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

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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. 12-375mg/ml of serially diluted ether extracts in DMSO and methanol extracts in water were tested using agar-well diffusion method against Neisseria gonorrhea 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 gonorrhea 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 antigonorrhoeal extracts. As expected Cassia alata plant in Central Uganda also has antibacterial activity.

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

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Introduction

Gonorrhea is a purulent infection of mucous membrane surfaces caused by Neisseria gonorrhoeae bacteria. According to the World Health Organization approximately 62 million new cases of gonorrhea disease occur globally each year[1]. The bacteria infect principally the urethra in men and endocervix in women. It may also infect extra-genital mucosal sites including the oropharynx, anorectum and ocular membranes. Genital infections in men usually present with a urethral discharge but asymptomatic infections and extra-genital infections are more common in women. However disseminated Gonoccocal Disease (DGI) is a rare occurance[2].

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There are three laboratory tests available for diagnosis of gonorrhea: gram staining, culturing and nucleic acid amplification tests[3,4]. In Uganda, diagnosis of gonorrhea is usually done clinically, based on patients' symptoms and since gonorrhea 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[5-10]. Poorly treated or undetected gonorrhea can cause complications like pelvic inflammatory disease in women; which can lead to ectopic pregnancy and infertility; and epididymitis and prostatitis in men[4,9,11]. These complications need prolonged treatment that becomes very expensive and often unattainable by resource limited countries including Uganda. Infections with gonorrhea have also been associated with increased Human immunodeficiency virus (HIV) shedding, which can lead to increased incidence of the HIV infections[12]. These coupled with the high incidences of adverse drug reactions (ADRs) of the current drugs used in treating gonorrhea, 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 living in developing countries use these plants for primary health care[13].

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 pantropics[14]. 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 includes: 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[15-26]. Though it has been used blindly in treating syphilis[23], 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 [25]. Few studies ventured to check its activity against gonorrhea, 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 gonorrhea and to determine the minimum inhibitory concentrations (MICs) of the extracts. The few documented actions of this plant against gonorrhea 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.

Whereas the previous studies focused on the leaves, this study included the roots and compared their activity with that of the leaves. Furthermore the previous studies did not emphasize the importance of the lipophilicity or hydrophilicity of the solvents used on the potency of extracts got; this study did and found out which solvent gave a more potent antibacterial extracts of the plant parts.

Besides from this study one can demonstrate whether Cassia alata from some part of Uganda and her environmental conditions are similar to other Cassia alata plants in the world, in that it also has antibacterial activity; albeit only one bacterial species was studied. Additionally from this study one can conclude about the qualitative difference or the similarity in the antibacterial constituents of Cassia alata due to its geographical source.

Materials and methods

Plant Collection and Identification Extraction Fresh whole-leaves and whole-roots of Cassia alata were collected from Mukono district, central Uganda, during the month of January 2011. For purposes of ensuring correct botanical identification, the plant herbarium specimen was prepared and submitted to the University herbarium, Department of Botany, School of Bio-Sciences, College of Natural Sciences, Makerere University, Uganda, for authentication.

Preparation of 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's 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-weighed 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 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-Martin 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. All the MTM agars used were prepared using Faur YC et al., 1973, NYC medium preparation method with slight modifications[27]. Instead of the horse blood, sheep blood was used. The sheep were from the School of veterinary and animal resources, college of veterinary medicine, animal resources and bio-security (COVAB), Makerere university, Uganda. The components used in the preparation included: Thayer Martin Medium Base (Oxoid Ltd), and the following antimicrobials: Vancomycin (Biolab Zrt), Colistin (BD & Co), Nystatin (Biolab Zrt) and Trimethoprim (BD & Co).

Screening extracts for antibacterial activity

The antibacterial activity screening used was the agar-well diffusion method used by Okunji et al., 1990 and Okeke et al., 200128-29, but changed to using a bigger well diameter, because the suitable well boring instrument was that big.

