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Research Article

IN VITRO ANTIMICROBIAL ACTIVITIES AND PHYTOCHEMICAL PROPERTIES OF THE COMMONLY USED CHEWING STICK IN WEST AFRICA (*ANOGEISSUS LEIOCARPUS)*

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ABSTRACT
The primary benefit of using plant part as chewing stick is that they are relatively safer and cheaper than the synthetic alternatives. A great interest has developed in searching for antimicrobial chewing sticks from natural plant products and this interest arises from the belief that the ones derived from plants are safe and dependable, compared to synthetic ones. Ethanol, ethyl acetate and chloroform extracts of stem of *Anogeissus leiocarpus* were screened for antimicrobial activities at various concentrations between C_1 = 500 mg/ml to C_5 = 25 mg/ml against *Escherichia coli, Pseudomonas aeruginosa*, *Klebsiella species*, *Streptococcus pneumonia*, *Bacillus cereus*, *Aspergillus flavus, Aspergillus niger, Trichoderma species* and *Candida species*. *Anogeissus leiocarpus* was active against the tested organisms. The result further revealed the effectiveness of the ethanol extract over ethyl acetate and chloroform extracts. The phytochemical screening of the chewing stick possesses all the tested phytochemical components except phlobatannins and reducing sugars.

Keywords: *Chewing Sticks, Natural Plant Product, Antimicrobial Activities, Phytochemical Components, Phlobatannins, Reducing Sugar*

INTRODUCTION
Anogeissus leiocarpus belongs to the family combretaceae; it is commonly called "Orin Ayin" in Yoruba language. It is a tall evergreen tree native to savannah of tropical Africa. It is the sole West African species of the genus Anogeissus, a genus otherwise distributed from tropical central and East Africa through the tropical South East Asia. The plant is also used in Nigeria as an antimicrobial agent against bacterial infections (Mann *et al*., 2008).
Recent interest in chewing sticks and their extracts has focused on their effects on organisms that are involved in oral infections. Africans that use chewing sticks have fewer carious lesions than those that use toothbrush and their use has been encouraged by the World Health Organization (Ndukwe *et al*., 2005). Most of the clinically important antibiotics have major setbacks, apart from their expensive costs. A good number of conventional antibiotics have been found to be neurotoxic, nephrotoxic and hypertensive, and few others cause severe damage to the liver and bone marrow depression (Chong and Pagano, 1997; Keay, 1989). The largest numbers of microorganisms are found on the tooth surfaces, especially at stagnant sites and are termed dental plaque, the composition of which varies at distinct surfaces (e.g. fissures, approximal surfaces, and the gingival crevice) due to the prevailing biological properties of the site. All surfaces in the mouth are colonised by a resident micro flora that is highly diverse in composition Adekunle and Odukoya, 2006; Akujobi *et al.*, 2004).
Herbal chewing stick is primarily important because they are relatively safer and cheaper than the synthetic alternatives (Aiyegoro and Okoh, 2009). Plants have the major advantage of still being the most effective and cheaper alternative source of safe chewing stick. In addition, herbal medicine is a complex mixture of different phytochemicals acting by different mechanisms, which makes it difficult for pathogens to develop resistance (Daferera *et al.*, 2003, Prescott, 2002). In recent years, multiple drug/chemical resistance in both human and plant pathogenic microorganisms have been developed due

to the indiscriminate use of commercial antimicrobial drugs/chemical commonly used in the treatment of infectious diseases (Davies, 1994). This situation forced scientists to the search for new antimicrobial (i.e. antibacterial and antifungal) substance from various sources like medicinal plants (Clark, 1996). For years, Man has used various parts of plants in the treatment and prevention of various ailments (Tanaka and Soto, 2002). Plant based natural constituents can be derived from barks, leaves, flowers, roots, fruits, seeds and stems of plants (Gordon and David, 2001) which in most cases contain active components (Jigna and Nehal, 2006).

Scientists estimated that about 10,000 different phytochemicals are effective against diseases like cancer and metabolic syndrome. Some phytochemical constituents found in plants are alkaloids, saponins, tannins, flavonoids, anthraquinone, steroids, terpernoids, phlobatannins, reducing sugar among others. In the attempt to evaluate the western Africans believe of using *Anogeissus leiocarpus* as chewing stick, *the* chemotherapeutic properties of *Anogeissus leiocarpus* was carried out in order to establish their antimicrobial activities against human pathogens and the bioactive compounds present in them.

