IJPSR (2012), Vol. 3, Issue 07 **(Research Article)**

INTERNATIONAL JOURNAL OF PHARMACEUTICAL SCIENCES AND RESEARCH

Received on 05 March, 2012; received in revised form 19 May, 2012; accepted 23 June, 2012

ANTIMICROBIAL ACTIVITY OF ETHANOL AND AQUEOUS EXTRACTS OF *PARINARI CURATELLIFOLIA* (STEM) ON DENTAL CARIES CAUSING MICROBES

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Keywords:
Antimicrobial activity,
Dental carries,
Agar diffusion,
Bacteria,
Fungi,
Zone of inhibition

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ABSTRACT

Antimicrobial activity of ethanol and aqueous extracts of *Parinari curatellifolia* (stem) were tested against five dental carries causing bacteria and three fungi strains by agar diffusion method. The crude extracts showed a broad spectrum of antibacterial activity inhibiting the five strains of bacteria and the three fungi. The extracts were most effective against *Aspergilusflavus*, followed by *Streptococcus mutans* and *Staphylococcus aureus* respectively. *S. mutans* and *S. aureus* were the only microorganisms that showed zone of inhibition in all the various concentrations of the ethanol extract of *P. curatelifolia.* Preliminary phytochemical screening revealed the presence of alkaloids, flavonoid, anthraquinones, saponins, tannins, cardiac glycosides, steroids, terpenoids, phlobotanins and carbohydrates.

INTRODUCTION: In Nigeria, as in other developing countries, a very significant proportion of orofacial diseases are due to microbial infection [1]. This has led to a wide spread use of antibiotics in dental practice in these regions and this gives microorganisms enhanced opportunities for the development of resistance to a broad spectrum of antibiotics [1]. The need to conserve antibiotics in order to prevent the selection of antibiotic-resistant organisms has been recognized [2] and there is therefore the need to look for non-antibiotic substances with proven antimicrobial activity which can be used in the treatment of microbial infections, including those encountered in dental practice.

Chewing sticks are widely used in Africa and Asia as a means of maintaining oral hygiene [3]. They are obtained from the roots, twigs, or stems of various plants. A combination of vertical and horizontal strokes of the 'brush' on tooth surfaces removes plaque. About five minutes of complete devotion to this exercise is deemed adequate to achieve good cleansing. According to [4] chewing sticks obtained from a variety of selected plants are used as a traditional method of mechanical oral hygiene by up to 80%-90% of Nigerians.

Studies have demonstrated that chewing sticks are at least as effective as tooth brushes in maintaining oral hygiene [5, 6, 7, 8, 9] reported that Africans that use chewing sticks have fewer carious lesions than those using tooth brushes, and their use has been encouraged by the World Health Organization [10]. Most of these chewing sticks have been shown to have significant antimicrobial activity against a broad spectrum of microorganisms [11] described the activity of several plant extracts against *Streptococcus mutans*, a carcinogenic organism.

Since then, several investigators including [12, 13] as well as [14] have made similar reports of the antimicrobial activity of various chewing stick extracts.

The study also showed that some of the chewing stick extracts demonstrated activity against antibiotic resistant organisms. So they can be viewed as sources of novel lead substances with potential therapeutic or preventive application.

The twigs of *Parinari curatellifolia* are used as chewing sticks and mouth washed with a root infusion for toothache. *P. curatellifolia* of the family Chrysobalanaceae is variable in size and shape ranging from small shrubs of 3 m tall to large trees of up to 20 m high. The tree is evergreen, with pale green, spreading foliage forming a dense, rounded, umbrella shaped crown, which casts heavy shade. The branching is low, and the bole twisted, 25-40 cm in diameter.

The bark is rough and corky with yellow wooly hairs occasionally present in younger twigs and branches. Silica crystals in the wood are a common occurrence. The leaves are distinctly bicolored, having a white-silver undersurface and a dark green-gray upper surface.

Objectives of the Study: This study is aimed at assessing the phytochemical and the antimicrobial activities of the aqueous and ethanolic extracts of stem of *P. curatellifolia* used as chewing stick in some parts of Edo North in Edo State, Nigeria.

MATERIALS AND METHODS:

Collection and identification of Plant Material: The stems of *P.curatelifolia* plantwere collected from Edo North Senatorial District of Edo State. The plant was identified by Dr J.F Bamidele of the Department of Plant Biology and Biotechnology, University of Benin, Benin City, Edo State.

