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Supra-additivity, antagonism and antibacterial activity of *Cassia abbreviata, Combretum hereroense Schinz* and *Acacia polyacantha:* An alternative therapeutic approach against *Neisseria Gonorrhoea* infections

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Abstract
In Zambia, the yearly incidence of sexually transmitted diseases (STDs) is 34 instances per 10,000 people, with men being afflicted nearly twice as often as females. *Neisseria gonorrhoea* has been reported to have developed chromosomally or plasmid-mediated antimicrobial resistance (AMR) towards most first of line treatment options. In this study aqueous root extracts of *Cassia abbreviate, Combretum hereroense Schinz*, and *Acacia polyacantha* were tested individually and in combination for antibacterial activity against *Neisseria gonorrhoea* strain. Ciprofloxacin, Doxycycline and Floxacin were used as standard drugs.
Neisseria gonorrhea was cultured from a swab sample on chocolate agar first to allow the bacteria to grow in an environment that is optimal and specific for its growth. Additional biochemical testing, such as oxidase testing and gram staining were done to confirm the identity of *Neisseria gonorrhea*. Disc diffusion and Well methods were used to conduct susceptibility tests.
The results showed that bacterial growth in combined extracts against *Neisseria gonorrhoea* strain was less than the bacterial growth when individual extracts were used. A combination of *C. abbreviate* + *C. hereroense Schinz* + *A. polyacantha* was most effective (++++), while *C. abbreviate* + *A. polyacantha* was effective (+++) whereas *C. hereroense Schinz* + *A. polyacantha* was least effective(++) and *C. abbreviate* + *C. hereroense Schinz* was not effective (+). The zones of inhibition of 20.75 mm (susceptible), 19 mm and 20 mm were observed using disc diffusion method whereas 21 mm, 15 mm and 19 mm were recorded via the Well method for *C. abbreviate*, *C. hereroense Schinz*, and *A. polyacantha* respectively. These finding support the continued use of medicinal plants to manage *Neisseria gonorrhea* with a combination of the three plant extracts proving more effective.

Keywords: *Neisseria gonorrhea*, synergy, antagonism, *Cassia abbreviate, Combretum hereroense Schinz, Acacia polyacantha*

Introduction
According to the World Health Organization (WHO), an estimated 82.4 million cases of gonorrhea were recorded worldwide in 2020 among adults aged 15-49 years [1]. In Zambia, the yearly incidence of sexually transmitted diseases (STDs) is 34 instances per 10,000 people, with men being afflicted nearly twice as often as females. STD-related problems are the third most prevalent reason individuals seek medical treatment for, putting a further burden on the already scarce healthcare resources especially in developing nations. STD's are a major health concern in Zambia, accounting for roughly 10% of all adult outpatient visits to hospitals and primary care facilities [2].
Neisseria gonorrhoeae, a bacterium that causes gonorrhoea, is often transmitted through unprotected sexual intercourse with an infected person through vaginal, oral or anal sex. More recently this pathogen has been reported to have developed chromosomally or plasmid-mediated antimicrobial resistance (AMR) towards most first of line treatment options has attracted as recommended by World Health Organization (WHO) [3]. A major challenge facing global healthcare systems is infectious diseases, especially the increase in antibiotic resistance [4]. Zambia's Ministry of Health guidelines recommends a single 500mg dose of ciprofloxacin and 100mg of Doxycycline twice a day for seven days to treat gonorrhea [5]. This line of treatment has recently been met with challenges.

For instance, a recent study conducted in 3 hospitals in Lusaka, Zambia, has revealed the emergence of superbugs of *Neisseria gonorrhoeae* showing high resistance to doxycycline, penicillin, and ciprofloxacin, thus presenting a therapeutic dilemma [6]. The overuse of antibiotics once effective in treating *Neisseria gonorrhoeae* is a primary reason for the rise in resistance to these drugs. The rise of antibiotic-resistant *Neisseria gonorrhoeae* highlights the need to revise treatment guidelines and underscores the urgency for scientists to develop new, safe, effective, and affordable antibiotics or explore alternative treatment options. One potent source readily available to answer this call involves screening of medicinal plants that are being used locally.