MATERIALS AND METHODS

Sample Collection and Preparation

The plant material was collected from the herbarium of the Department of Pure and Applied Biology, LAUTECH, Ogbomoso, Oyo State, Nigeria. The plant material was dried and grounded into powder, first with clean mortar and pestle. The powdered materials were also sieved through the mesh size of 20mm.

Extraction Method

Plant extracts were obtained by cold percolation method according to the method of Veeramuthu, 2006. Ethanol, ethyl acetate and chloroform were the solvents used in the extraction method. 50 g of the dried sample was percolated in 200 ml of the solvents in a covered plastic container. The mixtures were stirred intermittently and kept for two days (48 hours). The mixture was filtered, first through muslin cloth and then Whatman No.1 filter paper. The filtrates were allowed to dry at an ambient temperature. The residues were collected as the crude active compounds and stored at 4 ^{o}C for subsequent use.

Screening for Antimicrobial Activity

Preparation of Sensitivity Disc

Whatman No.1 filter paper was perforated using paper perforator of which discs of 6.0 mm in diameter were obtained. These were placed in sterile petri-dish and sterilized in a hot air oven at 160 °C for one hour.

Test Organisms

The test organisms used in the research were bacterial (*Escherichia coli, Pseudomonas aeruginosa, Klebsiella species, Bacillus cereus* and *Streptococcus pneumonia*) and fungal (*Aspergillus flavus, Aspergillus niger, Trichoderma* spp. and *Candida* spp.) isolates. They were obtained from Ladoke Akintola University of Technology (LAUTECH) Teaching Hospital, Ogbomoso, Oyo State.

Standardization of Inoculums

Each culture of the bacterial isolates was standardized by culturing on nutrient agar for 24 hours at 37 °C. The overnight bacterial cultures were sub cultured in nutrient broth for 2 hours to achieve turbidity that is according to standard. The fungal isolates on the other hand were sub-cultured in yeast extract medium and kept on incubator shaker for 48 hrs.

Antimicrobial Susceptibility

Disc diffusion method was employed to screen for antimicrobial activity of the plant extracts. Nutrient agar (bacteria) and potato dextrose agar (fungi) plates were used for the inoculation of organisms. The test organisms were swabbed evenly on the surface of the agar plates with the use of sterile swab sticks. With the aid of sterile pair of forceps, impregnated paper discs containing the extracts of materials at different concentrations 500 mg/ml, 250 mg /ml, 100 mg/ml, 50 mg/ml and 25 mg/ml were arranged radially with the control disc (i.e. disc impregnated with each solvent only) at the centre and pressed slightly and firmly to the inoculated agar surface to ensure even contact. The plates were incubated at 37 °C and 30 ^{0}C for bacteria and fungi respectively. Bacterial plates were incubated for 24 hours while fungal plates were

incubated for 2 days. The degree of sensitivity was determined by measuring the diameter in millimetre of the visible zone of inhibition of the microbial growth produced by the diffusion of the extracts (Lewis and Elvin-Lewis, 2005).

Antibiotic Susceptibility Test on the Collected Bacteria

Streptomycin and Gentamicin were used as positive control for gram negative and gram positive bacteria respectively. The standard antibiotic discs were placed on inoculated plates containing the isolates and incubated at 37 ^{0}C for 24 hours. The diameter of the inhibition zones were measured in millimetre after incubation.

Antibiotic Susceptibility Test on the Collected Fungi

Nystatin was used as positive control for the fungi tested. The discs impregnated with Nystatin were placed on already inoculated plates with the isolates and incubated at 30 ^{0}C for 48 hours. The diameter of the inhibition zones were measured in millimetre after incubation.

Phytochemical Screening

Qualitative screening of the phytochemical components of the chewing sticks was carried out according to the method of Adelowo and Oladeji (2016) to detect the presence of alkaloids, saponins, tannins, flavonoids, anthraquinone, steroids, terpernoids, reducing sugars and phlobatannins. 1 g of the each extract residue was dissolved in 25 ml of distilled water and filtered. The filtrate of each sample was used in testing for the active compounds;

Tannins 0.5 ml Ferric chloride was added to 1 ml of the filtrate, presence of blue black and blue green precipitate indicated the presence of tannin.

