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ANTBACTERIAL PROPERTY OF ETHANOLIC EXTRACT FROM THE LEAVES OF *Acacia nilotica* WILD. ON *Staphylococcus aureus* AND *Pseudomonas aeruginosa*

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Abstract
The traditional medicine involves the use of different plants or the bioactive constituents of different plants to cure diseases and this was done long time ago based on the history of human being *Acacia nilotica* is a multipurpose plant belonging to the family Mimosaceae. Commonly known as Prickly acacia in English and *Bagaruwa* in Hausa language, It have been used traditionally to treat infections. The present study aimed at investigating antibacterial activity of *Acacia nilotica* Wild. ethanolic leaves extract and its column fractions against selected multi drug resistant *Staphylococcus aureus* and *Pseudomonas aeruginosa*. Extract of *Acacia nilotica* were prepared using ethanol on the basis of their increasing polarity with varying concentrations and were screened for the antibacterial activity using disc diffusion assay. The crude extract was further subjected to column and thin layer chromatography (TLC) for bioassay guided fractionation; thus a total of 74 fractions were obtained. The fractions were screened for the antibacterial activity, fraction 6 (CF6) showed highest zone of inhibition of 12 mm and 9 mm against *S. aureus* and *P. aeruginosa* respectively. Therefore this study demonstrated the value of *A. nilotica* plant as alternative for the treatment of bacterial infections. The most active fraction can be further explored to isolate and characterize the bioactive components responsible for biological activity to develop new antibacterial drug.
Keywords: Antibacterial activity, *Acacia nilotica*, column chromatography, ethanol, extract, pathogenic.

INTRODUCTION

Medicinal plants used in the treatment of diseases are as old as civilization (Fabricant and Farnsworth, 2001). *Acacia nilotica* Wild. is a member of the family Mimosaceae and is known as Babul in Pakistan, Prickly acacia in English and *Bagaruwa* in Hausa language. According to World Health Organization (2011), medicinal plants would be the best source to obtain variety of drugs. About 80% of individuals from developed countries used traditional medicines, which have compounds derived from medicinal plants. Those plants used should be investigated to better understand their properties, safety and efficiency (Arunkumar and Muthuselvam, 2009).

Infectious diseases represent an important cause of morbidity and mortality among the general population, particularly in developing countries (Mattana *et al*., 2012). The development of resistance in microorganisms to presently available antibiotics has necessitated the search for new antimicrobial agents (Coates *et al*., 2002). Antibiotics provide the main basis for the therapy of microbial infections. Since the discovery of these antibiotics and their uses as antimicrobial agents there was a belief in the medical fraternity that this would lead to the eventual eradication of infectious diseases (Harbottle *et al*., 2006). However, overuse of antibiotics has become the major factor for the emergence and dissemination of multi drug resistant strains of several groups of microorganisms (Harbottle *et al*., 2006). Plants are rich in a wide variety of secondary metabolites such as tannins, alkaloids and flavonoids, which have been found *in vitro* to have antimicrobial properties (Seigler, 2003).

A number of researchers have investigated the antimicrobial activity of *A. nilotica*. However, the activity of solvents fractions has not been studied in detail. Thus, this study is aimed at investigating *in vitro* antibacterial activity of crude ethanolic leaves extract and solvents fractions of *Acacia nilotica* against multi drug resistant strains of *Staphylococcus aureus* and *Pseudomonas aeruginosa*

MATERIALS AND METHODS

Study Area

Aliero local government is located in the South-Eastern part of Kebbi State on the Al higher way2°C'16°C' 42°C' N4' 27' 066E.

It was created 1991 with a total land mass of 412Km^2, the local government is bounded in the North-East by Gwandu local government area, in the south by Jega local government, in the East by Tambuwal local government area of Sokoto State, in the North-West by Birnin Kebbi local government area. Kebbi State share boundary with Sokoto State in the North-Eastern axis, Zamfara State on the Eastern part, Niger State in Southern part and Republic of Niger on Western part, Kebbi State has the total population of 3,238,628 according to NPC, (2006).

Plant Materials

Leaves of the *A. nilotica* plant Wild. were collected from Aliero Town in Kebi State. The plant was authenticated and voucher specimen (Voucher number 284) was deposited to Botany unit, Department of Biological Sciences, Kebbi State University of Science and Technology, Aliero. Kebbi State, Nigeria.