Medicinal plants have long been used as potential therapeutic agents in Zambian communities to manage illness including STI's [7]. One medicinal plant, native to the Zambia terrain is *Cassia abbreviate. Cassia abbreviata* is a shrub that grows to a height of 10 meters. It is characterized by a light brown bark, rounded crown, and yellowish foliage. The shrub has compound leaves of 5 to 12 pairs, and its cylindrical pods are brown-black in hue [8]. In Zambia, the plant is prominently called Umunsokansoka. A decoction of the bark is used to treat stomach aches, malaria, while a mixture of leaves, roots and stem bark is used to manage dysentery, menstrual cycles problems, venereal diseases, gastrointestinal complications and as an arbotifacient [9-12]. In Kenya, various parts of *C. abbreviate* have been used as a treatment options for epilepsy, syphilis, gonorrhea, hernia, jaundice, infertility and many more complications [13,14]. Extracts of dry roots have also previously shown to be active against clinical isolates of *N. gonorrhoeae* [15].

Acacia polyacantha is a tall deciduous tree with a straight, cylindrical trunk that can reach heights of 10 to 15 metres. It is endemic to Tropical Africa, covering Gambia to Ethiopia and expanding southerly to Kenya, Zimbabwe and Zambia [16,17]. Root and bark extracts from this plant are used to treat venereal infections, diarrhea, and gastrointestinal problems. In addition, an infusion of the stem bark is used particularly for treating jaundice, and a combination of powdered root and honey is used to reduce cough and asthma symptoms [18,19].

Combretum hereroense is typically a deciduous shrub with arching stems, which is more frequently described as a tiny tree with a compact crown that can grow to be 9 to 12 meters tall. The plant is widely spread in Southern and Eastern African counties. Previously, the plant has been cited to be effective in treatment of various ailments such as chest pains, stomach complications, bad coughs and tonsillitis [20]. This study therefore aimed at analyzing the antibacterial potential of the aqueous root extracts of *Cassia abbreviate, Combretum hereroense Schinz, Acacia polyacantha* used individually and in combination (synergy) against *N. gonorrhoeae* clinical isolate.

Materials and Methods

Collection of plant materials: The plant materials were collected from Lufwanyama district, Copperbelt province of Zambia. The leaves of each plant collected were identified by Zambia forestry department in Kitwe, Zambia.

Preparation of plant extracts: The roots of *Cassia abbreviate, Combretum hereroense Schinz, Acacia polyacantha* were initially washed under running tap water and shade dried for a period of 2 weeks. The dried roots were chopped into small pieces and brought into powder form using a clean mortar and pestle. Thereafter, the aqueous extract of each plant sample was obtained via Soxhlet extraction method using 500 mL of distilled water and 35 g of powdered root sample in a thimble. The extracts obtained after multiple cycles of extraction were then filtered using whatman's Grade 1 qualitative filter paper to exclude solid particles. The obtained filtrate was then put on a water bath at 45℃ to obtain a final greasy material (crude extract). The greasy extracts were then treated further in according with Prakash, 2006 by dissolving 100 mg of crude extract into 100 mL of distilled water to maintain a concentration of 1mg/mL [21]. For the antibacterial activity tests using individual crude extracts the stock solution was used while for combined activity tests, different prepared concentrations of extracts were mixed ensuring stock solution concentration was maintained [21].