The dried leaf and root extracts were reconstituted in two different solvents; double-distilled water (made locally from our laboratory in Pharmacy Department) and DMSO (Sigma) (pure). Different quantities of the ether and methanol extracts were dissolved in different quantities of DMSO and water respectively to give final concentrations of 375, 187.5, 93.75, 46.875, 23.438 and 11.719mg/ml of the leaf and root extracts – see Table 2 below. Ceftriaxone (BioLab.) 100µg/ml, was included as a positive control while DMSO (100%) and water served as negative controls – see Table 1 below.

Fifteen fresh plates of MTM agar were brought to room temperature and to be used as follows: six plates for varying concentrations of ether and methanol leaf extracts, another six for ether and methanol root extracts and the remaining three for the negative and positive controls. Using a sterile loop isolated colonies of Neisseria gonorrhoeae were added to 4mls of sterile normal saline and the saline stirred using a sterile stainless steel rod. The addition of the colonies and stirring was repeated until the saline solution became turbid. A sterile swab was dipped into the stirred turbid cell suspension then used to inoculate the entire surface of the MTM plates by seeding first horizontally then vertically and left to dry/settle while covered at room temperature for about 30 min.

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% ofCO2 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 thus radius of zone 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 1: Antibacterial activity of controls

Showing radius of zones of inhibition of the controls run together with the extracts

Control	Final Concentration in the wells	Mean diameter of Zones of inhibition (mm)	Mean radius of Zones of inhibition (mm)
Ceftriaxone (Positive)	100**mg/ml OR µg/µl**	38.00	14.00
Water (Negative)	Pure	00.00	00.00
DMSO (Negative)	Pure	00.00	00.00

Mean radius (mm) = [{Mean diameter – inclusive of well diameter (mm)} – {Well diameter (10mm)}] / 2

Table 2: Antibacterial activity of crude plant extracts

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

Final Concentration in the well (mg/ml OR µg/µl)	Log concentration	Mean radius of zones of inhibition (mm)			
		Root extracts		Leaf extracts	
		Ether	Methanol	Ether	Methanol
11.719	1.069	02.09	00.00	00.00	00.00
23.438	1.370	03.67	00.00	00.00	00.00
46.875	1.671	05.00	00.00	00.84	00.00
93.75	1.972	06.09	00.92	02.17	00.84
187.5	2.268	07.25	02.00	03.67	02.25
375	2.574	08.34	03.67	07.17	03.25

NB: 1mg/ml ≡ 1 µg/µl.
Mean radius (mm), X = [{Mean diameter – inclusive of well diameter (mm)} – {Well diameter (10mm)}] / 2.
NB: 1mg/ml ≡ 1µg/µl.

