Antimicrobial and Antifungal Activities of Ethanol and Aqueous Crude Extracts of *Hymenocardia acida* Stem Against Selected Dental Caries Pathogens

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**ABSTRACT**

The ethanol and aqueous crude extracts of *Hymenocardia acida* stem showed inhibitory activity against all the tested microorganisms, *Staphylococcus aureus*, *Staphylococcus auricularis*, *Streptococcus pyogenes*, *Streptococcus mutans*, *Candida albicans*, *Aspergillus flavus*, *Microsporum gypseum* and *Bacillus subtilis*. The largest zone of inhibition was obtained with the ethanolic extract (100 mg/ml) against *S. mutans* (16.73 ± 0.07 mm) and the least zone of inhibition was obtained with the aqueous extract (100 mg/ml) against *S. pyogenes* (5.83 ± 0.03 mm). Minimum inhibitory concentrations (MIC) and minimum bactericidal concentrations (MBC) were also determined against the selected microorganisms showing zones of inhibition ≥ 6.25 mm for MIC and ≥ 12.25 mm for MBC. Phytochemical screening indicates the availability of secondary metabolites in the plant. This study reveals that the stem of *H. acida* possess antifungal and antibacterial activities respectively and can be used as a potential source of antimicrobial ingredients to cure dental carries pathogens.

**Key words:** Bactericidal, inhibition, secondary metabolites.

**INTRODUCTION**

Reported[1] that some of the chewing sticks being used are obtained from the following plants: *Garcinia manni*, *Musalaria acuminate*, *Terminalia glaucescens*, *Anogeissus leiocarpus*, *Pseudocedrela kotschyi*, *Zanthoxyllum gilleti* and *Azadirachta indica*.

Investigation further revealed that some of these chewing sticks possess anti-microbial activity against oral microbial flora such as *Staphylococcus aureus* and *S. auricularis*,[3] *Candida albicans*, *Aspergillus flavus*, *Microsporum gypseum* and *Trichophyton metagrophytes*.[5]

Confirmed[3] that chewing sticks have potential of preventing oral ailments. A majority of plants tested in his study revealed that chewing sticks are capable of inhibiting gram-positive and negative bacteria such as *Bacillus subtilis*, *Porphyromonas gingivalis* and *Fusobacterium nucleatum*. Chewing sticks with antimicrobial activity could become a potential source of new drugs for oral diseases. Oral microorganisms are known for their pathogenesis in tooth decay, gingivitis, periodontitis, and their ability to cause teeth loss[4]. Utilization of non-timber forest products (NTFP) are widely used as chewing sticks for dental cleaning in tropical Africa, they impact varying taste and sensation such as a tingling peppery taste, a bitter taste and numbness[5,6]. Posited that chewing sticks, in addition to providing mechanical stimulation of the gums, also destroy microbes; these advantages of the chewing sticks over the conventional toothpaste and brush has been attributed to the strong teeth of Africans[7].

*Hymenocardia acida* belong to the family Euphorbiaceae is a small savannah tree or shrub about 9 m high. Branchlets become rusty brown as the bark peels. The bole is short, often flattened and usually crooked. The branches form a fairly heavy, somewhat rounded crown. The bole is short, often flattened and usually crooked. Leaves thin, leathery, elliptic-oblong up to 8.75 cm long and 3.75 cm broad, apex obtuse to rounded, base obtuse; petiole slender, up to 1.8 cm long.

**OBJECTIVES OF THE STUDY**

Chewing sticks with antimicrobial activity could become a potential source of new drugs for oral diseases, hence
this study is aimed at testing the phytochemistry, antimicrobial activities, Minimum Inhibitory Concentration (MIC) and Minimum Bactericidal Concentration (MBC) levels of the aqueous and alcoholic extracts of stem of Hymenocardia acida used as chewing sticks collected from Edo state, Nigeria.

MATERIALS AND METHODS

Collection and identification of plant material
The stems of Hymenocardia acida plant were collected from Edo North Senatorial District of Edo State. The plants were 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 Hymenocardia acida were cut from the plants, 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 and root materials were soaked in 600 mls 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 stems and root materials were boiled in 600 mls of water for 24 hours after which the resultant solutions were 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.

Phytochemical screening
The phytochemical tests were carried out on the aqueous and ethanolic extracts using standard procedures as described by8,9.

Source of microorganisms
Pure stock cultures of Staphylococcus aureus, Staphylococcus aueruginas, Streptococcus pyogenes, Streptococcus mutans, Candida albicans, Aspergillus flavus, Microsporum 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 organisms10. 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.


