INTERNATIONAL JOURNAL OF
Clinical and Pharmaceutical Innovations
An International Peer Reviewed Open Access Journal

Research Article

ISSN (Online): 3142-8665

2026, Volume 1, Issue 3, Page No: 1-8.

SYNERGISM AND ANTIMICROBIAL ACTIVITIES OF *KIGELIA AFRICANA, CERATONIA SILIQUA* AND *KALAHARIA UNCINATA* AGAINST *NEISSERIA GONORRHOEAE*

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Article Info: **Received:** 14 April 2026, **Revised:** 04 May 2026, **Accepted:** 24 May 2026

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Citation:
Malimba Chileshe[1]*, Masule Emmanuel[2], Muzyamba Sidney[3], Mwafulilwa Laston[4], Brandy Deman[5], Kalirajan Arunachalam[6], Brian Chileshe[7], Siabbweka Maimbo[8], Siankuku Munsaka[9], Mukupa George[10]. (2026). Synergism And Antimicrobial Activities Of *Kigelia Africana, Ceratonia Siliqua* And *Kalaharia Uncinata* Against *Neisseria Gonorrhoeae*. International Journal of Clinical and Pharmaceutical Innovations, 1(3), 1-8.
DOI: https://doi.org/10.5281/zenodo.20474364

ABSTRACT
Crude extracts were obtained via Maceration for 4 days. To test for antimicrobial activity, a pure culture was initially sourced. Chocolate agar was prepared by dissolving 9.19 g in 100 mL distilled water prior to autoclaving. The sample was then streaked onto chocolate agar and incubated at 37°C in a 5-10 % CO_2 incubator for 24 hours. Isolated colonies were transferred to Mueller-Hinton Agar and incubated again under similar conditions to obtain a fresh culture for testing. Disc diffusion and Agar well diffusion method were adopted to carry out susceptibility tests. Disc diffusion method showed inhibition zones (diameters) of 26 mm, 26 mm and 28 mm while Well method showed inhibition zones (diameters) of 26 mm, 28 mm and 40 mm for *Kigelia africana, Ceratonia siliqua* and *Kalaharia uncinata* respectively. The combination of *Kigelia africana* and *Kalahari uncinata* exhibited the greatest antibacterial activity, while the pairing of *Kigelia africana* and *Ceratonia siliqua* showed the lowest activity. Interestingly, the combination of all three extracts did not result in the highest inhibition zone, suggesting possible antagonistic interactions or reduced potency due to compound dilution. These findings highlight the importance of specific plant pairings in enhancing antimicrobial activity and support the potential for synergistic effects between certain extracts, warranting further investigation into their bioactive compounds and mechanisms of action.

KEYWORDS: Antimicrobial resistance, *Ceratonia siliqua, Kalaharia uncinata, Kigelia africana, Neisseria gonorrhoeae.* Synergism.

INTRODUCTION

Despite all the tremendous efforts that have been made in the fight against diseases, one real threat that has been reported to be on the rise is Antimicrobial Resistance (AMR) owing to the over use and abuse of over-the-counter drugs. From a distance, it appears that perhaps the old saying that too much of anything is bad has finally been deciphered by pathogens and found a way to be resistant to first line empirical treatment options. At the center of the controversy is the infamous drug resistant-*Neisseria gonorrhoeae* the bacterium that is responsible for the sexually transmitted infection (STI) known profoundly as gonorrhea. Numerous reports have been cited conforming that *Neisseria gonorrhoeae* is steadily turning into a superbug. According to the World Health Organization (WHO) report of 2020, as many as 82.4 million new infections were recorded worldwide compared to 78 million recorded in 2012 with adults in the age bracket of 15-49 years forming those statistics.[1] gonorrhea appears to be a globally shared problem.[2]

For example, studies have shown that even those countries with good surveillance systems such as the United Kingdom reported increase of 11% between 2014-2015, while 29%-149% was reported in all Australian states between 2010-2014.[3-4] Unsurprisingly the vice is common in high burden countries with Africa shouldering much of the highest infection rates globally with 50-100 new reported infections for every 1000 women and men. In Zambia, sexually transmitted infections are a serious source of concern.[5]