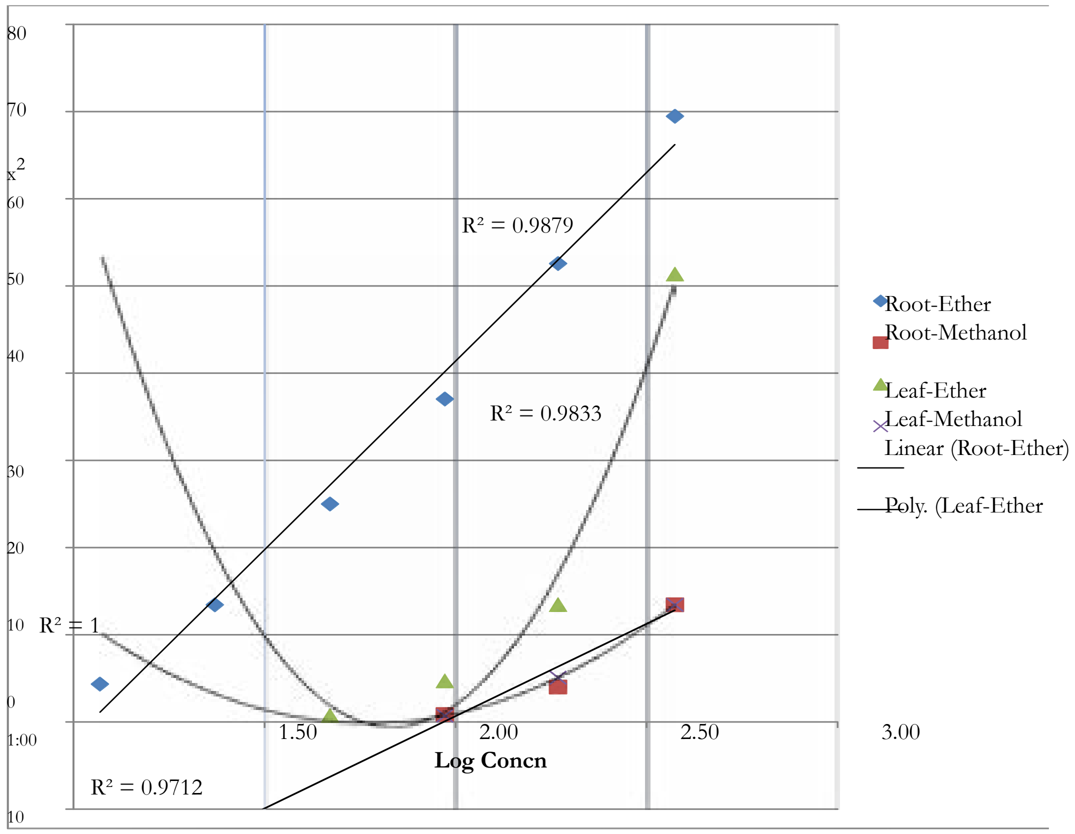
Table 3: Antibacterial activity of crude plant extracts

Showing logs concentration against X^2 (mm^2) for crude plant extracts

Final Concentration in the well (mg/ml OR µg/µl)	Log concentration	Mean square radius of zones of inhibition (mm^2)			
		Root extracts		Leaf extracts	
		Ether	Methanol	Ether	methanol
11.719	1.069	04.347	00.000	00.000	00.000
23.438	1.370	13.432	00.000	00.000	00.000
46.875	1.671	25.000	00.000	00.697	00.000
93.75	1.972	37.027	00.837	04.687	00.706
187.5	2.268	52.563	04.000	13.432	05.063
375	2.574	69.472	13.432	51.337	13.432

Where X mm= [{Mean diameter – inclusive of well diameter (mm)} – {Well diameter (10mm)}]/ 2. NB:

Figure 1: Showing Excel plots of logs concentration against X2 (mm2) for the crude plant extracts



1mg/ml ≡ 1 µg/µl.
The X-intercepts were got from equations of the trend lines from excel plotsof X2against Log concentrations of crude extracts (leaving out the coordinates with 0 values of X2) and used to calculate the MICs in the table 4 below.

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

	X – Intercept of the Extracts		MIC (mg/ml OR µg/µl) of the Extracts	
Plant Part	**Ether**	**Methanol**	**Ether**	**Methanol**
Roots	1.043	1.975	11.0	94.4
Leafs	1.925	1.969	84.1	93.1

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 potencies 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[19-22]. From previous studies, and since ether is more organic than methanol solvent, the ethereal extracts were as expected to be more active than the methanol extracts[19,21,22]. In this study the superiority of the activity of the solvents observed was as expected; ethereal extracts were more active than methanolic extracts. The greater activity of the ethereal extracts versus the methanolic extracts may suggest any one of the following:
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.

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[19,20,22]. This may explain why the ethereal root extract showed higher antibacterial activities at all the concentrations compared to the leaf extract of the same solvent, ether. Suggesting that the active principles maybe the same but are probably more concentrated in the roots than in the leaves. It can also be that the chief active principle(s) in the two parts of the plants are actually different in structures and potencies.

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]25-26. 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 Nejad Ebrahimi et al., 2008,31. 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 gonorrrhoeae (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[20,21]. 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 antigonococcal 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 gonorrhea strains and isolates and bacteria 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 thus 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.

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

Contributors

RBDO and BO were supervisors and designed the studies. RBDO and SA carried out laboratory investigations of 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 the authors confirm that they have no interest to declare.

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