revealed the presence of flavonoids, glycoside, protein, saponins tannins, carbohydrate, reducing sugar and steroidal aglycone and traces of cardiac glycoside. No clinical signs of acute toxicity were observed in mice given 250-500 mg kg⁻¹ bw of *Breynia nivosus* extract after 24 h. Histopathological studies of rats fed with 500 and 1000 mg kg⁻¹ bw of *Breynia nivosus* showed no signs of chronic toxicity as evidenced by the liver and kidney of the rats. This study justifies the use of *Breynia nivosus* as chewing stick by the people of South- Eastern Nigeria.

Key words: Antimicrobial screening, *Breynia nivosus*, *Ageratum conyzoides* dental caries *Streptococcus mutans*

INTRODUCTION

Microbial activity at tooth surface often results to progressive destruction of enamel dentine and cementum (Silverstone *et al.*, 1981). This often results to dental caries. Various factors including plaque microorganisms, suitable carbohydrate (mainly sucrose), susceptible tooth surface and time are amongst factors necessary to produce dental caries (Kidd *et al.*, 1990). *Streptococcus mutans* has been reported by various researches as the major and most efficient cariogenic bacteria (Samaranayake, 1996; Loesche, 1986; Rowe *et al.*, 1989; Prabhu *et al.*, 1992). Dental caries is still prevalent in most African countries, Nigeria inclusive (Manji *et al.*, 1991).

Various local preparations are used in different communities for the management of infections of the oral cavity. Medicinal plants have since ancient times been employed for prophylactic and curative purposes. (Ray and Majumdar, 1976; Ogunyemi, 1979; Iwu, 1993).

Herbal preparations have been used by local practitioners to treat various ailments (Sofowora, 1982). Stems and leaves of certain herbs are commonly used in cleaning and treatment of dental diseases (Okafor, 2001). This study reports the antibacterial effects of *Breynia nivosus* and *Ageratum conyzoides* on dental caries organisms. Phytochemical properties of the effective herb and its toxicity on experimental animals are also reported.

MATERIALS AND METHODS

Collection and preparation of plants: Fresh leaves of *Breynia nivosus* and *Ageratum conyzoides* were collected at Abakpa Nike, Enugu, Enugu State, Nigeria and were certified by a plant taxonomist in the department of Applied Biology, Ebonyi State University, Abakaliki. The leaves were washed and dried at room temperature. They were pulverized using a mechanical grinder (Victoria grain mill fondicion corona). Exactly 50 g of each macerated plant material were added to 250 mL of cold water and

95% ethanol, respectively for cold water and ethanol extraction. Another 50 g of plant material was introduced into 250 mL of water and boiled for 10 min for the hot water extraction. Each mixture was shaken at 2 h intervals for 24 h and then filtered using whatman filter paper (No. 2) and evaporated to dryness in a water bath at 40°C (Ibrahim *et al.*, 1997). The extracts were stored at 4°C and reconstituted to various concentrations when required.

Isolation and identification of isolates: Specimens were collected from 40 patients attending the federal dental clinic Enugu who had carious lesions, using sterile swab sticks and dental probe. Each of the specimen were inoculated into crystal violet blood agar (Collins *et al.*, 1995) and incubated anaerobically at 37°C for 24 h. The colonies were subcultured by the streak plate technique to obtain pure cultures (Ingraham *et al.*, 2001). The isolates were identified using colony morphology, haemolytic activity, grams reaction, motility test and biochemical tests namely catalase, bile solubility, litmus milk, arginine hydrolysis, hippurate hydrolysis, voges-prausker (V-P) and fermentation of the sugars-mannitol, Lactose, raffinose, trehalose and sorbitol (Collins *et al.*, 1995; Cheesbrough, 2002; Collins *et al.*, 1999; Baker *et al.*, 1998).

