Assessment of antibacterial activity of crude leaf and root extracts of Cassia alata against Neisseria gonorrhoea.

Robert B.D Otto, Sarah Ameso, Bernadina Onegi

Department of Pharmacy, School of Health Sciences, College of Health Sciences, Makerere University, P.O. Box 7072, Kampala, Uganda.

Abstract

Background: Gonorrhea is a major sexually transmitted disease worldwide and for its control, effective treatment is essential. However as more strains of Neisseria gonorrhoeae continuously develop resistance to several drugs, this strategy obliges scientists to discover newer effective drugs.

Objectives: To ascertain whether crude leaf and root extracts of Cassia alata (Caesalpiniaceae) have antimicrobial activity against clinically resistant Neisseria gonorrhoeae bacteria. To determine and compare the MICs of their ether and methanol extracts.

Materials and methods: Ether and methanol extracts were prepared from the plant parts. 12.375-5mg/ml of serially diluted ether extracts in DMSO and methanol extracts in water were tested using agar-well diffusion method against Neisseria gonorrhoea clinical isolate cultured on MTM agar. MICs were determined from corresponding concentration-response curves. Ceftriaxone was used as positive control, whereas DMSO and water as negative controls.

Results: All the crude extracts showed concentration-dependent Neisseria gonorrhoea inhibition. Ether extracts for both leaves and roots gave lower MICs compared to those of methanol. Ether root extract showed the highest potency.

Conclusions: Both the leaf and the root of Cassia alata plant have activity against clinically resistant Neisseria gonorrhoeae; the root having the higher activity. Lipophilic solvent, ether, give more potent antibiotic extracts. As expected Cassia alata plant in Central Uganda also has antibacterial activity.

Key words: Cassia alata, Extracts, MIC, Neisseria gonorrhoea, Resistance, Treatment

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Introduction

Gonorrhoea is a purulent infection of mucous membrane surfaces caused by Neisseria gonorrhoeae bacterium. According to the World Health Organization approximately 62 million new cases of gonorrhoea disease occur globally each year. The bacteria infect principally the urethra in men and endocervix in women. It may occur asymptomatic but extra-genital infections in men usually present with a urethral discharge and asymptomatic infections and extra-genital infections are more common in women. However disseminated Gonococcal Disease (DGI) is a rare occurrence. There are three laboratory tests available for diagnosis of gonorrhoea: gram staining, culturing and nucleic acid amplification tests. In Uganda, diagnosis of gonorrhoea is usually done clinically, based on patients’ symptoms and since gonorrhoea is usually asymptomatic especially in women, most cases go undetected. There is an increasing rise in resistance to drugs used for treatment of gonococcal infections with most patients left poorly treated. Poorly treated or undetected gonorrhoea can cause complications like pelvic inflammatory disease in women, which can lead to ectopic pregnancy and infertility; and epididymitis and prostatitis in men. These complications need prolonged treatment that becomes very expensive and often unattainable by resource limited countries including Uganda. Infections with gonorrhoea have also been associated with increased Human immunodeficiency virus (HIV) shedding, which can lead to increased incidence of the HIV infections. These coupled with the high incidences of adverse drug reactions (ADRs) of the current drugs used in treating gonorrhoea, calls for urgency in looking for alternative sources of potential antibacterial drugs, especially among plant species.

Medicinal plants have been used widely around the world to treat various infectious diseases and ailments, with the World Health Organization (WHO) estimating that 65-80% of the world’s population in developing countries use these plants for primary health care. Cassia alata, also known as ringworm bush, candle bush, candle stick and empress candle, is an erect annual herb that grows 3-4 meters tall and has dark green leathery compound leaves on stout branches. It was originally from South America but spread throughout the tropics. Uganda, being a tropical country, has this plant growing wildly, and in abundance.

This plant; especially its leaves is widely used in tropical regions as home remedies and sometimes cultivated for medicinal purposes. The uses include: ayurvedic medicine; treatment of constipation, stomach pain, ringworm, skin disease, inguinal hernia, intestinal parasitosis and diabetes. The antimicrobial activity reported is diverse; acting on; bacteria, fungi and amoeba. Though it has been used blindly in treating syphilis, the specific bacteria against which the leaves have been found to be active are: Vibrio cholerae, Bacillus subtilis, Staphylococcus aureus, Streptococcus sp. and Escherichia coli. Few studies ventured to check its activity against gonorrhoea, the disease but not on the bacterium Neisseria gonorrhoea, and they only examined the leaves.