Having observed the steady rise in AMR cases globally, the WHO is encouraging innovativeness in finding new drug led sources to help combat this growing threat. It is therefore not surprising that medicinal plants can play a crucial role in this direction having previously inspired a host of new and past drug innovations as herbal medicine (HM), which includes plants herbal ingredients, preparations and finished products have come a long way in treating various diseases.[23] Various medicinal plants have been cited to possess antimicrobial properties among others. These properties are attributed to the secondary metabolites such as alkaloids, tannins, terpenoids, Phenolics and many more that plants synthesize for their purpose defensive purposes. These phytochemicals whether in crude extracts or as isolated pure compounds have also shown therapeutic potential against most bacterial infections that affect humans.

Zambia is blessed with plentiful vegetation. For this reason and also for lack of synthetic drugs in the hospitals, people in local set up still depend heavily on traditional medicines to manage numerous ailments among them STI's.[6] The leaves, roots or stem bark of herbs are taken either individually or as concoctions. It is claimed locally that these plants work to effective lengths in management of STI's, however, most are yet to be profiled or subjected to any clinical trials making their efficacy all but a claim. It is thus imperative that in the fight against AMR, plant -based solutions are encouraged. *Kigelia africana*, a semi deciduous tree has been fun favorite in African set up for a long time. Commonly known as sausage tree or cucumber tree because of the strikingly long characteristic fruits, the leaves, fruit, stem bark and roots have previously used as in management of anemia, diabetes, venereal diseases, eczema, waist pain, jaundice and many other while *Ceratonia Siliqua* has also been cited to show antimicrobial activities against certain selected pathogens.[7-10]

This study thus aimed at evaluating the individual and synergistic antimicrobial properties of the aqueous root extracts of *Kigelia africana*, *Ceratonia siliqua* and *Kalaharia uncinata* against *Neisseria gonorrhoeae*. To the best of our knowledge, no effort has ever been made to evaluate the combined antimicrobial activities of the three named plants against *Neisseria gonorrhoeae.* While *Kalaharia uncinata* is yet to receive any noticeable attention.

MATERIALS AND METHODS

In vitro experiments were conducted to evaluate the antimicrobial potential of the aqueous plant extracts for both individual and combined extracts against the clinical isolate *Neisseria gonorrhoeae.*

Plant collection and criteria of plant selection

The leaves and root parts of *Kigelia africana*, *Ceratonia siliqua* and *Kalaharia uncinata* were collected from Central Province of Zambia, Kabwe district. The leaves of each plant were used to authenticate them by a botanist at Zambia Forestry department before extraction process. The three plants were selected based on their frequent usage to manage various aliments among them STI's in Kabwe.

Preparation of solvent extracts

The root material of the three plants was cut into smaller pieces and shade dried for 21 days, Once the root samples were dry, they were brought into powder form using a clean mortar and pestle. 30g of powdered plant material of each plant was then transferred into a bottle and 100 mL of water was then added to it. The material was then allowed to stay for 4 days after which filtration was done to obtain the filtrate. This filtrate was then reduced to a volume of 35 mL using a rotatory evaporator and kept at 4°C for usage.

Antimicrobial activity determination

Preparation of *Neisseria Gonorrhoeae* Culture

A pure culture of *N. gonorrhoeae* was obtained from the University of Zambia research center. The sample was then streaked onto chocolate agar for purity and selectivity. It was incubated at 37°C in a CO_2 incubator for 24 hours (fig 1). Isolated colonies were then transferred and inoculated onto Mueller-Hinton Agar and incubated again under similar conditions to obtain a fresh culture for testing.

Fig 1: Appearance of *N. gonorrhoeae* on chocolate agar after a 24-hour incubation.

Confirmatory Tests

Oxidase test

The oxidase test result was positive which was indicated by a purple color change, This test confirmed the pathogen as *N. gonorrhoeae.*

Superoxol test

The superoxol test was positive. The purpose of the superoxol was to detect the presence of cytochrome C oxidase. A few drops of superoxol reagent were added to a colony of *Neisseria gonorrhoeae*. A positive result was indicated by a rapid color change. This test confirmed the pathogen as *N. gonorrhoeae.*

Analytical profiling index (API)

N. gonorrhoeae was confirmed using API. API is an identification system that uses biochemical tests to confirm different pathogens. The *Neisseria gonorrhoeae* was inoculated into the API NH strips. Then the system interpreted the biochemical reactions and provided for an identification.