Preliminary antimicrobial screening: Preliminary antimicrobial activities of the different plant extracts were determined using the agar-well diffusion method (Perez *et al.*, 1990). Exactly 0.06 mL of overnight culture of *Streptococcus mutans* adjusted to 0.5 Macfarland standard (Cheesbrough, 2002) was seeded on blood agar medium. Four wells each 6 mm diameter were made using sterile cork borers in the already seeded blood agar media. The herbal extracts were reconstituted to concentration of 400 mg mL⁻¹ and 0.06 mL of each extract was placed in the wells, respectively. The remaining wells were filled with 0.06 mL of erythromycin tablet suspension (2.5 mg mL⁻¹) and distilled water to serve as positive and negative controls. The plates were then incubated anaerobically at 37°C for 24 h. The degree of antimicrobial activity of each extract was measured as the Inhibition Zone Diameter (IZD) in millimeters.

Determination of minimum inhibitory concentration: Minimum inhibitory concentration was determined for only the extracts that showed inhibitory activity. The agar well diffusion method was also employed (Perez *et al.*, 1990). Exactly 0.06 mL of each herbal extract reconstituted to concentrations of 12.5, 25, 50, 100, 200 and 400 mg mL⁻¹ were introduced into the wells on blood agar media seeded with 0.06 mL of the overnight culture

of the test organisms (Macfarland standard 0.5). (Cheesbrough, 2002). The plates were incubated anaerobically at 37°C for 24 h. The lowest concentration of the extract that showed noticeable inhibition was recorded as Minimum Inhibitory Concentration (MIC).

Antimicrobial activity of herbal combinations: Equal volumes of the 400 mg mL⁻¹ concentration of extracts from the two herbs were mixed thoroughly and used for antimicrobial screening. The screening was done as described earlier (Perez *et al.*, 1990).

Phytochemical analysis of extract: The phytochemical screening of the herbal extracts that showed visible inhibitory activity against the test organism was carried out using standard methods described by Iwu and Chiori (1984).

Toxicity testing: Acute and chronic toxicity test was carried out on the herb that showed noticeable inhibitory effect. Acute toxicity test was done with 70 (8-10 week old) male mice weighing 18-22 g. The experimental animals were randomly divided into 7 groups of 10 mice each (A-G) The mice groups A-F were administered a single dose of 250, 500, 750, 1500, 3000 and 5000 mg kg⁻¹ body weights, respectively of *Breynia nivosus* extract through intra-peritoneal route. The group G mice were given 2 mL of distilled water to serve as control. The mice were observed for clinical signs of toxicity and mortality during a 24 h period.

Chronic toxicity test was carried out with 15 (8-10 week old) male albino rats weighing between 60-120 g. The rats were divided into three groups of five each (A-C). Groups A rats were fed with 500 and 1000 mg kg⁻¹ body weights of *Breynia nivosus* extract. Exactly 1.0 mL of blood collected from each rat using heparinised capillary tubes via retro-bulba plexus of the medial cantus of the eye prior to the introduction of the herbal extract was used to establish the normal haematological parameters namely, packed cell volume, red blood cell counts, haemoglobin concentration, erythrocyte sedimentation rate and total leukocyte count. At the end of 30 days, 1.0 mL of blood was collected from the experimentally fed rats and used for post-treatment haematological studies. The rats were then sacrificed and used for histopathologic examination. The liver and kidney tissues were stained and observed with a light microscope (Nwajagu and Okoli, 2002).

Statistical analysis: Results obtained from the assessment of haematological parameters was analyzed using the analysis of variance (ANOVA) $p \leq 0.01$.