56
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\(^{[11]}\). 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 ± 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\(^{[10]}\).

**Minimum bactericidal concentration (MBC)**

The Minimum Bactericidal Concentration (MBC) of the plant extracts were determined by the method described by\(^{[12,13]}\). 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 ± 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\(^{[14]}\) was used for the determination of microbial sensitivity. The antibiotic discs employed were: septrin, chloranphenicol, sparflxacin, 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.

**RESULTS AND DISCUSSION**

In Table I the results of the phytochemical analysis of aqueous and ethanolic stem extracts of *H. acida* revealed the presence of some secondary metabolites such as alkaloids, flavonoids, cardiac glycosides, anthraquinones, phlobotannins, tannins, saponins and carbohydrates. Phlobotannins and terpenoids were absent in ethanol extracts of *H. acida*. Table II
shows the antimicrobial properties of the ethanol extract of the *H. acida* on the test microorganisms. The entire test organisms were sensitive to the ethanol extracts at a concentration of 100 mg/ml. 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. pyogenes* (gram +ve) and *M. gypseum* (fungus) respectively. Table III revealed that the antimicrobial activity of the aqueous extract of the *H. acida* plant extracts were significantly different from one to another on each organism. The ethanol extract has the highest antibacterial and antifungal activity against all the tested oral microorganisms with inhibition diameters of 17.20 ± 0.06 mm and 5.83 ± 0.03 mm respectively at 100 mg/ml. Table IV presents the Minimum Inhibitory Concentration (MIC) and the Minimum Bactericidal Concentration (MBC) values of the ethanol extracts. The ethanol extract of *H. acida* showed minimum inhibitory concentration (MIC) of 12.5 mg/ml against *B. subtilis* and *S. auricularis* while for the fungi 6.25 mg/ml concentration was sensitive to *M. gypseum* and it is fungicidal (MFC) at 12.5 mg/ml to *M. gypseum* (fungus) respectively. The presence of bioactive compounds (qualitative phytochemicals) has been known to show medicinal activity as well as exhibit and regulate some physiological activity[15,16]. Saponins have been reported to be an antifungal agent, while tannins prevent the development of microorganisms by precipitating microbial protein and making nutritional proteins unavailable to them[17] and tannins have been traditionally used on inflamed surfaces of mouth and treatment of catarrh[18,19]. Also reported that tannins have antioxidant properties.

Table 3: Zone of inhibition in mm of various concentrations of the ethanol extract of *Hymenocardia acida* on test organisms

<table>
<thead>
<tr>
<th>Test organisms</th>
<th>Concentration of extract (mg/ml)</th>
<th>Sterile distilled water</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>3.125</td>
<td>6.5</td>
</tr>
<tr>
<td><em>S. aureus</em></td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td><em>S. auricularis</em></td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td><em>S. pyogenes</em></td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td><em>S. mutans</em></td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td><em>M. gypseum</em></td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td><em>B. subtilis</em></td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td><em>A. flavus</em></td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td><em>C. albicans</em></td>
<td>–</td>
<td>–</td>
</tr>
</tbody>
</table>

NB: Means ± S.E.M; n=3, Means ± S.E.M within a row are significantly different, P < 0.01.

= No inhibition.

Table 4: Minimum Inhibitory Concentrations (MICs) and Minimum Bactericidal Concentrations (MBCs) in mg/ml of the ethanol extracts of *Hymenocardia acida* plant against the test bacteria

<table>
<thead>
<tr>
<th>H. acida (stem) (mg/ml)</th>
<th>Test Bacteria</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td><em>S. aureus</em></td>
</tr>
<tr>
<td>MIC</td>
<td>12.50</td>
</tr>
<tr>
<td>MBC</td>
<td>25.00</td>
</tr>
</tbody>
</table>

Concentrations (MBCs) in mg/ml of the ethanol extracts of *Hymenocardia acida* plant against the test bacteria

Means ± S.E.M; n=3, Means ± S.E.M within a row are significantly different, P < 0.01.

= No inhibition.

Table 5: Minimum Inhibitory Concentrations (MICs) and Minimum fungicidal Concentrations (MFCs) in mg/ml of the ethanol extracts of *Hymenocardia acida* plant against the test fungi

<table>
<thead>
<tr>
<th>H. acida (stem) (mg/ml)</th>
<th><em>M. gypseum</em></th>
<th><em>A. flavus</em></th>
<th><em>C. albicans</em></th>
</tr>
</thead>
<tbody>
<tr>
<td>MIC</td>
<td>6.25</td>
<td>100.00</td>
<td>50.00</td>
</tr>
<tr>
<td>MFC</td>
<td>12.50</td>
<td>100.00</td>
<td>50.00</td>
</tr>
</tbody>
</table>

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 VI 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 VII 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 mm and *A. flavus* 17 mm.