This present study was undertaken to determine the in vitro antibacterial activity of crude ether and ethanol leaf as well as root extracts of Cassia alata on Neisseria gonorrhoea and to determine the minimum inhibitory concentrations (MICs) of the extracts. The few documented actions of this plant against gonorrhoea were about this plant and its efficacy or effectiveness in treatment of the clinical disease; without checking the bacteria themselves in vitro. Unlike those studies, this study used gonococcal isolates and assessed the action of this plant against the bacteria. In addition, the bacteria used were a clinical isolate from a resistant case.

Preparing the extracts

The whole-leaves and whole-roots of the plant were washed in water and air-dried under a shade for a period of two weeks after which they were ground to powders with a mechanical grinder. 190g of the root powder and 300g of the leaf powder were macerated separately at room temperature with occasional shaking using ether as the solvent for 24 hours after which the extract was decanted and filtered using a Whatman No 1 filter paper. The procedure was repeated twice at the end of which the marc was left to dry to remove more traces of the solvent. The above process was repeated this time using methanol. In each case the obtained concentrates were then transferred to pre-weighted Petri dishes and allowed to dry in a warm oven (about 37OC), until constant weight was got – this took about six hours. The dried extracts were kept in the dried oven and used within five days in screening the extracts for antibacterial activity described far below.

Test bacteria and culture media

A urethral swab was obtained from a chronic resistant case at Uganda, Mulago National Referral and Teaching hospital, sexually transmitted diseases clinic, in an

Corresponding author: Robert Otto.

Department of Pharmacy, School of Health Sciences, College of Health Sciences, Makerere University, P.O. Box 7072, Kampala, Uganda.

Mobile: +256-(0)-71-111-44-000, +256-(0)-70-11-44-000, +256-(0)-555-44-000.

E-mail addresses: rbdotto@yahoo.com, rbdotto@ehs.mak.ac.ug

African Health Sciences Vol 14 Issue 4, December 2014 840
ethical manner, with the assistance of the laboratory of Department of Microbiology, College of Health Sciences, Makerere University, Uganda. The urethral swab was used to inoculate a freshly prepared Modified Thayer-Martín agar, and the inoculum was spread using a sterile loop, across the entire plate surface in a Z-pattern.

The inoculated plate was incubated immediately at 35-37°C in 3.5% carbon dioxide (CO2) for 48-56 hrs. The resultant growth (colonies) was used in two ways: Some was spent (without further re-isolation or passage) in making the cell suspension used during screening the extracts for antibacterial activity as described below, whilst some was used in Gram-stained smears to reveal the characteristic gram negative diplococcic, as a way of confirming that the growth is actually N. gonorrhoeae.

After the drying, two wells, 10mm diameters each were bored in each plate with an aseptic cork borer. As soon as the wells were prepared, they were filled with 200µl of extracts and controls using a sterile pipette. All these together were left to stand in a sterile environment for 1 hour to let the extracts diffuse into the agar before incubation.

After incubating the plates at 35-37°C at 3-5% of CO2 for 32-8hrs., diameters of zones of inhibition were measured using a calibrated ruler. For each concentration, three replicates were used and the corresponding mean diameter and radius of zones of inhibition calculated.

Determination of Minimum Inhibitory Concentrations of the extracts

A plot of the square of the radius diameter of the zones of inhibition against log concentration of the dilutions was done and a suitable curve drawn from the plots for each extract. Extrapolation of the curve was done to determine the log of MIC. From this log the MIC was calculated as the antilog.

Results

Table 1 shows the radii of zones of inhibition of Ceftriaxone (positive control) and of water and DMSO (negative controls). Table 2 shows the different concentrations of the crude leaf and root ether and methanol extracts and their corresponding radii of zones of inhibition. Table 3 shows the different log concentrations of the crude leaf and root ether and methanol extracts and their corresponding square of the radii of zones of inhibition.

The negative controls i.e. the solvents; DMSO and water had no inhibition of the bacteria, whereas the positive control i.e. the Ceftriaxone had marked inhibition of the gonococci.

<table>
<thead>
<tr>
<th>Control</th>
<th>Final Concentration in the wells</th>
<th>Mean diameter of Zones of inhibition (mm)</th>
<th>Mean radius of Zones of inhibition (mm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ceftriaxone</td>
<td>100mg/ml OR µg/µl</td>
<td>38.00</td>
<td>14.00</td>
</tr>
<tr>
<td>(Positive)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Water (Negative)</td>
<td>Pure</td>
<td>00.00</td>
<td>00.00</td>
</tr>
<tr>
<td>DMSO (Negative)</td>
<td>Pure</td>
<td>00.00</td>
<td>00.00</td>
</tr>
</tbody>
</table>

\[
\text{Mean radius (mm)} = \frac{\{\text{Mean diameter} \times \text{inclusive of well diameter (mm)}\} - \{\text{Well diameter (10mm)}\}}{2}
\]