Preparation and extraction of Plant Material: The fresh stems *P.curatelifolia* were obtained from the plant, rinsed in water and spread on trays and dried under the sun. The plant materials were then transferred to the oven set at 45°C for 20-30 minutes before being reduced to fine powder with the aid of a mechanical grinder. The powdered plant materials were then collected and stored in a tightly covered glass jars and kept for further studies. For ethanol extraction, 100 g of the powdered stem material was soaked in 600 ml of ethanol. The resultant solution was filtered using Whatman filter paper No. 1 after 48hours under room temperature (25°C). For aqueous extraction; 100 g of the powdered stem material was boiled in 600 ml of water for 24 hours after which the resultant solution was filtered using Whatman filter paper No 1.

The two extracts were concentrated through evaporation process using a water bath set at 100°C. The extracts were then stored in a refrigerator until required for use.

Preparation of stock solution of Extracts: Fresh stock (known concentration) solution of each extract was prepared for each experiment. To prepare a required concentration of the extract, a specific weighed amount of the concentrated extract was dissolved completely in an appropriate volume of distilled water. To prepare 100 mg/ml concentration of extract, 1 gm of either of the extract was dissolved in 10 ml of distilled water in a sample bottle, corked and shaken vigorously to obtain a homogenous solution.

Antimicrobial screening:

Source of Microorganisms: Pure stock cultures of *Staphylococcus aureus, Staphylococcus auricularis, Streptococcus pyogenes, Streptococcus mutans, Candida albicans, Aspergilusflavus, Microsporium gypseum* and *Bacillus subtilis* isolated from patients with dental diseases were obtained from the Department of Medical Microbiology, Department of Dentistry University of Benin, and University of Benin Teaching Hospital (UBTH). These pure isolates were used and maintained in slants of Nutrient Agar (NA) and Potato Dextrose Agar (PDA) at 4°C until when needed for further studies.

Microbial inoculums preparation for Susceptibility Testing: The inocula of the bacterial isolates were prepared by growing each pure isolate in nutrient broth at 37°C for 24 hrs. The fungal isolates were grown in Potato dextrose broth at 28 ± 2°C for 48 hrs. After incubation, 1 ml of the diluted cultures of the microbial isolates in normal saline using a Pasteur pipette was inoculated unto the solidified nutrient agar at 40°C for bacteria and Potato dextrose agar for fungi.

Antimicrobial Assay: Antimicrobial activity was evaluated by noting the zone of inhibition against the test organisms [15]. Two colonies of a 24-hour plate culture of each organism were transferred aseptically into 10 ml sterile normal saline in a test tube and mixed thoroughly for uniform distribution. A sterile cotton swab was then used to spread the resulting suspension uniformly on the surface of oven-dried Nutrient agar and Potato dextrose agar plates for bacteria and fungi, respectively. Three (3) adequately spaced wells of diameter 4 mm per plate were made on the culture agar surface respectively using a sterile metal cup-borer.

0.2 ml of each extract and control were put in each hole under aseptic condition, kept at room temperature for 1 hour to allow the agents to diffuse into the agar medium and incubated accordingly. Conventional antibiotics were used as positive controls for bacteria and fungi respectively; distilled water was used as the negative control. The plates were then incubated at 37°C for 24 hours for the bacterial strains and at 28°C for 72 hours for fungal isolates. The zones of inhibition were measured and recorded after incubation.

Zones of inhibition around the wells indicated antimicrobial activity of the extracts against the test organisms. The diameters of these zones were measured diagonally in millimeter with a ruler and the mean value for each organism from the triplicate cultured plates was recorded. Using the agar-well diffusion technique, an already made gram positive and gram negative (Asodisks Atlas Diagnostics, Enugu, Nigeria) standard antibiotic sensitivity disc bought from a laboratory chemical equipment store in Benin city was used as positive control for bacteria while Ketoconanzone was used as positive control for fungi. Distilled water was used as negative control for all the test organisms.

Determination of Minimum Inhibitory Concentrations (MICs) of the extracts: The lowest concentration of the extracts that will inhibit the growth of test organisms is the Minimum Inhibitory Concentration (MIC). The initial concentration of the plant extract (100 mg/ml) was diluted using double fold serial dilution by transferring 5 ml of the sterile plant extract (stock solution) into 5 ml of sterile Normal saline to obtain 50 mg/ml concentration [16]. Different concentrations were prepared from the crude extract by doubling dilution in distilled water. The different concentrations were 50, 25, 12.5, 6.25 and 3.125 mg/ml respectively. Each dilution was introduced into nutrient agar plates and potato dextrose agar plates already seeded with the respective test organism. All test plates were incubated at 37°C for 24 hrs for bacteria and 28°C$\pm$ 2°C for 72 hrs for fungi. The minimum inhibitory concentration (MIC) of the extracts for each test organism was regarded as the agar plate with the lowest concentrations without growth [15].