Collection, culture inoculation of isolates: A sterile swab was used to collect a clinical specimen from the suspected site of infection (genital) on a patient at Kabwe general hospital, Zambia. The swabs were placed in a transport medium to maintain the viability of the bacteria during transportation to Mulungushi University laboratory (the swabs were placed in ice packs in a cooler box to arrest microbial growth). In order to obtain a pure culture of *Neisseria gonorrhoea*, the bacteria was first grown on Chocolate agar. This allowed the bacteria to grow in an environment that is optimal and specific for its growth. After the initial growth on Chocolate agar, the bacteria were then sub cultured onto Nutrient agar to isolate a pure colony of *Neisseria gonorrhoea*.

Preparation of Chocolate Agar Plates: 45.5 grams was suspended in 495ml distilled water. Thereafter the mixture was heated to boiling and occasionally stirred to dissolve the media completely. After the media fully dissolved, the mixture was sterilized by autoclaving at 121℃ for 15 minutes. The mixture was then cooled in a water bath. Haemoglobin solution was then aseptically added to the media, in order to further enhance the growing environment for the bacteria. The media and blood was mixed well and poured into sterile petri dishes.

Streaking the Plate: A swab was used to streak from the clinical specimen onto the media plates. The lid of the agar plate was opened just sufficiently enough to streak the plate with the swab.

Zig-zag Streak (hockey stick): A bacterial sample was placed at the border of the plate and inoculated using the swab to produce the agar plate. After that, the entire plate was scanned using a swab in a back-and-forth zig-zag pattern, beginning at the location where the bacteria was injected. The swab was kept flat against the agar surface.

Inoculation of Chocolate Agar: The bottom of each agar plate was labelled with necessary details to help identify them. A sterile loop was used for dilution method and swab from the clinical specimen was used to streak onto the Chocolate agar plate.

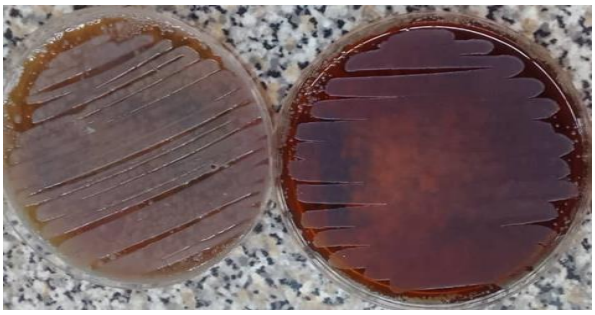

Fig 1: Shows growth of *Neisseria gonorrhoea* on chocolate agar plate

Incubation: The inoculated Chocolate agar plates were placed into a 35-37 °C incubator with 5-10% CO_2 (enclosed space with a candle). These conditions were maintained throughout the incubation period. The media plates were incubated for 18-24 hours and checked periodically for colony growth. After the incubation period, the plates were examined for colony growth (Figure 1).

Nutrient Agar: 13g of nutrient agar powder was suspended in 300 mL of distilled water. Thereafter the mixture was heated and occasionally stirred to fully dissolve all the components. After the components fully dissolved, the mixture was autoclaved at 121℃ for 15 minutes. The mixture was then cooled in a water bath. The liquid agar was then dispensed into sterile petri dishes and left to solidify.

Sterilization of the transfer loop: The wire loop was heated in the light blue zone, just above the tip of a Bunsen burner flame, until it blazed red hot, sterilising metal transfer loops. The sterilised loop was held in place and allowed to cool.

Streak the Plate: Bacteria from the soup were streaked onto media plates using a sterile loop. The cover of the agar plate was momentarily opened to streak the plate, reducing environmental exposure to avoid contamination.

Three Sector Streak (t streak): A wire loop was sterilised, allowed to cool on the edge of a sterile agar plate, and then dipped into the bacterial broth culture. To implant the loop, the plate's lid was momentarily raised. The top third of the agar surface was then streaked with the loop in a zigzag pattern. The loop was sterilised once more, the plate was rotated 90℃, and the loop was dragged two or three times around the region that had already been streaked before completing the zigzag pattern over the other half of the plate. Following another sterilisation and a 90℃ rotation of the plate, this procedure was repeated.