RESULTS

The isolates were identified as *Streptococcus mutans*, *Streptococcus salivarius*, *Streptococcus sobrinus* and *Streptococcus mitior*. Their prevalence was *S. mutans* (88%), *S. salivarius* (6%), *S. sobrinus* (4%) and *S. mitior* (2%) in the carious lesions of the 40 patients attending federal dental clinic Enugu. The preliminary antimicrobial screening showed that only the ethanolic and hot water extracts of *Breynia nivosus* produced visible inhibitory activity on the test organism. The cold water extracts of *Breynia nivosus* and all the extracts of *Ageratum conyzoides* did not show any inhibitory effects. The Minimum Inhibitory Concentration (MIC) of the ethanolic and hot water extracts of *Breynia nivosus* was 25 mg mL⁻¹ (Table 1). The combination of the ethanolic extracts of the herbs however showed noticeable inhibitory effect (19 mm) while others did not (Table 2).

Phytochemical analysis of *Breynia nivosus* extract revealed the presence of flavonoids, glycosides, proteins, saponins, tannins, carbohydrate, reducing sugar, steroidal aglycone and cardiac glycosides (Table 3).

No mortality or signs of acute toxicity were observed among the mice groups administered 250-5000 mg kg⁻¹ body weights of *Breynia nivosus* extract after 24 h post treatment. No clinical signs of chronic toxicity were observed among the rats given 500 and 1000 mg kg⁻¹ bw of *Breynia nivosus* extracts after 30 days of observation (Table 4 and 5). No significant difference was observed ($p < 0.01$) in the haematological parameters analyzed.

Histopathological studies indicated congestion in the central vein of the liver tissues of the rat groups given 500 and 1000 mg kg⁻¹ bw of *Breynia nivosus* extract.

There was no degenerative changes or necrosis observed in the liver tissues and no significant changes in the kidney of the rat groups.

Table 1: Minimum inhibitory concentration of *Breynia nivosus* extracts on *Streptococcus mutans*

Concentration (mg mL ⁻¹)	Inhibition zone diameter (mm)		
	Cold water	Hot water	Ethanol
400	NI	15	15
200	NI	13	14
100	NI	12	13
50	NI	11	12
25	NI	10	11
12.5	NI	NI	NI
(Control erythromycin) 2.5 mg mL ⁻¹	20	ND	ND

NI = No Inhibition, ND = Not Determined

Table 2: The *in vitro* effects of the 400 mg mL⁻¹ herbal combinations of *Breynia nivosus* and *Ageratum conyzoides* on *Streptococcus mutans*

Extraction method	Inhibition zone diameter (12D) mm
Cold water	NI
Hot water	NI
Ethanol	19

NI = No Inhibition

Table 3: Phytochemical properties of *Breynia nivosus*

Phytochemical constituents tested	Present or absent
Alkaloids	-
Flavonoids	++
Glycosides	+++
Protein	++
Saponins	+
Tannins	++
Carbohydrate	+++
Cardiac glycoside	Tr
O- and C-glycoside	-
Cyanogenic glycoside	-
Anthracene glycoside	-
Reducing sugar	++
Steroidal aglycone	++

- = Not Present, Tr = Trace, + = Present in varying degrees

Table 4: Changes in body weight, packed cell volume and red blood cell counts of rats before and after administration of *Breynia nivosus*

Rat group treatment	Mean body weight (g)		Mean packed cell volume (%)		Mean red blood cell count (10 ⁶ cells/ μ L blood)	
	BT	AT	BT	AT	BT	AT
500 mg kg ⁻¹ bw	88.80 ^a	150.64 ^b	41.80 ^a	42.60 ^a	5.24 ^a	5.06 ^a
1000 mg kg ⁻¹ bw	82.30 ^a	146.36 ^b	41.80 ^a	42.80 ^a	5.27 ^a	5.12 ^a
Control	78.60 ^a	155.40 ^b	41.80 ^a	41.20 ^a	5.21 ^a	5.06 ^a

BT = Before Treatment, AT = After Treatment. Means on the same row with same superscripts are not significantly different ($p < 0.01$)

Table 5: Changes in haemoglobin concentration, erythrocyte sedimentation rates and total leukocyte counts of rats before and after administration of *Breynia nivosus*