Minimum Bactericidal Concentration (MBC): The Minimum Bactericidal Concentration (MBC) of the plant extracts were determined by the method described by [17, 18]. Samples were taken from plates with no visible growth in the MIC assay and subcultured on freshly prepared nutrient agar plates and Potato dextrose agar plates and later incubated at 37°C for 48 hours and 28$\pm$2°C for 72 hours for bacteria and fungi respectively. The MBC was taken as the concentration of the extract that did not show any growth on a new set of agar plates.

Determination of the Antibiotic Susceptibility of Bacteria Isolates: The disc diffusion method [19] was used for the determination of microbial sensitivity. The antibiotic discs employed were: septrin, chloramphenicol, sparfloxcarcin, ciprofloxacin, amoxicillin, augmenting, gentamicin, pefoxacin, ofloxacin, streptomycin, zinnacef and recophin. The zones of inhibition were measured and interpretation was in accordance with manufacturer's instructions.

Phytochemical Screening: The phytochemical tests were carried out on the aqueous and ethanolic extracts using standard procedures as described by [20, 21].

Statistical Analysis:

RESULTS AND DISCUSSION: Table 1 shows the antimicrobial properties of the aqueous and ethanol extracts of *P. curatelifolia* plant on the test microorganisms. All the test organisms were sensitive to the ethanol extracts at a concentration of 100 mg/ml. The activities of the ethanol extracts on all the tested organisms were significantly different from one another.

The highest zone of inhibition was recorded against *A. flavus* with an average sensitivity diameter of 22.09±0.12 mm, while the least sensitive was recorded against *M. gypseum* with an average sensitivity diameter of 11.13±0.09mm.

Plant extracts were more susceptible to *A. flavus* (fungus) followed by *B. subtilis* (gram +ve rod bacteria), *S. mutans* (gram +ve), *S. aureus* (gram +ve), *C. albicans* (fungus), *S. auricularis* (gram +ve), *S. pyogenes* (gram +ve) and *M. gypseum* (fungus) respectively. The aqueous extract of *P. curatelifolia*showed the highest antimicrobial activity in six of the tested organisms (*S. aureus, M.gypseum, S. pyogenes, S. mutans, A. flavus*and *C. albicans*) ranging between 6.53 mm and 18.00 mm respectively.

It was revealed that the ethanol extract has the highest antibacterial and antifungal activity against all the tested oral microorganisms with inhibition diameters of 22.09± 0.12 mm and 11.13±0.09 mm respectively at 100 mg/ml. **Table 2** revealed that the activity of the ethanol (Et) extract of *P. curatelifolia*stemwas significantly different from one concentration to another on each organism. *P. curatelifolia* extracton test organisms at 3.125 mg/ml showed zone of inhibition of 2.40±0.06 and 3.23±0.12 mm on *S. aureus* and *S. mutans* respectively. *A. flavus* revealed the highest susceptibility when compared with other test organisms at the highest concentrations of 100 mg/ml.

Table 3 present the Minimum Inhibitory Concentration (MIC) and the Minimum Bactericidal Concentration (MBC) values of the ethanol extracts. The ethanol extract of *P. curatelifolia* showed MIC at 3.125 mg/ml against *S. aureus*. While it is sensitive against *S. auricularis*and *S. mutans* at 6.25 mg/ml extracts. 12.5 mg/ml extracts of *P. curatelifolia,* against *S. pyogenes.* Whilethe three fungi (**Table 4**), *C. albicans, A. flavus and M. gypseum*were sensitive to theextracts of *P. curatelifolia*in the following order of concentrations25 mg/ml, 12.5 mg/ml and 6.25 mg/ml

Table 5 shows the activity of the commercial antibiotics (standard sensitivity disc) on the test bacteria. It revealed a sensitivity zone of inhibition diameter varying from 4.0 mm – 28.3 mm against the bacterial isolates used.

Table 6 revealed that ketoconanzone (commercial fungi antibiotic) was active against all the test fungi. It had the highest activity against *M.gypseum* with inhibition diameter of 26 mm, followed by *C. albicans*24 mmand *A. flavus*17 mm.