Anthraquinone 10 ml of chloroform was added to 1 ml of the filtrate, greenish yellow precipitate indicated the presence of anthraquinone.

Steroids 0.5 ml acetic anhydride was added to 1 ml of the filtrate few drops of conc. H_2SO_4; bluish green precipitate indicated the presence of steroid.

Flavonoids 2 ml NaOH was added to 2 ml of the filtrate to give a yellow colour which turns to colourless on addition of dilute acid as an indication for the presence of flavonoid.

Terpernoids 2 ml of chloroform was added to 5 ml of filtrate, 3 ml concentrated H_2SO_4 was then carefully added to form a layer with reddish brown colour at the interface which is a positive result for the presence of terpernoids.

Alkaloids 2 ml of chloroform with few drops of Wagner reagent were added to 1 ml of the filtrate, reddish brown precipitate indicated the presence of alkaloid.

Saponins: This was carried out by frothing test, 2 ml of the filtrate was vigorously shaken in the test tube for two minutes, presence of frothing in the test tube indicated positive test.

Reducing Sugars 1 ml of water and 5 to 8 drops of Fehling's solution were added to 0.5 ml of the filtrate at hot condition, brick red precipitate shows the presence of reducing sugar.

Phlobatannins Few drops of 1% HCl were added to 1 ml of the filtrate, a reddish brown colour indicated the presence of phlobatannins.

Statistical Analysis

The Statistical Package for Social Scientists (SPSS, version 19.0) was used for the analysis of the data obtained. Two way ANOVA test was used to determine the level of significance of the crude extracts at different concentrations i.e. 500 mg/ml, 250 mg/ml, 100mg/ml, 50 mg/ml and 25 mg/ml. Also, comparison of the different solvent extractions (i.e. ethanol, ethyl acetate and chloroform) were made statistically.

The general antimicrobial effects of the extracts were also compared with the standard antibiotics and antifungal disc statistically.

RESULTS AND DISCUSSION

Antimicrobial Activity of Anogeissus Leiocarpus

The *in vitro* antimicrobial activity of the ethanol, ethyl acetate and chloroform extracts of *A. leiocarpus* was evaluated statistically against nine pathogenic organisms. The organisms include both gram-positive

and gram-negative bacteria and fungi. The antimicrobial activity of various solvent extract of the chewing sticks by disc diffusion method was analysed statistically (Table 1a and 1b).

Table 1a: Diameter Zones of Inhibition (mm) of the Crude Extracts of *Anogeissus Leiocarpus* against Selected Bacteria

		Ethanol		Ethyl Acetate		Chloroform	
P. aeruginosa	C	8.50	± 0.707	7.50	± 0.707	7.50	± 0.707
	C_1	18.00	± 1.414*	13.50	± 2.121*	11.50	± 2.121
	C_2	15.00	± 1.414*	10.00	± 1.414	9.50	± 0.707
	C_3	14.00	± 1.414*	9.00	± 1.414	8.50	± 0.707
	C_4	11.50	± 0.707	9.00	± 1.414	8.50	± 0.707
	C_5	10.50	± 0.707	7.50	± 0.707	8.00	± 1.414
Klebsiella sp	C	9.00	± 1.414	8.00	± 1.414	8.00	± 1.414
	C_1	16.50	± 0.707*	10.50	± 0.707	10.50	± 2.121
	C_2	15.00	± 1.414*	9.50	± 0.707	9.50	± 0.707
	C_3	14.00	± 1.414*	10.00	± 1.414	8.50	± 0.707
	C_4	11.50	± 0.707	9.00	± 1.414	8.00	± 1.414
	C_5	10.00	± 1.414	8.00	± 1.414	8.50	± 2.121
E.coli	C	7.50	± 0.707	8.50	± 0.707	8.50	± 0.707
	C_1	16.00	± 1.414*	12.00	± 1.414	11.50	± 2.121
	C_2	13.50	± 0.707*	9.50	± 0.707	10.00	± 1.414
	C_3	12.50	± 0.707*	9.50	± 0.707	10.00	± 1.414
	C_4	10.00	± 1.414	9.00	± 1.414	9.50	± 2.121
	C_5	8.00	± 1.414	8.50	± 0.707	8.00	± 1.414
S. pneumonia	C	8.50	± 0.707	7.50	± 0.707	7.00	± 1.414
	C_1	21.00	± 1.414*	15.50	± 0.707*	14.00	± 1.414*
	C_2	18.00	± 1.414*	13.00	± 1.414*	12.00	± 1.414
	C_3	14.00	± 1.414*	12.00	± 1.414*	12.50	± 2.121
	C_4	12.50	± 0.707	10.50	± 0.707	10.00	± 1.414
	C_5	10.00	± 1.414	10.00	± 1.414	9.00	± 1.414
B. cereus	C	8.00	± 1.414	9.00	± 1.414	9.00	± 1.414
	C_1	12.00	± 1.414	13.00	± 1.414	10.00	± 1.414
	C_2	11.00	± 1.414	12.00	± 1.414	9.00	± 1.414
	C_3	10.00	± 1.414	9.50	± 0.707	9.00	± 1.414
	C_4	10.00	± 1.414	9.00	± 1.414	8.00	± 1.414
	C_5	9.00	± 1.414	9.00	± 1.414	9.00	± 1.414

