IJMPR 2024, 8(7), 13-18

International Journal of Modern Pharmaceutical Research
www.ijmpronline.com

ISSN: 2319-5878
IJMPR
Research Article

SJIF Impact Factor: 6.669

ANTIBACTERIAL ACTIVITY PROFILE OF CRUDE LEAF EXTRACTS OF *ACACIA NILOTICA* USED FOR THE TREATMENT OF HUMAN DIARRHEA IN NASARAWA WEST, NASARAWA STATE, NIGERIA

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Article Received on: 08/05/2024
Article Revised on: 29/05/2024
Article Accepted on: 18/06/2024

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ABSTRACT

Plants have been used in treatment of diseases from time immemorial. This study evaluated the Antibacterial Activity Profile of crude leaf extracts of *Acacia nilotica* used for the treatment of human diarrhea in Nasarawa West, Nasarawa State, Nigeria. Leaf of the plant were collected, air-dried and powdered. The powdered material was extracted by cold maceration with water, ethyl-acetate, methanol and n-Hexane. The *in vitro* antibacterial activities of the crude extracts were evaluated against diarrhea-causing bacteria *Escherichia coli*, *Salmonella paratyphi* and *Shigella dysenteriae* by the agar diffusion, dilution, and time-kill assay methods. The percentage yield of the methanolic extract was highest at 30.37% while n-hexane was the least at 0.49%. The methanolic, n-hexane, and ethyl-acetate extracts showed antibacterial activity comparable to the standard antibiotic (ofloxacin) with inhibition zone diameter of 28.00 ± 2.00 mm against *E. coli* and *S. paratyphi*. Ethyl-acetate extract was inactive against *S. dysenteriae*. The minimum inhibitory concentrations (MICs) range from 1.56 - 12.50 mg/ml; and the minimum bactericidal concentrations (MBCs) range from 0.00 - 50.00mg/ml. At 1/2 x MIC, all the test bacteria grew well without inhibition or kill. At 1 x MIC, all the test bacteria were killed to various extent, with the highest killing effect (0.987 $\log_{10}$ reduction) on *S. dysenteriae* at 8h. At 2 x MIC, all the bacteria were killed the more, with greatest killing-effect (-0.425 $\log_{10}$ reduction) on *S. paratyphi* at 8h. The results have demonstrated the phytopharmaceutical potential of the leaf extracts of *Acacia nilotica* as a bactericidal agent.

KEYWORDS: Antibacterial, *Acacia nilotica*, Diarrhea.

INTRODUCTION

Diarrhea is the frequent and profuse discharge of intestinal content in loose and fluid form.[1] It occurs due to the increased movement of the intestine. It may be acute or chronic. In diarrhea, the fluid is not absorbed sufficiently, resulting in watery bowel discharge. The cause of acute diarrhea may be temporary problems like infection and the chronic diarrhea may be due to disorders of the intestinal mucosa. Waterborne diseases including infectious or noninfectious diarrhea is one of the leading causes of morbidity and mortality in developing countries[2] in which Nigeria and Africa are not exempted. The major causative agents of diarrhea in man include *Shigella flexneri*, *Staphylococcus aureus*, *Escherichia coli*, *Salmonella typhi* and *Candida albicans*. Diarrhea is commonly defined by medicinal practitioners as having more than three watery or loose bowel movements per day. The disease can arise from multiple causes including bacterial, viral, or parasitic infections as well as simple causes like indigestion arising from overeating. In 2019 an estimated 5.2 million children under 5 years died mostly from preventable and treatable causes. Children aged 1 to 11 months accounted for 1.5 million of these deaths while children aged 1 to 4 years accounted for 1.3 million deaths. Newborns (under 28 days) accounted for the remaining 2.4 million deaths.[3]