Inoculation of Nutrient Agar: The bottom of each agar plate was labelled with necessary information for easier identification. A sterile loop was used to streak the swab from the Chocolate agar plate onto the Nutrient agar plate.

Incubation: The nutrient agar plates that had been inoculated were kept in an incubator set between 35 and 37°C for the duration of the incubation period. The plates were incubated for 18 to 24 hours, and the growth of the colonies was routinely monitored (fig 2) were examined for colony growth.

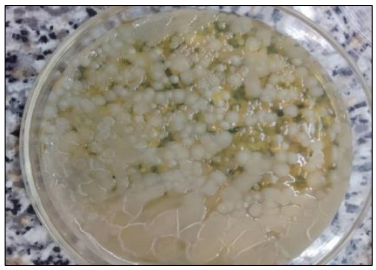

Fig 2: Shows a sub cultured growth of *Neisseria gonorrhoea* on Nutrient agar

Gram Staining: A Gram stain was performed on suspect colonies to confirm the presence of Gram-negative diplococci characteristic of Neisseria gonorrhoea.

Procedure: A slide of cell sample (Figure 3A) to be stained was made and heat fixed by carefully passing the slide with a drop or small piece of sample on it through a Bunsen burner three times. The primary stain crystal violet was added to the slide for 1 minute by flooding the slide with stain and then rinsed with water. Iodine solution was applied to the slide for 1 minute by flooding and rinsed with water. The gram stain decolourizer was carefully used to decolourize the slide for 3 seconds and rinsed off with a gentle stream of water. The slide was then flooded with a couterstain, Safranin for 30 seconds and rinsed with water. The slide was then allowed to air dry. The slide was the viewed under the microscope (fig 3B) starting with the high dry objective lens marked 40x then the magnification was switched to the high power oil immersion objective lens marked 100x in order to observe the results.

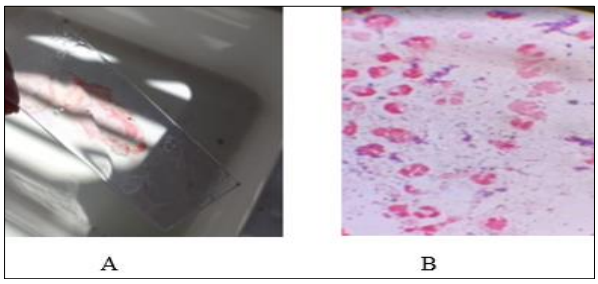

Fig 3: Shows the gram stain on the microscopic slide (A) and when viewed using a microscope (B)

Biochemical Testing: This was done to confirm the identity of *Neisseria gonorrhoea* through additional biochemical testing, such as oxidase testing. Kovacs Reagent Oxidase Test was used.

Kovacs Reagent: The oxidase test uses Kovac's reagent to detect the presence of cytochrome c in a bacterial organism's respiratory chain.

Swab method for Kovac's oxidase test: Suspect colonies were selected from the chocolate and nutrient agar culture plate with the swab (fig 4A). A Pasteur pipette was then used to add one drop of kovacs reagent to the swap. A positive purple reaction after 5 minutes indicates *N. gonorrhea*

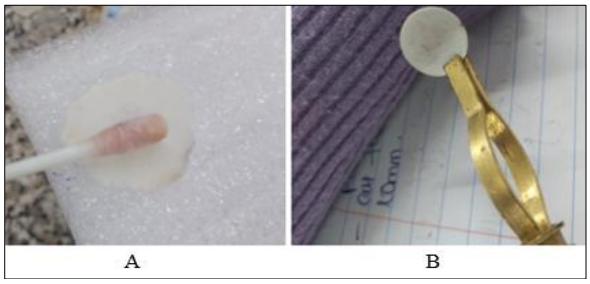

Fig 4: Shows the positive reaction on the swab (A) and filter paper disc (B)

Moistened filter paper method for Kovac's oxidase test: A petri dish was filled with a piece of filter paper. The filter paper (fig 4B) was then treated to one to two drops of oxidase reagent, which was then left to absorb for some minutes. After the reagent was absorbed, care was taken to make sure the filter paper was damp but not wet. A part of the colonies to be tested were picked up with a plastic loop and rubbed onto the moistened filter paper. Five minutes later, a positive purple reaction suggested N. gonorrhoea [22].