Values are expressed as mean ± SD for n=2 for each concentration. Values with (*) are significantly higher than the control at $p < 0.05$.

The inhibitory action was observed in terms of diameter of inhibition zone formed around each disc caused by the disc diffusion of antimicrobial substances from the paper discs into the surrounding medium. The significances of the ethanol extracts of the chewing sticks with standard antibiotics (Streptomycin, Gentamicin and Nystatin) were analysed statistically (Table 2).

The bioactive compounds present in the crude extracts of the chewing sticks tested against the microorganisms. The diameter zone of inhibition (mm) of crude ethanol extracts of *A. leiocarpus* against *A. flavus* (33.50 mm) (Figure 1). The diameter zone of inhibition (mm) of standard antibiotic disc (Gentamicin) on *Streptococcus pneumoniae* (Figure 2) ((Table 1a and 1b).

Table 1b: Diameter Zones of Inhibition (mm) of the Crude Extracts of *Anogeissus Leiocarpus* against Selected Fungi

		Ethanol		Ethyl Acetate		Chloroform	
A. flavus	C	7.50	± 0.707	7.00	± 1.414	6.50	± 0.707
	C_1	14.00	± 1.414	13.50	± 0.707*	11.00	± 1.414
	C_2	13.00	2.828	12.00	± 2.828	10.00	± 1.414
	C_3	11.50	± 2.121	9.50	± 0.707	8.00	± 1.414
	C_4	9.00	± 1.414	10.00	± 1.414	8.00	± 1.414
	C_5	7.50	± 0.707	8.50	± 0.707	7.50	± 0.707
A. niger	C	10.00	± 1.414	8.50	± 0.707	9.00	± 1.414
	C_1	16.00	± 1.414*	14.00	± 1.414*	11.00	± 1.414
	C_2	13.50	± 0.707	12.00	± 1.414	10.00	± 1.414
	C_3	13.00	± 1.414	10.50	± 0.707	8.00	± 1.414
	C_4	10.50	± 0.707	8.50	± 0.707	8.00	± 1.414
	C_5	10.00	± 1.414	8.00	± 1.414	8.00	± 1.414
Trichoderma sp	C	7.50	± 0.707	7.50	± 0.707	6.50	± 0.707
	C_1	18.00	± 1.414	17.00	± 1.414*	12.50	± 0.707*
	C_2	16.00	± 1.414	13.50	± 2.121*	12.00	± 1.414*
	C_3	12.50	± 0.707	12.00	± 1.414	9.50	± 0.707
	C_4	6.00	± 8.485	10.00	± 1.414	9.50	± 2.121
	C_5	9.50	± 0.707	8.50	± 0.707	8.00	± 1.414
Candida sp	C	8.50	± 0.707	8.50	± 0.707	7.50	± 0.707
	C_1	20.50	± 0.707*	18.50	± 0.707*	15.00	± 1.414*
	C_2	18.00	± 1.414*	14.50	± 0.707*	13.00	± 1.414
	C_3	16.00	± 1.414*	11.50	± 0.707	12.00	± 1.414
	C_4	12.50	± 0.707	11.50	± 0.707	10.50	± 0.707
	C_5	10.00	± 1.414	9.00	± 1.414	9.50	± 2.121