Diarrhea is most commonly due to viral gastroenteritis with rotavirus, it is responsible for 90% of epidemic diarrheal cases worldwide and approximately 50% of all viral gastroenteritis cases. It accounts for 19 to 21 million cases of diarrheal illness annually in the United States alone.[4] However, the disease can also affect children above five years of age and adults, who accounted for another 1.5 million deaths in the same year. Norovirus is the most common cause of diarrhea in adults. Adenovirus and astroviruses have also been implicated in diarrhea. Various bacterial species, which have been found to cause diarrhea are species belonging to the *Aeromonas*, *Cryptosporidium*, *Campylobacter*, *Salmonella*, and *Shigella* genus as well as *E. coli*.[5] Parasites like *Giardia* can also cause diarrhea. Chronic diarrhea can be due to several chronic medical conditions affecting the intestine. Common causes include ulcerative colitis, Crohn's disease, microscopic colitis,

celiac disease, irritable bowel syndrome and bile acid malabsorption. Diarrhea can also be a symptom of another disease like cholera, caused by *Vibrio* species.[6] Antibiotics are the major remedy of infections like diarrhea, however significant increase in antibiotics resistance has been observed in common human pathogens worldwide.[7] Similarly, Oral Rehydration Therapy (ORT) has been widely identified as a key factor in the decline of child mortality due to diarrhea. However, the attack rate of the disease has remained unchanged, and this treatment often fails in the high stool output state. The use of herbal drugs in the treatment of diarrhea is a common practice in many countries of Africa. These plants, which abound in the environment, enjoy wide acceptability by the population and serve as alternatives to orthodox medicines.

Diarrhoea is said to be an endemic disease in many of developing Asian countries, considered one of the major public health concerns that leads epidemic cause of high degree of morbidity and mortality in rural communities.[8] It is one of the most common diseases for all age groups with a symptom of having 3 or more loose or liquid bowel movements per day or more frequently than normal for the individual.[9] Diarrhoea is a condition of gastrointestinal infection, which can be caused by a variety of bacterial, viral, and parasitic organisms and infection spreads through contaminated food or drinking water, or from person to person because of poor hygiene. In every year, many children in developing countries are suffering from malnutrition caused by this serious epidemic disease. According to World Health Organization (WHO), Diarrhoeal disease is the second leading cause of death in children under five years old, and was responsible for the deaths of 370,000 children in 2019. The most severe threat posed by diarrhoea is dehydration. During an episode of diarrhoea, water and electrolytes including sodium, chloride, potassium and bicarbonate are lost through liquid stools, vomit, sweat, urine and breathing. A person with diarrhoea becomes dehydrated when these losses are not replaced. In addition, diarrhoea is a major cause of malnutrition, making the person more susceptible to future bouts of diarrhoea and to other diseases. Whereas, dysentery is an intestinal inflammation causing diarrhea with blood, i.e. *Shigella* spp (bacillary dysentery) or *Entamoeba histolytica* (amoebic dysentery) are most often cause for dysentery in which the loose or watery stools contain visible red blood. Amoebic dysentery is more severe state than bacillary dysentery.[5] There were about 15% of all deaths from dysentery.

Importance of the traditional indigenous medicines greatly emphasized by WHO, as these medicines are being used by many rural people in the developing countries for the first safety in health care till now. A diarrhoeal disease control programme, including indigenous medical therapy along with evaluation of health education and prevention approaches, has recently been launched by WHO.[10] There is sufficient support of national and international organizations for the studies on treatment of diarrhoeal diseases where medicinal plants are becoming hopeful source of antidiarrhoeal drugs Therefore, indigenous medicinal plants are playing significant alternative role to antibiotics.[11] This certain aspect of using medicinal plants as a remedy or home cure for diarrhoea is applied in this research.[12]

MATERIALS AND METHODS

Collection, Identification and Processing of Plant Material

The plant used were collected from Nasarawa West Senatorial District (Nasarawa West), Nasarawa State; and identified by a taxonomist as *Acacia nilotica* at the National Institute for Pharmaceutical Research and Development (NIPRD), Abuja. Leaves of the plant collected were transported to the Department of Microbiology at NIPRD, air-dried at room temperature, chopped into small pieces and ground into powder.[13]

Extraction of Plant Material

Powdered leaf of *Acacia nilotica* was extracted by cold maceration technique. Exactly 5g of plant material was macerated with water and other absolute solvents (ethyl acetate, methanol and n-Hexane). Solvents were changed daily, and the collected solvent stored in closed containers in the fridge. This process continued until the solvent appeared clear. Extracts obtained were then filtered twice through a Whatman No.1 under vacuum pressure. Extracts were concentrated by vacuum rotary evaporator, and then freeze-dried to completely eradicate solvents. The dried crude extracts were stored in glass containers and kept in the freezer at -20°C until needed for the antibacterial activity profile studies.