Table 2: Antibacterial activity of crude plant extracts

Showing radius of zones of inhibition with concentration of crude plant extracts

<table>
<thead>
<tr>
<th>Final Concentration in the well (mg/ml OR µg/µl)</th>
<th>Log concentration</th>
<th>Mean radius of zones of inhibition (mm)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Root extracts</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Ether</td>
</tr>
<tr>
<td>11.719</td>
<td>1.069</td>
<td>02.09</td>
</tr>
<tr>
<td>23.438</td>
<td>1.370</td>
<td>03.67</td>
</tr>
<tr>
<td>46.875</td>
<td>1.671</td>
<td>05.00</td>
</tr>
<tr>
<td>93.75</td>
<td>1.971</td>
<td>06.09</td>
</tr>
<tr>
<td>187.5</td>
<td>2.268</td>
<td>07.25</td>
</tr>
<tr>
<td>375</td>
<td>2.574</td>
<td>08.34</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Final Concentration in the well (mg/ml OR µg/µl)</th>
<th>Log concentration</th>
<th>Mean radius of zones of inhibition (mm)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Leaf extracts</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Ether</td>
</tr>
</tbody>
</table>

African Health Sciences Vol 14 Issue 4, December 2014
Where \( X \) \( \text{mm} = \frac{\text{Mean diameter} - \text{inclusive of well diameter (mm)} - \text{Well diameter (10mm)}}{2} \).

NB: 1mg/ml \( \equiv \) 1 µg/µl.

Table 3: Antibacterial activity of crude plant extracts

<table>
<thead>
<tr>
<th>Final Concentration in the well (mg/ml) OR µg/µl</th>
<th>Log concentration</th>
<th>Mean square radius of zones of inhibition (mm²)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Root extracts</td>
<td>Leaf extracts</td>
</tr>
<tr>
<td></td>
<td>Ether</td>
<td>Methanol</td>
</tr>
<tr>
<td></td>
<td>Ether</td>
<td>Methanol</td>
</tr>
<tr>
<td>11.719</td>
<td>1.069</td>
<td>04.347</td>
</tr>
<tr>
<td>23.438</td>
<td>1.370</td>
<td>13.432</td>
</tr>
<tr>
<td>46.875</td>
<td>1.671</td>
<td>25.000</td>
</tr>
<tr>
<td>93.75</td>
<td>1.972</td>
<td>37.027</td>
</tr>
<tr>
<td>187.5</td>
<td>2.268</td>
<td>52.563</td>
</tr>
<tr>
<td>375</td>
<td>2.574</td>
<td>69.472</td>
</tr>
</tbody>
</table>

Where \( X \text{mm} = \frac{\{\text{Mean diameter} - \text{inclusive of well diameter (mm)}\} - \{\text{Well diameter (10mm)}\}}{2} \).

Figure 1: Showing Excel plots of logs concentration against \( X^2 \) (mm²) for the crude plant extracts

Table 4: Antibacterial activity of crude plant extracts showing MICs of the crude plant extracts

<table>
<thead>
<tr>
<th>Plant Part</th>
<th>Ether Intercept of the Extracts</th>
<th>MIC (mg/ml OR µg/µl) of the Extracts</th>
</tr>
</thead>
<tbody>
<tr>
<td>Roots</td>
<td>1.043</td>
<td>11.0</td>
</tr>
<tr>
<td></td>
<td>1.975</td>
<td>94.4</td>
</tr>
<tr>
<td>Leaves</td>
<td>1.925</td>
<td>84.1</td>
</tr>
<tr>
<td></td>
<td>1.969</td>
<td>93.1</td>
</tr>
</tbody>
</table>

From the Figure 1 and Tables 2, 3 & 4 above, it could be observed that there was concentration-dependent inhibition of the gonococci. The methanol extracts of both the leaves and roots had similar inhibition potentials and were lower than those of the ether extracts. The roots-ether extract had the highest potency of the inhibition at the concentrations used and a linear relationship of the \( X^2 \) with the log concentration. The leaves-ether extract however showed an exponential relationship.

Discussion

Both the ethereal and methanolic leaf and root extracts of Cassia alata exhibited variable antibacterial activity against the clinical isolate of Neisseria gonorrhoeae. However, the ether extracts generally exhibited better antibacterial activity as compared to the methanol extracts; Compare the positions of the curves in Figure 1 and MICs in Table 4. Differences in activities of extracts from different solvents are not new, and for Cassia alata extracts it had also been observed in other solvents.8

Conversely one may argue that compared to the leaves, the roots maybe had more amounts of active principles, or more potent active principles, or less antagonistic impurities. Distribution of active principles within a plant vary from part to part of the plant and this was also witnessed in Cassia alata.25-26

First, the active principles responsible for the antibacterial activity could be identical in structures in both extracts, only that they could be more soluble in ether than in methanol i.e. perhaps they are more lipophilic.