The presence of bioactive compounds (qualitative phytochemicals) has been known to show medicinal activity as well as exhibit and regulate some physiological activity[15,16]. Saponins have been reported to be an antifungal agent, while tannins prevent the development of microorganisms by precipitating microbial protein and making nutritional proteins unavailable to them[17] and tannins have been traditionally used on inflamed surfaces of mouth and treatment of catarrh[18,19]. Also reported that tannins have antioxidant properties.

It was observed that susceptibility increased with increased concentration of the extracts and that ethanol extracts exhibited more activity, potency and consistency than the...
aqueous extract. These results support earlier studies which observed that plant extracts in organic solvent provided more consistent antimicrobial activity compared with those extracted in water\textsuperscript{20,21}.

It was also observed that the extracts were active when compared with the negative control (sterile distilled water) against all the test organisms (Table III). The control recorded no visible activity. The positive control (standard sensitivity disc) used on the test bacteria revealed that the extracts were active when compared with the commercial antibiotics (ketoconazole and ciprofloxacin). Rocephin, Amoxacillin, Gentamycin, Ofloxacin, Ciprofloxacin, Erythromycin, and Septrin had inhibitory effects on all the test bacteria (Table VI). Amoxacillin has activity against S. aureus, S. pyogenes, S. mutans, and S. aureus with zone of inhibition of 27.0 mm for ciprofloxacin and 21.6 mm for perfloxacin. Rocephin, to the commercial antibiotics (Table VI). Amoxacillin has activity against S. mutans and erythromycin had inhibitory effects on all the test bacteria (Table VI). Amoxacillin has activity against S. aureus, S. pyogenes, S. mutans, and S. aureus with zone of inhibition of 27.0 mm for ciprofloxacin and 21.6 mm for perfloxacin. Rocephin, to the commercial antibiotics (Table VI). Amoxacillin has activity against S. mutans and S. aureus with zone of inhibition of 27.0 mm for ciprofloxacin and 21.6 mm for perfloxacin. Rocephin, to the commercial antibiotics (ketoconazole and ciprofloxacin).

**CONCLUSION**

Diet plays a major role in preventing dental caries, the practice of dental hygiene is also important. Even when people would prefer to use toothbrushes, they do not have access to toothpaste due to high cost or remoteness. 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 use of the tested plant as chewing sticks.

**REFERENCES**


### Table 6: Sensitivity zone of inhibition of commercial antibiotics (standard sensitivity disc) on the test bacteria

<table>
<thead>
<tr>
<th>Test isolates</th>
<th>Zone of inhibition (in mm) for commercial antibiotics</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>CN</td>
</tr>
<tr>
<td>S. aureus</td>
<td>28.3</td>
</tr>
<tr>
<td>S. auricularis</td>
<td>27.0</td>
</tr>
<tr>
<td>S. pyogenes</td>
<td>19.7</td>
</tr>
<tr>
<td>S. mutans</td>
<td>20.6</td>
</tr>
<tr>
<td>B. subtilis</td>
<td>24.8</td>
</tr>
</tbody>
</table>

**PEF = Perfloxacin (10 µg/ml), CN = Gentamicin (20 µg/ml), OFX = Ofloxacin (30 µg/ml), AM = Ampiclox (30 µg/ml), SXT = Septrin (30 µg/ml), APX = Ampiclox (10 µg/ml), E = Erythromycin (10 µg/ml), X = Ciprofloxacin (10 µg/ml), R = Rocephin (25 µg/ml), S = Streptomycin (30 µg/ml), T = Tetracycline (30 µg/ml), B = Benzylpenicillin (30 µg/ml).**

### Table 7: Sensitivity zone of inhibition of commercial fungi antibiotics (ketoconazole) on the test fungi

<table>
<thead>
<tr>
<th>Test fungi</th>
<th>Ketoconazole (200 mg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Aspergillus flavus</td>
<td>17 mm</td>
</tr>
<tr>
<td>Candida albicans</td>
<td>24 mm</td>
</tr>
<tr>
<td>Microsporum gypseum</td>
<td>26 mm</td>
</tr>
</tbody>
</table>