Nitrate Reduction test

The nitrate reduction test determines the ability of *N. gonorrhoeae* to reduce nitrate into nitrite. The *N. gonorrhoeae* was inoculated into the nitrate broth and the result was negative because there was no reduction of the nitrate. This test confirmed the pathogen as *N. gonorrhoeae.*

Gram Staining

A Gram stain was performed on *Neisseria gonorrhoeae* a gram-negative diplococcus. The procedure Of Gram stain was performed on colonies to confirm the presence of gram-negative diplococci characteristics of *Neisseria gonorrhoeae.*

Preparation of paper discs

Paper discs were made from filter papers using a perforator. The discs were autoclaved for 15 minutes and then impregnated with the crude extract and allowed to dry for an hour.

Antimicrobial Susceptibility Testing (Disc and well methods)

For disc diffusion method, the dry impregnated discs were picked using sterile forceps and placed on the inoculated media. The plates were incubated at 37°C in a 5-10% CO_2 incubator for 24 hours prior to reading zones of inhibition.

For Well diffusion method a sterile surgical blade was used to cut a well at the center of the Mueller Hinton and 10 mL of plant extracts was gently introduced into the well. For the analysis of synergetic potential, equal amounts of extracts were initially combined in a beaker from which 10 mL was pipetted out and introduced into the well. The plates were then allowed to stay for 120 minutes for the media to absorb the extract. The bacteria suspension was then swabbed onto the media and the plates incubated for 24 hours prior to reading the results (fig 2). Ciprofloxacin was used as a control drug.

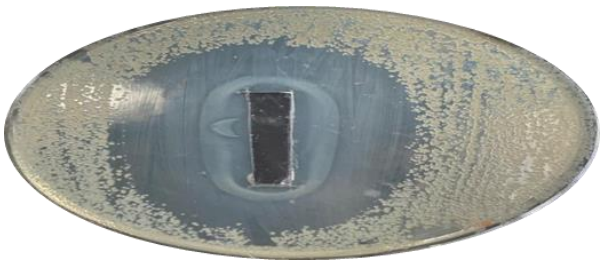

Fig 2: Zone of inhibition after a 24-hour period.

RESULTS

Disc diffusion method results from individual extracts

The zone diameters were compared to interpretive criteria provided by the clinical and laboratory standards institute and other relevant guidelines for zones and were classified as susceptible, intermediate and resistance based on established breakpoints for each antibiotic.

Table 1: **Zones of inhibition (diameter mm) of individual extracts of *Kigelia africana, Kalahari uncinata,* and *Ceratonia siliqua against Neisseria gonorrhoeae* observed via disc diffusion method.**

	Antibiotic tested	***Zone of inhibition***	***Sensitivity interpretation***	***Plant extract***	***Zone of inhibition***	***Sensitivity interpretation***
1	Ciprofloxacin	30 mm	Intermediate	*Kigelia africana*	26 mm	Intermediate
2	Ciprofloxacin	28 mm	Intermediate	*Kalahari uncinata*	26 mm	Intermediate
3	Ciprofloxacin	29.2 mm	Intermediate	*Ceratonia siliqua*	28 mm	Intermediate

Table 1 above shows results of zones of inhibition (in mm) of individual plant extracts using Ciprofloxacin against *Neisseria gonorrhoeae* using disc diffusion method**.**

Table 2: Analysis of Variance of Zones of inhibition (diameter mm) of individual extracts of *Kigelia africana, Kalahari uncinata,* and *Ceratonia siliqua* against *Neisseria gonorrhoeae* observed via disc diffusion method.