ANTIBACTERIAL ACTIVITY SCREENING

Bacterial Test Strains

The bacterial Test Strains used in the study, *Escherichia coli* (ATCC 25922), *Salmonella paratyphi* and *Shigella dysenteriae*, were obtained from the Department of Microbiology at NIPRD. All isolates were preserved on Micro bank beads (Prelab Diagnostics Ltd.) in Tryptone Soy Broth (TSB: Oxoid Ltd., England) with 15% glycerol and stored at -80°C until they were needed. These beads were revived on Tryptone Soy Agar (TSA: Oxoid Ltd., England) under standard conditions. After obtaining the culture, the test organisms were streaked on nutrient agar plates and incubated at 37°C for 24 hours. From the isolated colony Gram staining was performed. The organism thus obtained were tested for their purity and confirmed by their morphological, cultural and biochemical characteristics.

Preparation of Inoculum

Fresh cultures were prepared from frozen stocks every two weeks on TSA overnight at 37°C. A single colony was inoculated into a 10 ml TSB media and incubated at 37°C for 24 h. The suspension was standardized according to the Clinical and Laboratory Standards Institute (CLSI) with sterile normal saline (0.9% NaCl in

water) to turbidity equivalent to 0.5 McFarland scale approximately1-2×10^8cfu/ml[14] and diluted further to 1 x 10^6cfu/ml.

Disc Diffusion Assay

The disc diffusion assay was described by.[15] Discs impregnated with extracts was prepared and used for the assay. Briefly, four concentrations (200 mg/ml, 100 mg/ml, 50 mg/ml and 25 mg/ml of crude extracts) were prepared in 10% dimethylsulfoxide (DMSO) and water for non-aqueous and aqueous extracts respectively and filtered through 0.22 µm syringe filter. Sterile filter paper discs (Whatman, 6 mm) was impregnated with 100 µl of the reconstituted extract and dried completely under sterile conditions in a laminar flow. Each disc was gently pressed down to ensure complete contact with Mueller-Hinton agar (MHA) inoculated with standardized (approx. 10^4cfu) test culture and incubated for 24 h at 37°C. Discs with 10% DMSO and water saturated assay discs was used as negative control; while chloramphenicol 30 µg discs were used as positive control. The experiment was done in triplicate. The inhibitory activity of the compounds was determined by comparing the average diameter of inhibition zones (mm) of the different extracts with those of the controls.

Determination of Minimum Inhibitory Concentration of Active Extracts

The minimum inhibitory concentrations (MIC) of antibacterially active extracts were determined by microbroth dilution method in sterile 96-well microplates containing Mueller-Hinton broth.[15] The wells were filled with 100 µl of sterile H_2O and 100 µl of the plant extracts was added to the wells by serial two-fold dilution from the suspension of plant extract stock solution to achieve varying concentrations of the extracts (200 mg/ml, 100 mg/ml, 50 mg/ml, 25 mg/ml, 12.5 mg/ml, 6.25 mg/ml, 3.125 mg/ml and 1.562 mg/ml). Each well was inoculated with 100 µl of standardized culture containing 10^5cfu of bacteria. Controls with 0.5 ml of only culture medium (media sterility control) or others with plant extracts (extract sterility control) or cultures on the agar (organism viability control) was used in the tests. The plates were covered and incubated at 37°C for 24 h. In this study, the MIC was the lowest concentration of plant extracts that exhibited no growth of the microorganism.[15]

Determination of Minimum Bactericidal Concentration of Active Extracts

To determine minimum bactericidal concentration (MBC), 100 µl of the culture from each of two wells above MIC, MIC and two wells below the MIC was sub-cultured on MHA plates and incubated for 24 h. The lowest concentration of extracts which exhibited no bacterial growth was taken as MBC. The experiments were repeated in triplicate.