Values are expressed as mean ± SD for n=2 for each concentration. Values with (*) are significantly higher than the control at $p < 0.05$.
Key; C=Concentration C_1 = 500 mg/ml C_2 = 250 mg/ml C_3 = 100 mg/ml C_4 = 50 mg/ml C_5 = 25 mg/ml.

The results obtained for the antimicrobial activity of *Anogeissus leiocarpus* on the test organisms, the data was analysed statistically and the significance level was obtained at $p<0.05$. Values are expressed as mean ± SD for each concentration. Values with (*) are significantly higher than the control at $p < 0.05$ (Table 2).
The ethanol extract effectively inhibited all the test organisms. The highest zone of inhibition at C_1 (500 mg/ml) which is the highest concentration used, was recorded from *S. pneumoniae* with a diameter of 21.00 mm.
The lowest zone of inhibition at the same concentration was recorded from *B. cereus* with a diameter of 12.00 mm. From the ethyl acetate extract, the highest zone of inhibition at C_1 (500 mg/ml) was recorded from *Candida sp* with a diameter of 18.50 mm while the lowest zone of inhibition at the same

concentration was recorded from *Klebsiella sp.* with a diameter of 10.50 mm. The chloroform extract was ineffective against all the test organisms (Table 1a and 1b).

Table 2: Significance of *Anogeissus Leiocarpus* Ethanol Extract to Standard Antibiotics and Antifungal

Bacterial Isolates		**Ethanol**	**Extract**	**Fungal Isolates**		**Ethanol**	**Extract**
P. aeruginosa	STR	11.50	± 0.707	*A. flavus*	NYS	15.00	± 0.000
	C_1	18.00	± 1.414*		C_1	14.00	± 1.414
	C_2	15.00	± 1.414		C_2	13.00	± 2.828
	C_3	14.00	± 1.414		C_3	11.50	± 2.121
	C_4	11.50	± 0.707		C_4	9.00	± 1.414
	C_5	10.50	± 0.707		C_5	7.50	± 0.707
Klebsiella sp	STR	10.50	± 0.707	*A. niger*	NYS	16.50	± 0.707
	C_1	16.50	± 0.707*		C_1	16.00	± 1.414
	C_2	15.00	± 1.414*		C_2	13.50	± 0.707
	C_3	14.00	± 1.414		C_3	13.00	± 1.414
	C_4	11.50	± 0.707		C_4	10.50	± 0.707
	C_5	10.00	± 1.414		C_5	10.00	± 1.414
E. coli	STR	10.00	± 0.000	*T. species*	NYS	14.00	± 0.000
	C_1	16.00	± 1.414*		C_1	18.00	± 1.414
	C_2	13.50	± 0.707		C_2	16.00	± 1.414
	C_3	12.50	± 0.707		C_3	12.50	± 0.707
	C_4	10.00	± 1.414		C_4	6.00	± 8.485
	C_5	8.00	± 1.414		C_5	9.50	± 0.707
S. pneumoniae	GEN	13.50	± 0.707	*Candida sp*	NYS	14.00	± 0.000
	C_1	21.00	± 1.414*		C_1	20.50	±0.707*
	C_2	18.00	± 1.414		C_2	18.00	± 1.414
	C_3	14.00	± 1.414		C_3	16.00	± 1.414
	C_4	12.50	± 0.707		C_4	12.50	± 0.707
	C_5	10.00	± 1.414		C_5	10.00	± 1.414
B. cereus	GEN	13.00	± 0.000				
	C_1	12.00	± 1.414				
	C_2	11.00	± 1.414				
	C_3	10.00	± 1.414				
	C_4	10.00	± 1.414				
	C_5	9.00	± 1.414				

Values are expressed as mean ± SD for n=2 for each concentration. Values with (*) are significantly higher than the standard at $p < 0.05$.
Key; STR=Streptomycin, GEN=Gentamicin, NYS=Nystatin; C=Concentration

Phytochemical Screening
Ethanol, ethyl acetate and chloroform extracts of *Anogeissus leiocarpus* showed the presence of Steroids, Terpernoids, Saponins and Alkaloids were present in the three extracts. Tannins and Flavonoids were present in the ethanol and ethyl acetate extracts and absent in the chloroform extract. Anthraquinones, Phlobatannins and Reducing sugars were absent in all the extracts (Table 3).