Source of variation	d.f.	s.s.	m.s.	f.r.	F pr.
Plant extract	2	0.02613	0.01307	2.733	p<0.05
Rep.	3	0.10720	0.03573		
Total	5	0.13333			

Grand mean: 27.87mm

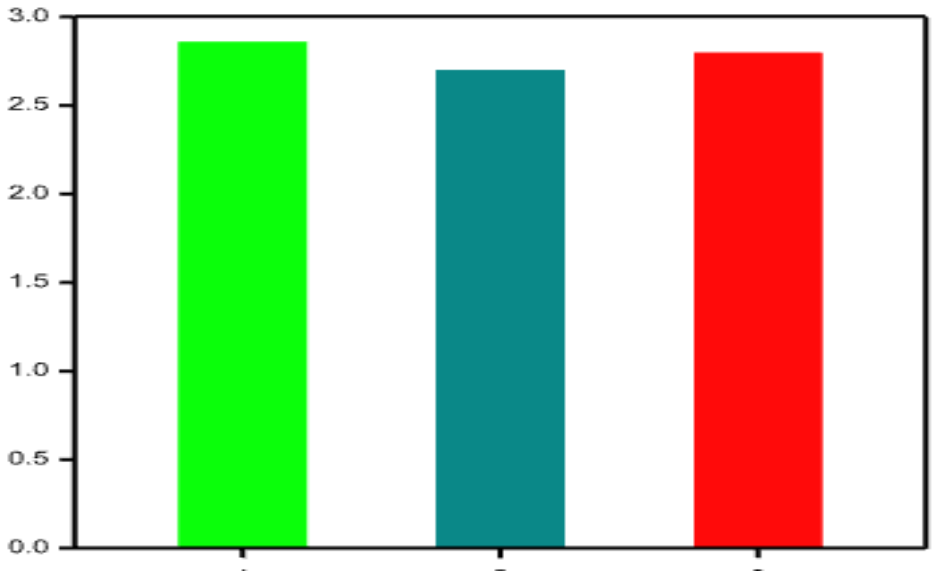

Figure 3: above shows a bar graph of individual plant extracts of *Ceratonia siliqua* (Green), *Kalahari uncinate* (Blue) and *Kigelia africana* (Red) against *Neisseria gonorrhoeae* via disc diffusion method. *Ceratonia siliqua* from the above analysis exhibited the highest antimicrobial activity against *Neisseria gonorrhoeae*. the data was analyzed using GenStat 18th edition, 2015 version.

Well disc diffusion method Results of individual extracts

Table 3: **Zones of inhibition (diameter mm) of individual extracts of *Kigela africana, Kalahari uncinata,* and *Ceratonia siliqua against Neisseria gonorrhoeae* observed via Well diffusion method.**

Plant extract	***Zones of inhibition (diameter mm)***	***Sensitivity***
Kigelia africana	26	Intermediate
Kalahari uncinata	40	Susceptible
Ceratonia silique	28	Intermediate

Table 3 above shows results of zones of inhibition (in mm) of individual plant extracts using Ciprofloxacin against *Neisseria gonorrhoeae* using well diffusion method.

Table 4: Analysis of variance of Zones of inhibition (diameter mm) of individual extracts of *Kigelia africana, Kalahari uncinata,* and *Ceratonia siliqua against Neisseria gonorrhoeae* observed via Well diffusion method.

Source of variation	d.f.	s.s.	m.s.	f.r.	F pr.
Plant extract	2	0.4368	0.2184	1.23	p<0.05
Rep	3	0.8072	0.2691		
Total	5	1.2440			