Secondly, the active principles could be different in structures and those extracted by ether are perhaps more potent compared to those extracted by methanol. Thirdly, there could be impurities that are more soluble in methanol than in ether which may be responsible for the lower activity by diluting the active principles in the methanolic extract. Lastly, maybe there are different impurities with different antagonism against the active principles and those impurities dissolved by the ether extracts could be less antagonistic compared to those taken up by methanol.

Cassia alata, being a tropical plant and also Mukono, Uganda, from where the plant was collected being geographically tropical, one could not expect absence of antimicrobial activity, albeit qualitatively, since previous studies had found them.25-26-28. This was predictable as explained by, arguably, a comparative study by Zouari et al., 2012,30.
Getting antibacterial activity in the plant in this study, without concern in the phenological stage of the plant could have well been by chance, because plant components and antibacterial activities can differ depending on the phenological stage of the plant, as illustrated by the study of Nejald Ebrahiemi et al., 2008. It could also be that this plant Cassia alata, makes substantial amounts of antibacterial components at whatever phenological stage of its life.

Ceftriaxone (positive control), the standard therapeutic agent, exhibited greater antibacterial activity against Neisseria gonorrhoeae (38.00mm) as compared to both the root and leaf extracts of Cassia alata. This is to be expected since the extracts have various impurities as compared to the drug that is a purified synthetically processed molecule[22]. Also the concentration used was high.

Conclusion
Both leaf and root extracts of Cassia alata have antibacterial activity against Neisseria gonorrhoeae, but the root extracts are more potent than the leaf extracts, irrespective of the solvent used for the extraction. However using non-polar solvents, like ether for extraction, most likely give rise to more potent extracts against the bacteria. Since both the roots and the leaves of the plant have antitumor activity, other parts of the plant may also have the antigonococcal activity.

The study also confirms the validity of the use of the plant in traditional medicine for the treatment of gonococcal infections. Any Cassia alata plant, growing anywhere within the tropics most likely have some antibacterial activity, provided the plant parts are harvested at the right phenological stage or stages.

Recommendations
This is the first study of its kind in Uganda ascertaining the in-vitro antibacterial activity of Cassia alata crude extracts against Neisseria gonorrhoeae.

More different solvents, conditions and types of extractions should be performed on different parts of the plants and their corresponding extracts studied again on the gonococci, so as to find the best solvent for extraction of the plant, and the most active part of the plant for optimal utilization of the plant crudely or for isolation of the active ingredients.

Other resistant gonorrhoea strains and isolates and bacterial species should also be tested for antagonism by this plant.

If the possible the active components should be isolated and elucidated to ascertain whether it is a new antimicrobial agent or not, followed by clinical and toxicological studies. This will pave the way for continuation with advanced drug development thereby widening the scope of drug-based treatment of infectious diseases.

In line with conservation efforts, the lipophilic active ingredients from both roots and leaves, obtained by using a wider range of lipophilic solvent systems, should be structure elucidated and assessed for antibacterial activity. Depending on the results, the raw material for further drug processing and development should be obtained from the leaves and not roots as the latter leads to destruction of the plants at harvest.

Studies should be done to find out the best phenological stage(s) of the Cassia alata plant for harvesting that give rise to the most potent and best antimicrobial activities. Knowing the best phenological stage can result in the plant being used sparingly, since small amounts would be enough for treatment.

Acknowledgements
We would like to acknowledge the parents of Ameso who gave financial support for the project, Makerere University, College of Health Sciences, Department of microbiology for giving us its laboratory for use and Mr.Baluku of the same for his assistance during the laboratory work in this study.

Abbreviations: DMSO, Dimethyl sulphoxide; MTM, Modified Thayer-Martin agar; MIC, minimal inhibitory concentration and STD, sexually transmitted disease.

Contributors
RBDO and BO were supervisors and designed the study. SA collected the samples of both the gonococcal and plant parts. All authors were involved in the preparation of the manuscript.

Ethical considerations
The Department of Microbiology, School of Biomedical Sciences, College of Health Sciences, Makerere University, from whom the Gonococcal isolate was obtained, assured the study that, as usual, all ethical issues were sorted out at the time of getting the isolates from the patients in Mulago University Teaching Hospital.

Declaration of conflict of interest
All authors confirm that they have no interest to declare.

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