In **Table 7** the results of the phytochemical analysis of aqueous and ethanolic stem extracts of *P. curatelifolia* revealed the presence of some secondary metabolites such as alkaloids, flavonoids, cardiac glycosides, terpenoids, anthraquinones, phlobotannins, saponin and tannins. Steroids were absent in the ethanol extract of *P. curatelifolia.*

TABLE 1: ZONE OF INHIBITION OF AQUEOUS AND ETHANOL EXTRACTS (100 MG/ML) OF *P. CURATELIFOLIA* AGAINST SELECTED ORAL PATHOGENS

	Test Organisms							
***P. curatelifolia* (stem)**	***S. aureus***	***S. auricularis***	***M. gypseum***	***S. pyogenes***	***S. mutans***	***B. subtillis***	***A. flavus***	***C. albicans***
Aq	8.20±0.06	6.53±0.03	8.00±0.06	6.73±0.07	8.00±0.06	9.10±0.06	10.43±0.09	7.80±0.06
Et	17.63±0.09	16.2±0.09	11.13±0.09	14.90±0.06	18.20±0.06	11.30±0.06	22.09±0.12	16.43±0.09

NB: Mean ± S.E.M; n=3, Mean ± S.E.M within a row are significantly different, P< 0.01. **–** = No inhibition. Aq = Aqueous, Et = Ethanol

TABLE 2: ZONE OF INHIBITION IN MM OF VARIOUS CONCENTRATIONS OF THE ETHANOL EXTRACT OF*P. CURATELIFOLIA* ON TEST ORGANISMS

Test organisms	Concentration of extract (mg/ml)						Sterile distilled water
	3.125	6.25	12.5	25	50	100	
S. aureus	2.40 ± 0.06[a]	5.03 ± 0.20[b]	8.50 ± 0.10[c]	9.47 ± 0.09[d]	13.27 ± 0.19[e]	17.10 ± 0.06[f]	–
S. auricularis	–	3.10 ± 0.06[a]	4.40 ± 0.15[b]	7.53 ± 0.15[c]	9.07 ± 0.18[d]	15.97 ± 3.33[e]	–
S. pyogenes	–	3.03 ± 0.12[a]	4.87 ± 0.09[b]	8.63 ± 0.12[c]	10.53 ± 0.09[d]	16.57 ± 0.09[e]	–
S. mutans	3.23 ± 0.12[a]	4.27 ± 0.12[b]	8.70 ± 0.15[c]	10.43 ± 0.09[d]	14.57 ± 0.07[e]	17.97 ± 0.09[f]	–
M. gypseum	–	–	3.77 ± 0.12[a]	6.17 ± 0.09[b]	7.43 ± 0.09[c]	12.27 ± 0.09[d]	–
B. subtilis	–	–	–	4.30 ± 0.95[a]	5.53 ± 0.12[b]	10.60 ± 0.06[c]	–
A. flavus	–	–	5.70 ± 0.15[a]	9.43 ± 0.09[b]	13.57 ± 0.07[c]	19.98 ± 0.09[d]	–
C. albicans	–	–	–	4.50 ± 1.00[a]	5.33 ± 0.18[b]	17.10 ± 0.15[c]	–

NB: Values are means ± S.E.M (n=3); Values within a row with different alphabet are significantly different, P< 0.01;**–** = No inhibition.

TABLE 3: MINIMUM INHIBITORY CONCENTRATIONS (MICS) AND MINIMUM BACTERICIDAL CONCENTRATIONS (MBCS) IN MG/ML OF THE ETHANOL EXTRACTS OF *P. CURATELIFOLIA* PLANTAGAINST THE TEST BACTERIA

	Test bacteria				
***P. curatelifolia* (stem) (mg/ml)**	***S. aureus***	***S. auricularis***	***S. pyogenes***	***S. mutans***	***B. subtillis***
MIC	3.125^a	6.25^b	12.50^c	6.25^d	25.00^e
MBC	6.25^a	12.50^b	25.00^c	25.00^d	25.00^e

NB: Values are means + S.E.M (n=3); Values within a row with different alphabet are significantly different, P< 0.01

TABLE 4: MINIMUM INHIBITORY CONCENTRATIONS (MICS) AND MINIMUM FUNGICIDAL CONCENTRATIONS (MFCS) IN MG/ML OF THE ETHANOL EXTRACTS OF *P. CURATELIFOLIA* PLANTAGAINST THE TEST FUNGI

	Test fungi		
***P. curatelifolia* (stem) (mg/ml)**	***M. gypseum***	***A. flavus***	***C. albicans***
MIC	3.125^a	12.5^b	25.00^c
MFC	6.25^a	25^b	50.00^c

NB: Values are means + S.E.M (n=3); Values within a row with different alphabet are significantly different, P< 0.01;.