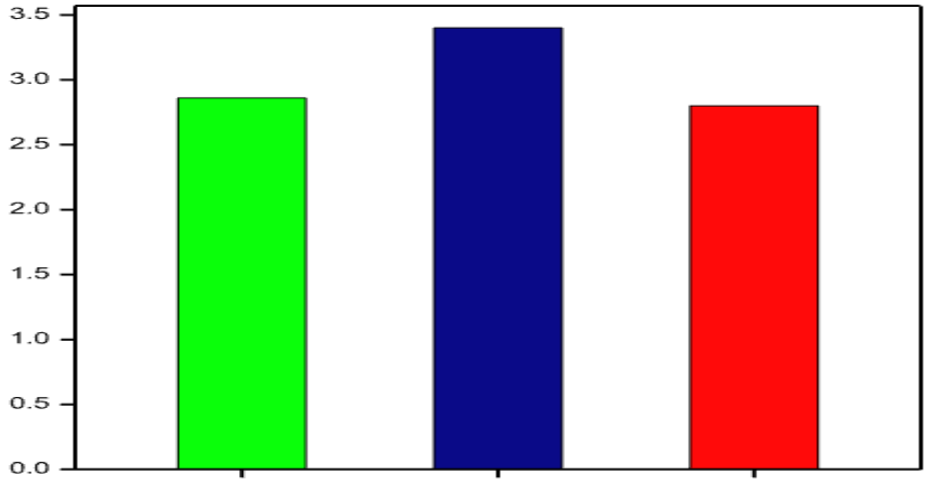

Figure 4: above shows a bar graph of individual plant extracts of *Ceratonia Siliqua* (Green), *Kalahari uncinata* (Blue) and *Kigelia africana* (Red) against *Neisseria gonorrhoeae* via well diffusion method. From the analysis *Kalahari uncinata* displayed the highest antimicrobial activities. The data was analyzed using GenStat 18th edition, 2015 version.

Table 5: **Zones of inhibition (diameter) of combined effect of *Kigelia africana, Kalahari uncinata,* and *Ceratonia silique against Neisseria gonorrhoeae.***

Plant extract	***Zones of inhibition (diameter) mm***	***Sensitivity***
Kigelia africana + Kalahari uncinata	30	Intermediate
Kigelia africana + Ceratonia siliqua	22	Intermediate
Ceratonia silique + Kalahari uncinata	28	Intermediate
Kigelia Africana + Kalahari uncinata + Ceratonia siliqua	24	Intermediate

Table 5 above shows results of zones of inhibition (in mm) of combined plant extracts using Ciprofloxacin against *Neisseria gonorrhoeae.*

Table 6: Analysis of Variance of Zones of inhibition (diameter) of combined effect of *Kigelia africana, Kalahari uncinata,* and *Ceratonia siliqua against Neisseria gonorrhoeae.*

Source of variation	**d.f.**	**s.s.**	**m.s.**	**f.r.**	**F pr.**
Plant extract	3	0.27040	0.09013	1.14	p<0.05
Rep.	4	0.31520	0.07880		
Total	7	0.58560			

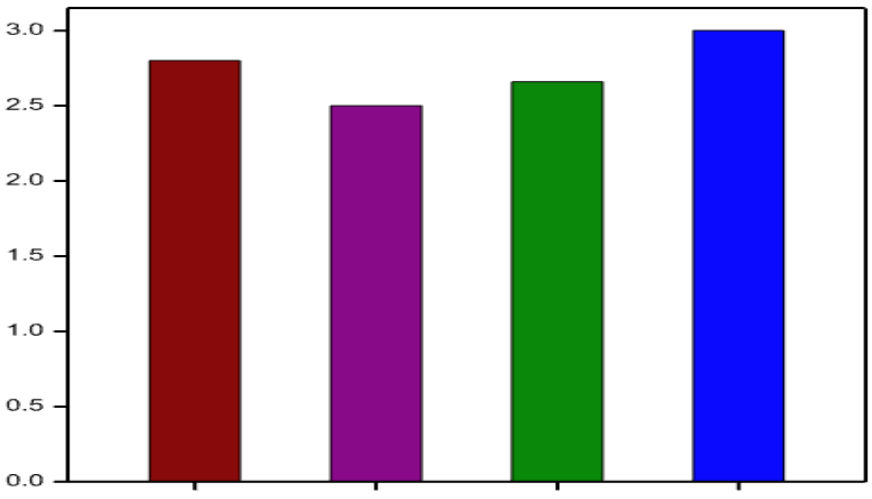

Figure 3: above shows a Bar Graph of combined plant effects of *Ceratonia silique* + *Kalahari uncinata* (maroon), *Kigela africana* + *Ceratonia siliqua* (Purple), *Kigelia africana*+ *Kalahari uncinata* + *Ceratonia siliqua* (Green), *Kigelia africana* + *Kalahari uncinata* (Blue). From the analysis above, *Kigelia africana* + *Kalahari uncinata* had

the greatest effect against *Neisseria gonorrhoeae*. Then, *Ceratonia siliqua* + *Kalahari* uncinata and then followed by *Kigela africana* + *Kalahari uncinata* + *Ceratonia siliqua* and then the least effect on gonorrhea was *Kigela* + *Ceratonia siliqua.*