Determination of time-kill kinetics of the leaf extracts

The method of[16] was adapted to determine the rate of kill of each test bacteria by the leaf extracts. One milliliter (1.0 ml) of standardized culture (10^6 cfu/ ml) of *E. coli*, *Salmonella paratyphi* and *Shigella dysenteriae* were separately added to 9.0 ml of different MIC concentrations (1/2 MIC, 1x MIC and 2x MIC) of the extracts in a sterile universal bottle such that the final test suspension contained approximately 10^5cfu of each test organism. The admixtures were kept in water bath at 37°C. At pre-determined time intervals of 0, 30, 60, 120, 180, 240, 720 and 960 minutes, 1 ml of the admixtures were withdrawn and diluted ten-fold with 9 ml sterile normal saline containing 3% Tween 80, 5% yeast extract and 0.3% egg lecithin. Exactly 0.1 ml of each dilution was aseptically plated out in duplicates using pour-plate method for viable counts after incubation at 37°C for 24 hours. Colony counts were plotted against time intervals on a semi-log graph paper to obtain the killing curve for each selected fixed concentration of the various extracts. The percentage reduction and log reduction from initial microbial population for each time point was calculated to express the change (reduction or increase) of the microbial population relative to a starting inoculum.

The change was determined as follows:

$$\% \text{ Reduction} = \frac{\text{Initial Count-Count at x interval}}{\text{Initial count}} \times 100$$

The log reduction was calculated as follows

Log_{10} (initial count) — Log_{10} (x time interval) = Log_{10} reduction.[17][18]

Data Analysis

Statistical analysis of antimicrobial activity was done using statistical program for social sciences (statistical analysis software). Using the software, ANOVA was used to determine whether there were significant differences in the mean diameter of inhibition zones at various concentrations. Once the means were found to be different from each other, Dunnett test was then used for multiple comparisons of inhibition to determine whether inhibition arising from the various treatments were different from the inhibition induced by the positive controls. The significance level used in the analysis was 0.05 (Alpha Level ≤ 0.05).

RESULTS AND DISCUSSION

Weight recovered and percentage yield of the crude leaf extracts of *Acacia nilotica*

The amount (in grams) and the percentage (%) yield of the four crude extracts are presented in Table 1. The percentage yield of extract decreased in the order: methanolic (30.37%) > ethyl acetate (3.18%) > aqueous (1.35%) > n-hexane (0.49%) extract was and the highest at while n-hexane extract was the least at 0.49%.

Antibacterial activity of the leaf extracts and fractions

The *in vitro* antibacterial activities of the crude leaf extracts against test isolates show different degrees of activity, as shown in Table 2. The methanolic and ethyl acetate extracts showed comparable activity with the standard antibiotic (ofloxacin), and comparatively higher activity against the entire test isolates than the other extracts. For aqueous extract, highest and lowest activities were observed against *S. paratyphi* and *E. coli* respectively; the ethyl acetate extract showed comparable activity against *E. coli and S. paratyphi*, but inactive against *S. dysenteriae*; the n-hexane extract also showed comparable activity against *E. coli and S. paratyphi*, but lowest against *S. dysenteriae*.

Table 1: Weight recovered and percentage Yield of the crude leaf extracts of *Acacia nilotica.*

Extract	Weight (g)	Percentage yield (%)
Methanolic	195.80	30.37
Aqueous	13.34	1.35
n-Hexane	13.80	0.49
Ethyl acetate	32.68	3.18

Table 2: Antibacterial activity of crude leaf extracts of *Acacia nilotica.*

S/N	Bacterial isolates	Zone of Inhibition (mm)				
		Aqueous	Ethyl acetate	n-hexane	Methanolic	Ofloxacin
1	*E. coli*	9.12 ± 2.22	28.00 ± 2.00	19.33 ± 0.58	28.00 ± 2.00	28.00 ± 1.00
2	*S. paratyphi*	10.21 ± 1.34	25.00 ± 1.00	20.33 ± 0.58	28.00 ± 2.00	25.37 ± 1.53
3	*S. dysenteriae*	10.00 ± 0.25	0 ± 0.00	11.33 ± 0.58	20.00 ± 1.00	28.00 ± 2.00

E. coli = Escherichia coli; *S. paratyphi = Salmonella paratyphi*; *S. dysenteriae = Shigella dysenteriae*. The values are mean and standard deviation of three (3) replicates, 0 ±0.00 = No activity