Table 3: Phytochemical Components of Ethanol, Ethyl Acetate and Chloroform Extracts of *Anogeissus Leiocarpus*

Active Compound	Ethanol Extract	Ethyl Acetate Extract	Chloroform Extract
Anthraquinones	-	-	-
Tannins	+	+	-
Phlobatannins	-	-	-
Steroids	+	+	+
Flavonoids	+	+	-
Terpenoids	+	+	+
Saponins	+	+	+
Alkaloids	+	+	+
Reducing Sugars	-	-	-

Key; + = present, - = absent

Figure 1: Diameter Zone of Inhibition (mm) of the Crude Ethanol Extracts of *Anogeissus Leiocarpus* against *Aspergillus Flavus*

Figure 2: Diameter Zone of Inhibition (mm) of Standard Antibiotic Disc on *Streptococcus Pneumoniae* Plate

The antimicrobial activities of the crude extracts of *Anogeissus leiocarpus* like the other chewing sticks tested also appeared to be a broad spectrum since both Gram negative and Gram positive bacteria and fungi were sensitive to the extracts. From the result, it shows that the ethanol extract of the chewing stick is the most effective in inhibiting the growth of the test organisms recording the highest zone of inhibition at concentration C_1 (500 mg/ml), a diameter of 20.5 mm against *Candida sp.* compare to ethyl acetate extract which recorded 18.5 mm against *Candida sp*. and chloroform extract with 15.0 mm against *Candida sp.* This justifies their use in the reduction or suppression of tooth ache on gums (Ndukwe *et al.*, 2005). The ethanol extract was compared statistically with the standard antibiotics and the results showed that C_1 (500 mg/ml) was significantly higher than Nystatin for *Candida sp.*, C_1 and C_2 were significantly higher than Streptomycin against *Klebsiella sp*. but only concentration C_1 was higher than Streptomycin against *P. aeruginosa* and *E. coli* and Gentamicin against *S. pneumoniae* while Gentamicin was more effective than the extract against *B. cereus* and Nystatin against *A. flavus, A. niger* and *Trichoderma sp*.
Medicinal plants contain physiologically active components which over the years have been exploited in the traditional medical practices for the treatment of various ailments (Ajibesin, 2011; Ngulefack *et al.*, 2005). Phytochemicals such as saponins, terpenoids, flavonoids and alkaloids have been shown to have anti-inflammatory effects (Cherian and Augustine, 1995). The ethanol, ethyl acetate and chloroform extracts of *A. leiocarpus* as shown in the result obtained from preliminary phytochemical screening carried out in study has revealed the presence of anthraquinone, tannins, steroids, flavonoids, terpernoids, saponins and alkaloids. Flavonoids and tannins have been reported to be antioxidants used to neutralize highly unstable and extremely reactive molecules like free radicals that attack the cells of human body (Karthishwaran *et al.*, 2010; Owoyale *et al.*, 2005; Stauth, 2007).

Conclusion

The result obtained from this study showed that stem of *Anogeissus leiocarpus* used as chewing sticks are effective in treating both bacterial and fungal infections caused by some pathogenic organisms. The antimicrobial activities of the chewing stick can be due to the presence of secondary metabolites or phytochemicals such as tannins, saponins and anthraquinone. The presence of secondary metabolites, singly or in combination with others could be responsible for the antimicrobial activity of the chewing sticks. From the study, ethanol extract of *A. leiocarpus* are the most effective when compared to other solvents due to the solvent polarity. Therefore, the presence of phytochemicals is responsible for the antimicrobial activity of the stem of *A. leiocarpus* as chewing sticks.

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Compliance with Ethnical Standards

Conflict of Interest The authors declare that there is no competing interests or conflict or conflict of interest regarding the publication of this article.

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