DISCUSSION

The zones of inhibition recorded in this study (26–40 mm) are notably large when compared with those reported in similar investigations of plant extracts against *Neisseria gonorrhoeae*.[20] screened 50% ethanolic extracts of 16 medicinal plants against clinical isolates and WHO strains of *N. gonorrhoeae,* including multidrug-resistant (MDR) strains, and reported that 60% of the extracts exhibited high activity, though specific zone sizes varied widely across species. Similarly,[22] demonstrated significant zones of inhibition *for Securidaca longipedunculata* root and bark ethanol extracts against two standard *N. gonorrhoeae* isolates using the disc diffusion method, further supporting the anti-gonococcal potential of plant-derived compounds. The antimicrobial activities observed in the present study can be attributed to the bioactive secondary metabolites known to be present in the tested species. *Kigelia africana* has been extensively profiled and shown to contain flavonoids, tannins, alkaloids, phenolic compounds, glycosides, and terpenoids[15-16] all of which are capable of disrupting bacterial membrane integrity and inhibiting key enzymatic functions. *Ceratonia siliqua* is similarly rich in polyphenols, with aqueous extracts yielding total polyphenol content as high as 43.55 ± 0.22 mg GAE/g, and HPLC-MS analysis has identified gallic acid derivatives with documented antibacterial properties against both Gram-positive and Gram-negative organisms[17-19] Given that *N. gonorrhoeae* is a Gram-negative diplococcus with a lipopolysaccharide-rich outer membrane, these polyphenolic compounds and tannins may exert their effects by chelating metal ions essential for bacterial metabolism or by directly compromising outer membrane permeability. A noteworthy finding of this study was that the combination of all three extracts (26.6 mm) produced a smaller zone of inhibition than the *Kigelia africana* + *Kalaharia uncinata* pair (30.0 mm), suggesting possible antagonistic interactions. This observation is consistent with established principles in Phyto synergy research;[21] emphasized that synergy, additivity, indifference, and antagonism can all occur within a single combinatorial system and recommended the use of the fractional inhibitory concentration index (ΣFIC) and isobologram analyses for rigorous characterization of interaction types. The reduced activity of the triple combination may be attributable to chemical antagonism whereby compounds in *C. siliqua* interfere with bioactive metabolites in the other extracts, competitive binding at bacterial target sites, or a simple dilution effect that reduces the effective concentration of the most potent components. Additionally, the discrepancy between the disc diffusion and well diffusion results, particularly for *Kalaharia uncinata* (28 mm disc vs. 40 mm well), is methodologically significant, as the well diffusion method permits direct contact between a larger volume of extract and the agar medium, thereby allowing greater diffusion of high-molecular-weight polyphenols and glycosylated compounds that may diffuse poorly through the cellulose matrix of paper discs. Furthermore, the present findings can be directly compared with our group's previous work[18] in which aqueous root extracts of *Cassia abbreviata*, *Combretum hereroense*, and *Acacia polyacantha* were tested against *N. gonorrhoeae* using the same methodology, and both supra-additive and antagonistic interactions were similarly observed. With respect to the broader AMR context, the present study is particularly relevant given that[20] reported that plant extracts showed greater inhibition of MDR *N. gonorrhoeae* strains than susceptible ones, suggesting that plant-derived antimicrobial compounds may operate through mechanisms distinct from those targeted by conventional antibiotics and could therefore retain efficacy against resistant strains. Nevertheless, several limitations of this study must be acknowledged, including the absence of Minimum Inhibitory Concentration (MIC) and Minimum Bactericidal Concentration (MBC) determinations, the use of a single clinical isolate rather than multiple strains encompassing characterised resistance profiles, the lack of phytochemical profiling of the extracts used, and the need for FIC index calculations using checkerboard or microdilution assays to formally distinguish synergistic from merely additive interactions. These are planned as next steps in our ongoing programme of research into plant-based alternatives against drug-resistant *N. gonorrhoeae.*