Rat group treatment	Mean haemoglobin (g dL ⁻¹)		Mean erythrocyte sedimentation rate (mm h ⁻¹)		Mean leukocyte count (10 ³ cells/ μ L blood)	
	BT	AT	BT	AT	BT	AT
500 mg kg ⁻¹ bw	13.08 ^a	13.16 ^a	0.58 ^a	0.34 ^b	9.60 ^a	15.41 ^b
1000 mg kg ⁻¹ bw	13.02 ^a	13.20 ^a	0.68 ^a	0.36 ^b	9.43 ^a	15.38 ^b
Control	13.04 ^a	13.14 ^a	0.62 ^a	0.38 ^b	9.61 ^a	15.62 ^b

BT = Before Treatment, AT = After Treatment. Means on the same row with same superscripts are not significantly different ($p < 0.01$)

DISCUSSION

The isolation of *Streptococcus mutans* from over 80% of the caries patients in this study agrees with previous reports that *S. mutans* is the major agent of dental caries in man (Kristofersson *et al.*, 1985; Loesche, 1986). *Streptococcus mutans* is used as a microbial assessment tool for dental caries (Samaranayake, 1996) and has been employed in a semi-quantitative method to assess the risk of oral infection in pre school children (Linossier *et al.*, 2003).

The preliminary screening indicated that only the hot water and ethanolic extracts of *Breynia nivosus* had visible inhibitory effects on *Streptococcus mutans*. *Breynia nivosus* is one of the major plants whose stems are commonly used as chewing stick in southeastern Nigeria (Sofowora, 1982). Similar studies had reported the *in vitro* antibacterial and anticaries properties of some local medicinal plants. Linke and Le Geros (2003) reported the effect of black tea.

Although the ethanolic, cold and hot water extracts of *Ageratum conyzoides* had no visible inhibitory activity against *S. mutans*, equal proportions of the ethanolic extract of this herb and that of *Breynia nivosus* inhibited *Streptococcus mutans* producing Inhibition Zone Diameter (IZD) of 19 mm which is higher than the affect of *Breynia nivosus* ethanolic extract alone. This enhanced inhibitory activity could be as a result of synergistic interaction between the two herbs. Previous studies had reported increased activities of herbal combinations (Adetunji, 1999; Amadi *et al.*, 2004).

The phytochemical analysis of *Breynia nivosus* showed the presence of flavonoids, saponins, tannins and steroidal aglycone amongst others. Alkaloids, tannins saponins, glycosides and steroid derived from plants has been shown to have antimicrobial affect and pharmacological activities (Trease and Evans, 1983; Leven *et al.*, 1979; Ghani, 1985; Gill, 1992; Frel *et al.*, 1998). The antimicrobial activity of *Breynia nivosus* observed in this study could be attributed to the presence of these compounds. It has also been reported that isoflavonoids isolated from *Erythrina variegata* had antibacterial affect against *S. mutans* (Sato *et al.*, 2003). Uzel *et al.* (2003) reported that the main compound in propolis extract which shows anticaries activities were flavonoids.

The finding that mice subjected to acute toxicity test using *Breynia nivosus* extract showed no clinical signs of acute toxicity or death is noteworthy. Moreso the chronic toxicity test with rats showed that the extract is non toxic at 500 and 1000 mg kg⁻¹ bw. Similar low potential for acute toxicity has been reported for *Uncaria tomentosa* and *Lepidium meyenii* (Valerio and Gonzales, 2005).

The result of this study confirms the ethnomedical use of the plant *Breynia nivosus* as one of the commonly used chewing stick in southeastern part of Nigeria. It also confirms the fact that herbal preparations can be used for cleansing of the oral cavity, maintenance of oral hygiene and prevention of dental caries. There is therefore need for interest in study on medicinal herbs and the validation of herbs used in various localities in order to incorporate them into the health system especially since more people are placing emphasis on natural products over synthetic drugs.

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