The minimum inhibitory concentrations of crude leaf extracts of *Acacia nilotica*

The minimum inbitory concentrations (MICs) for the crude leaf extracts are presented in Table 3. The highest MIC of 12.5mg/ml was observed with crude extract against *E. coli* and *S. paratyphi*; with n-hexane fraction against *E. coli*; and with ethyl acetate fraction against *S. paratyphi*. The lowest MICs of 1.56 mg/ml (aqueous, ethyl acetate, n-hexane fractions) and 3.12 mg/ml (crude extract) were observed against *S. dysenteriae*.

The minimum bactericidal concentration of Crude methanolic leaf extract and fractions of *Acacia nilotica*

Table 4 shows minimum bacterial concentration (MBC) exhibited by the crude leaf extracts of *A. nilotica* against test bacterial isolates. The crude extract showed the highest (50.00 mg/ml) MBC against all the test isolates. Of the extracts, ethyl acetate extract showed the highest MCB of 25.00 mg/ml. The aqueous extract showed a zero MBC against *S. dysenteriae*.

Table 3: The minimum inhibitory concentrations of crude leaf extracts of *Acacia nilotica*.

S/N	Bacterial isolates	Minimum Inhibitory Concentration (mg/ml)				
		Aqueous	Ethylacetate	n-hexane	Crude	Ofloxacin
1	*E. coli*	3.12	1.56	12.50	12.50	0.78
2	*S. paratyphi*	3.12	12.50	3.12	12.50	0.39
3	*S. dysenteriae*	1.56	1.56	1.56	3.12	0.25

E. coli = Escherichia coli; *S. paratyphi = Salmonella paratyphi*; *S. dysenteriae = Shigella dysenteriae*.

Table 4: The minimum bactericidal concentrations of crude leaf extracts of *Acacia nilotica.*

S/N	Bacterial isolates	Minimum Bactericidal Concentration (mg/ml)				
		Aqueous	Ethylacetate	n-hexane	Crude	Ofloxacin
1	*E. coli*	3.13	25.00	3.13	50.00	0.78
2	*S. paratyphi*	3.13	25.00	3.13	50.00	0.39
3	*S. dysenteriae*	0.00	25.00	3.13	50.00	0.25

E. coli = Escherichia coli; *S. paratyphi = Salmonella paratyphi*; *S. dysenteriae = Shigella dysenteriae*.

In vitro* Time-kill assessment of the crude leaf extracts of *Acacia nilotica

The results of the *in vitro* time-kill assay of the crude leaf extracts of *A. nilotica* against test bacteria presented in Table 5 indicated that the extract exhibited a significant bactericidal activity. Bactericidal activity was defined as being equal to $3\log_{10}$cfu/ml or greater reduction in the viable colony count relative to the initial inoculum.

At 1/2 x MIC, all the test bacteria grew well without inhibition or kill. At 1 x MIC, all the test bacteria were killed to various extent, with the highest killing effect (0.987 $\log_{10}$ reduction) on *S. dysenteriae* at 8 h. At 2 x MIC, all the bacteria were killed the more, with greatest killing effect (-0.425 $\log_{10}$ reduction) on *S. paratyphi* at 8 h.

Table 5: *In vitro* time-kill assessment of the crude leaf extracts of *Acacia nilotica.*

S/N	Test Isolates	1/2 x MIC Log_{10} kill			1 x MIC Log_{10} kill			2x MIC Log_{10} kill		
		0 h	4 h	8 h	0 h	4 h	8 h	0 h	4 h	8 h
1.	*Escherichia coli*	2.135	3.327	5.482	2.247	1.548	0.899	2.541	0.678	-679
2.	*Salmonella paratyphi*	2.399	3.648	4.887	2.368	0.976	0.637	2.465	0.394	-425
3.	*Shigella dysenteriae*	2.229	3.177	3.982	2.246	1.399	0.987	2.410	0.298	-526