Previously, numerous studies have demonstrated the synergism potential of medicinal plants. In in their study,[11] observed improved antimicrobial and synergistic effects when their combined selected plant extracts with aquatic extract while in another recent study by[12] a polyherbal formulation showed better potential against pathogens as compared when extracts were used individually. Similarly[13] observed synergism when *Senna alata*, *Ricinus communis*, and *Lannea barteri* were combined and tested against selected pathogens. Their findings validated the relevance of combining extracts or herbs in search for novel antibiotics. Weak synergistic effects were also observed in a study by[14] when they combined guava leaves and beetroot extracts. Previously, our work on synergism showed that certain herbals when combined can improve antimicrobial activity but certain combinations proved that combining extracts can also decrease their potency.[6]

In this study a one-way Analysis of Variance (ANOVA) was conducted to compare the mean zones of inhibition (in mm) of three individual plant extracts—*Ceratonia*

siliqua, *Kigelia africana*, and *Kalahari uncinata*—against *Neisseria gonorrhoeae* using the disc diffusion method. The mean inhibition zones were 28.6 mm for *Ceratonia siliqua*, 28.0 mm for *Kigelia africana*, and 27.0 mm for *Kalahari uncinata*. The ANOVA revealed a statistically significant difference among the group means ($p < 0.05$), indicating that at least one of the extracts produced a significantly different antibacterial effect. This rejects the null hypothesis of equal means and suggests that the antibacterial activity varied depending on the plant extract used. Although the differences in mean inhibition zones were relatively small (1.6 mm range), the statistical significance implies that the variation observed is unlikely to be due to random chance alone. The highest mean zone of inhibition was observed for *Ceratonia siliqua*, suggesting it may possess slightly greater efficacy against *N. gonorrhoeae* under the tested conditions.

An analysis of variance (ANOVA) was also conducted to evaluate the differences in antimicrobial activity of *Kigelia africana*, *Kalahari uncinata*, and *Ceratonia siliqua* extracts against *Neisseria gonorrhoeae*, as measured by the mean zones of inhibition (diameter in mm) using the well diffusion method. The ANOVA revealed a statistically significant difference among the three groups ($p < 0.05$), indicating that at least one extract had a significantly different effect. The mean zones of inhibition observed were 34.0 mm for *Kalahari uncinata*, 28.6 mm for *Ceratonia siliqua*, and 28.0 mm for *Kigelia africana*. These results suggest that *Kalahari uncinata* exhibited the highest antimicrobial activity, significantly outperforming the other two extracts, which showed relatively similar levels of inhibition. The statistical significance ($p < 0.05$) confirms that the observed differences are unlikely due to random variation, thereby supporting the potential of *Kalahari uncinata* as a promising source of bioactive compounds for managing *N. gonorrhoeae* infections.

An analysis of variance (ANOVA) was also performed to assess the differences in the zones of inhibition (diameter in mm) produced by various combinations of *Kigelia africana*, *Kalahari uncinata*, and *Ceratonia siliqua* against *Neisseria gonorrhoeae*. The mean inhibition zones for the combinations were as follows: *Kigelia africana* + *Kalahari uncinata* (30.00 mm), *Ceratonia siliqua* + *Kalahari uncinata* (28.00 mm), *Kigelia africana* + *Kalahari uncinata* + *Ceratonia siliqua* (26.60 mm), and *Kigelia africana* + *Ceratonia siliqua* (25.00 mm). The ANOVA revealed a statistically significant difference among the groups **(**$p < 0.05$), indicating that the efficacy of the different extract combinations against *N. gonorrhoeae* varied significantly.

CONCLUSION

The combination of *Kigelia africana* and *Kalahari uncinata* exhibited the greatest antibacterial activity, while the pairing of *Kigelia africana* and *Ceratonia siliqua* showed the lowest. Interestingly, the combination of all three extracts did not result in the highest inhibition zone, suggesting possible antagonistic interactions or reduced potency due to compound dilution. These findings highlight the importance of specific plant pairings in enhancing antimicrobial activity and support the potential for synergistic effects between certain extracts, warranting further investigation into their bioactive compounds and mechanisms of action.

ACKNOWLEDGEMENTS

Mulungushi University & The University of Zambia are acknowledged for offering laboratory services & resources that made this study possible to conduct. All authors are also acknowledged for their contributions made to this study.

Conflict of interest

The authors declare no conflict of interest.

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