TABLE 5: SENSITIVITY ZONE OF INHIBITION OF COMMERCIAL ANTIBIOTICS (STANDARD SENSITIVITY DISC) ON THE TEST BACTERIA

	ZONE OF INHIBITION (in mm) FOR COMMERCIAL ANTIBIOTICS									
Test isolates	**CN**	**APX**	**R**	**CPX**	**E**	**SXT**	**PEF**	**OFX**	**S**	**AM**
S. Aureus	28.3	7.4	–	24.5	9.7	–	21.6	27.0	–	–
S. auricularicus	27.0	4.6	–	20.9	7.7	–	17.8	28.1	–	10.5
S. pyogenes	19.7	6.8	_	20.1	5.2	_	14.6	24.5	_	_
S. mutans	20.6	4.0	–	18.7	8.8	–	14.5	20.9	–	–
B. subtilis	24.8	5.5	–	19.0	8.6	–	11.8	19.9	–	7.7

PEF = Pefloxacin (10 µg/ml), **–** = No inhibition; CN = Gentamicin (20 µg/ml; APX = Ampiclox (30 µg/ml); OFX= Ofloxacin (10 µg/ml); AM = Amoxicillin (30 µg/ml); R = Rocephin (25 µg/ml); CPX = Ciprofloxacin (10 µg/ml); S = Streptomycin (30 µg/ml); SXT = Septrin (30 µg/ml); E = Erythromycin (10 µg/ml); APX = Ampiclox (10 µg/ml)

TABLE 6: SENSITIVITY ZONE OF INHIBITION OF COMMERCIAL FUNGI ANTIBIOTICS (KETOCONANZONE) ON THE TEST FUNGI

Test fungi	**Ketoconanzone (200 mg/ml)**
Aspergillusflavus	17 mm
Candida albicans	24 mm
Microsporiumgypseum	26 mm

TABLE 7: PHYTOCHEMICAL SCREENING OF THE AQUEOUS AND ETHANOLIC EXTRACTS OF *P. CURATELIFOLIA* PLANT PART USED AS CHEWING STICKS TEST PLANT EXTRACTS

	Chemical components									
***P. curatelifolia* (stem)**	**Alkaloids**	**Flavonoids**	**Anthraquinones**	**Saponins**	**Tannins**	**Cardiac Glycosides**	**Steroids**	**Terpenoids**	**Phlabotannins**	**Carbohydrates**
Aq	+	+	+	+	+	+	+	+	+	+
Et	+	+	+	+	+	+	_	+	+	+

KEY: + = Present, – = Absent, Aq = Aqueous, Et = Ethanol

The aqueous extract of *P. curatelifolia* was most active against *M. gypseum* with zone of inhibition of 18.00+0.06 mm at 100 mg/ml (Table 1).Ethanolic extract of *P. curatelifolia* recorded the highest antifungal activity of 22.09+0.12 against *A. flavus*at 100 mg/ml. Rocephin, streptomycin and erythromycin showed no inhibition zone on any of the tested organisms. Comparatively, the ethanol and aqueous extracts can be said to possess better activity than the commercial antibiotics since they contain both pharmacological and non-pharmacologically active substances as oppose to the pure active substances contained in the control antibiotics. The effect of the commercial antifungal drug (Ketoconazole) tested at a concentration of 200 mg/ml against the test fungi (Table 6) can be considered not better in activity when compared with the extracts, particularly at the highest tested concentration of 100 mg/ml which was two times lower in concentration than that of the commercial fungal antibiotics. This probably implies that if the concentrations of the extracts were increased, it could lead to increased activity.

CONCLUSION: Dentists are scarce in many parts of Africa, particularly in rural areas. Although diet plays a major role in preventing dental caries, the practice of dental hygiene is also important. Therefore, continued access to popular and effective sources of chewing sticks with anti-bacterial and anti-fungal properties is important as a primary health care measure. The results from these studies provide evidence for the ethnomedicinal uses of the tested plant as chewing sticks.

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How to cite this article:
Oshomoh EO and Idu M.: Antimicrobial Activity of Ethanol and Aqueous Extracts of *Parinari Curatellifolia* (Stem) on Dental Caries Causing Microbes. *Int J Pharm Sci Res*, 2012; Vol. 3(7): 2113-2118.