Key = -ve bacteria reduction in log (cfu/ml) at sample times in hours

DISCUSSION

Medicinal plants have been a unique source of medicines and constituted the most common human use of biodiversity.[19] The traditional use of medicinal plants, being before the advent of antibiotics and other modern drugs[20] and their use worldwide for thousands of years with more than 80% of the world's population depending on traditional medicines for various diseases, implicated the anticipations of scientists that phytochemicals with adequate antibacterial efficacy will be useful for the treatment of bacterial infections. The amount (in grams) and the percentage (%) yield of the four crude extracts (are presented in Table 1. The weight and percentage yield of crude methanolic of *A. nilotica* were 195.80g and 30.37% respectively. Of all the four extracts, ethyl acetate has the highest percentage yield (3.18%), followed by aqueous (1.35%), and lastly n-hexane (0.49%).

Extraction of the plant material with methanol gave a higher yield of the bioactive components compared to others extract. Since methanol is less polar than water, it can easily penetrate the cell membrane thereby permitting the extraction of large amount of phytochemicals. Methanol can dissolve some non-polar molecules as well as bioactive compounds from plants which belong to various chemical groups such as tannins, alkaloids, and saponins.[21] Some of these phytochemical components have been implicated to possess analgesic and anti-inflammatory effects (Gupta, 1994). Tannins are known to have astringent properties and therefore could be used to treat diarrhea.[22]

The *in vitro* antibacterial activities of the crude methanolic leaf extract of *A. nilotica* against test isolates show different degrees of activity, as shown in Table 2 The crude extract showed comparable activity with the standard antibiotic (ofloxacin), and comparatively higher activity against the entire test isolates than the extracts. In aqueous fractions, highest and lowest activities were observed against *S. paratyphi* and *E. coli* respectively; the ethyl acetate extract showed comparable activity against *E. coli and S. paratyphi*, but inactive against *S. dysenteriae*; the n-hexane fraction also showed comparable activity against *E. coli and S. paratyphi*, but lowest against *S. dysenteriae*.

The minimum inhibitory concentration (MIC) was determined for the crude leaf extracts of *A. nilotica* are presented in Table 3. The highest MIC of 12.5mg/ml was observed with crude extract against *E. coli*, and *S. paratyphi*; with n-hexane fraction against *E. coli*; and with ethyl acetate against *S. paratyphi*. The lowest MICs of 1.56 mg/ml (aqueous, ethyl acetate, n-hexane fractions) and 3.12 mg/ml (crude extract) were observed against *S. dysenteriae*.

Table 4 shows minimum bacterial concentration (MBC) exhibited by the crude leaf extracts of *A. nilotica* against test bacterial isolates. The crude extract showed the highest (50.00 mg/ml) MBC against all the test isolates. Of the extracts, ethyl acetate showed the highest MCB of 25.00 mg/ml. The aqueous extract showed a zero MBC against *S. dysenteriae*.

The results of the *in vitro* time-kill assay of the crude leaf extracts of *A. nilotica* against test bacteria presented in Table 5 indicated that the extract exhibited a significant bactericidal activity. Bactericidal activity was defined as being equal to $3\log_{10}$cfu/ml or greater reduction in the viable colony count relative to the initial inoculum. At 1/2 x MIC, all the test bacteria grew well without inhibition or kill. At 1 x MIC, all the test bacteria were killed to various extent, with the highest killing effect (0.987 $\log_{10}$ reduction) on *S. dysenteriae* at 8 h. At 2 x MIC, all the bacteria were killed the more, with greatest killing effect (-0.425 $\log_{10}$ reduction) on *S. paratyphi* at 8 h.

The antibacterial susceptibility test results of the extract compared favorably with the positive control antibiotic as the difference between the inhibition zone diameter of each the test bacteria of the extract and the control drug was not statistically significant ($P>0.05$).

CONCLUSION

This study demonstrated the potential of *Acacia nilotica* leaf as a source of therapeutic agents by the bactericidal activity of its extracts.

ACKNOWLEDGEMENT

Gratitude to God Almighty for the immeasurable and unquantifiable mercies, love, blessings and wisdom in my life, and also for this divine guidance and protection.

I sincerely acknowledge with absolute sense of gratitude my supervisors Prof. Y. B. Ngwai and Prof. D. Ishaleku, Department of Microbiology, Nasarawa State University, Keffi.

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