Synergy Method: For combined activity tests, different prepared concentrations of extracts were mixed ensuring stock solution concentration (1000 ppm) was maintained. Each of the extracts were added together and a spread plate method was done. This is a method in which the appropriate amount of merged extracts was poured into the petri dish first then the liquid Muellar Hinton agar so they can solidify together. After solidification, sterile swabs that were dipped in

the broth were used to streak on each plate and incubated at 35-37^{0}C for 18-24 hours.

Antimicrobial Activity Determination

Preparation of Filter Paper: A filter paper disc was used to prepare the antibiotic discs. Small disc shapes were then cut out from the filter papers. The filter papers were then autoclaved. Thereafter the discs were impregnated with different concentrations of the plants extracts for a few minutes to allow them to absorb the medicinal extract. After soaking them in the extract stock solution they were allowed to dry before exposing them to the bacterial culture. Once the discs were dry they were stored in a sealed container at room temperature in a cool dark place to maintain their potency until they were needed for testing.

Placement of Antibiotic Disks: Sterile forceps were used to place antibiotic disks containing different medicinal herb extracts on the inoculated agar surface. The inoculated plates were then incubated in a 35-37°C incubator with 5-10% CO_2 for 18-24 hours.

Disc and Well-diffusion method: On Muellar Hinton agar plates, a deep enough well (fig 5) was cut using a sterile surgical blade in the centre of the agar plate. Afterwards each extract was placed in the well for 60 minutes. For disc diffusion method, filter papers (discs) were dipped into plant extracts for an hour in order to allow the absorption of the extracts. Thereafter excess extracts was dabbed off using sterile cotton wool in readiness for use.

A broth containing the bacteria was made and a sterile swab was dipped in the broth and streaked around the trench. The plates were then incubated at 35-37^{0}C for 18-24 hours. For the well-diffusion method, the zone of inhibition was measured using the radius of the well while for disc diffusion method, zones were read at point complete inhibition [23].

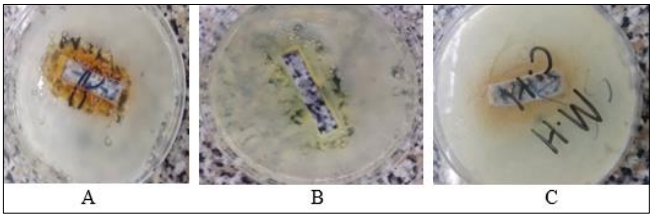

Fig 5: Shows zone of inhibition for *Cassia abbreviate* (A), *Acacia polycantha* (B), *Combretum hereroense* (C) respectively.

Ethical Consideration: Prior to conducting this study, ethical clearance was sought and obtained (Reference no.: SMHS-MU1-2024-02) from the research ethics committee (REC) at Mulungushi University, Zambia.

Results: The zone diameters were compared with the interpretive criteria provided by the Clinical and Laboratory Standards Institute (CLSI) or other relevant guidelines. Zones are classified as susceptible, intermediate or resistant based on established breakpoints for each antibiotic. The zone of inhibition for the aqueous root extract of *C. abbreviate* showed the maximum activity, followed closely by *A. polycantha* and *C. hereroense* against *Neisseria gonorrhoeae* respectively (table 1). Among the 3 standard antibiotics used, Ofloxacin EX5 showed the highest activity followed closely by Doxycycline DO30 and Ciprofloxacin CIP5. Similarly, in table 2, zones of inhibition obtained using Well method showed that *C. abbreviate* showed the maximum activity, followed closely by *A. polycantha* and *C. hereroense* against *Neisseria gonorrhoeae* respectively

Table 1: Effects of standard antibiotics and individual plant extracts against *N. gonorrhoeae* using disc diffuse method.

SI. No.	Antibiotics tested	Zone of inhibition (mm)	Sensitivity Interpretation	Plant extract tested	Zone of inhibition (mm)	Sensitivity Interpretation
1	Ciprofloxacin CIP5	25	Susceptible	*Cassia abbreviata*	20.75	Susceptible
2	Doxycycline DO30	25	Susceptible	*Acacia polycantha*	20	Intermediate
3	Ofloxacin EX5	26	Susceptible	*Combretum hereroense*	19	Intermediate

Table 2: Zones of inhibition in the Well-diffusion method

Plant extract tested	Radius from well (mm)	Diameter from well (mm)	Sensitivity Interpretation
Cassia abbreviata	10.5	21	Susceptible
Acacia polycantha	9.5	19	Intermediate
Combretum hereroense	7,5	15	Intermediate

Synergetic and antagonistic

Table 3 summarises the effectiveness of the combined (synergy) aqueous extracts of the 3 plants. A combination of *C. abbreviate* + *C. hereroense Schinz* + *A. polyacantha* was most effective (++++), while *C. abbreviate* + *A. polyacantha* was effective (+++) whereas *C. hereroense Schinz* + *A. polyacantha* was least effective(++) and *C. abbreviate* + *C. hereroense Schinz* was not effective (+).

Table 3: Showing bacterial growth on synergetic and antagonistic plates

Treatment	Effectiveness	Interpretation
Acacia polycantha + *Cassia abbreviata* + *Combretum hereroense*	++++	Most effective
Acacia polycantha + *Cassia abbreviata*	+++	Effective
Acacia polycantha + *Combretum hereroense*	++	Least effective
Cassia abbreviata + *Combretum hereroense*	+	Not effective

Discussion

From the study, it can be noticed that all the root aqueous extracts of the three plants showed antibacterial activity against *Neisseria gonorrhoeae*. *Cassia abbreviate* showed the maximum activity with a zone of inhibition of 20.75 mm while Well method showed zones of 21 mm. This result is in according with previous literature by [15,24,25]. The individual root of extracts *Combretum hereroense* showed maximum activity of around 19 mm for disc diffusion method and 15 mm for Well diffusion method. This showed the ability of *C.*

hereroense root extracts to inhibit grown of *Neisseria gonorrhoeae.* In the present study, aqueous root extracts of *Acacia polycantha* (1000 ppm) showed maximum inhibitory potential against *Neisseria gonorrhoeae* at 20 mm and 19 mm for disc and Well diffusion methods respectively. These potential to inhibit bacterial growth is consistent with previous studies by [26]. Compared to extracts, the standard antibiotics (Ciprofloxacin, Doxycycline and Ofloxacin) performed better with zone of inhibitions range from 25-26 mm. The present study also assessed the synergetic potential of the three extracts. The study showed that a combination of *C. abbreviate + C. hereroense Schinz + A. polyacantha* was most effective (++++), while *C. abbreviate + A. polyacantha* was effective (+++) whereas *C. hereroense Schinz + A. polyacantha* was least effective (++) and *C. abbreviate + C. hereroense Schinz* was not effective (+). Finding showed synergetic potential when all three extracts. Antagonistic effect was observed when *C. abbreviate + C. hereroense Schinz* was combined, as it was observed that more bacterial grow when extracts were combined whereas when used individually, they proved more effective.

Conclusion

The present study showed that *C. abbreviate* was the most active followed closely by *A. polycantha* and *C. hereroense* against *Neisseria gonorrhoeae*. The results showed that bacterial growth in combined extracts against *Neisseria gonorrhoea* clinical isolate was less than the bacterial growth when individual extracts were used.

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