Test Bacteria

The multi-drug resistant bacteria (*Staphylococcus aureus* and *Pseudomonas aeruginosa*) were obtained from Usmanu Danfodio University Teaching Hospital Sokoto. The bacterial strains were identified on the basis of cultural and morphological characteristics and the sensitivity test were conducted at microbiology laboratory Kebbi State University of Science and Technology, Aliero, to test their resistance to many antibiotics.

Preparation of *Acacia nilotica* leaves Extract

The leaves of the plant were washed thoroughly under running tap water to remove the surface dirt, followed by rinsing with sterilized distilled water. The plant sample was dried under shade in open air for 48 hours. The dried samples were grounded into finely divided powder by pestle and mortar.

The ethanolic extract of the plant was obtained according to the method described by Leonard *et al.*, (2013). One hundred gram (100g) of the dried powder of *A. nilotica* leaves sample was suspended in 75% ethanol. The mixture was gently stirred, tightly covered with cotton wools and foiled, then allowed to stand for 3 days at room temperature. The extract was decanted and filtered through muslin cloth. The filtrates obtained were concentrated to dryness on a water bath at 65°C.The extract were stored at 4°C for screening of antibacterial activity.

Media Preparation

The media used in this study were Mueller Hinton agar (MHA), nutrient agar, and nutrient broth, The media were prepared according to Manufacturer's instructions using standard aseptic technique Cheesbrough, (2000).

Phytochemical Screening of the Plant Extract

Standard screening test were used to detect the presence of phytochemicals such as saponnins, tannins, glycosides, phenols, flavonoids, steroids and terpeniods using standard analytical protocol Harborne, (1998).

Antibacterial Activity

The extracts were tested for antibacterial; activity using disc diffusion on Muller Hinton agar according to (Agarry *et al.*, 2005). The presence of zone of inhibition is an evidence of antibacterial activity. The ethanolic extract of *Acacia nilotica* leaves was tested against the test bacteria in triplicates at different concentration of 90 mg/ml, 120 mg/ml and 150 mg/ml. The sterilized medium was seeded with 0.1ml of the standard inoculum of the test bacteria and spread evenly over the surface of the media with a sterile swab. Discs incorporated with the extract were placed on the surface of the medium. The plates were allowed for the pre-diffusion time of 15 minutes after which they were incubated for 24 hours at 37°C. Diameter zone of inhibition was measured using millimeter ruler and the result was expressed in millimeter (mm).

Column Chromatography

Three grams (3g) of the crude extract of *A. nilotica* was further subjected to column chromatography to separate the extract into its component fractions. A 120g Silica gel for column chromatography (60-120 mesh) was used as the stationary phase, and the solvent system hexane: ethyl acetate: methanol as mobile phase. The extract (3g) was loaded on top of the packed column. The elution of the extract was done using the solvent system; hexane: ethyl acetate: methanol (100:0:0 % v/v) to (0:80:20 % v/v) respectively 100% each. The fractions were collected into the sterile sample bottles Davies and Johnson, (2007).

Thin Layer Chromatography (TLC)

Each fraction was subjected to TLC for homogeneity or otherwise. Hexane: ethyl acetate: methanol (60:30:10 % v/v) was used as the motile phase. The developed chromatograms were sprayed with 10% sulphuric acid and heated at 100^{0}C for 3 minutes for optimum spots visibility (Jonathan *et al.*, 2007). Thereafter, the fractions obtained were combined together based on their TLC profile or similarities.

Antibacterial Activity of the Column Fractions
The combined fractions obtained from column chromatography were allowed to stand at room temperature to concentrate. The fractions were subjected to *in vitro* antibacterial activity at 20 mg/ml concentration by disc diffusion assay on the MHA medium.

Determination of Minimum Inhibitory Concentration (MIC) and Minimum Bactericidal Concentration (MBC).
All the bacterial isolates used in this study showed inhibition zones, they are therefore considered sensitive to the extract. For MIC, two-fold serial dilutions of the extracts were performed. Each inoculum was prepared in its respective medium and density was adjusted to 0.5 Mcfarland standard (10^8 CFU/mL). The standardized inoculum was introduced in each concentration of extract. The test tubes were incubated at 37 ^oC and the MIC was recorded after 24 hours. The MIC is the lowest concentration of the extract at which the microorganism tested does not demonstrate visible growth. MBC were determined by sub-culturing the test dilutions on to a fresh solid medium and incubated further for 18 hours. The highest dilution that yielded no bacterial growth on solid medium was considered as MBC Suffredini *et al*. (2004).

Statistical analysis
All experiments were carried out in triplicates and results are expressed as mean values with standard deviation (±SD) of three replicates. One-way analysis of variance (ANOVA) and Duncan's multiple range tests were carried out to determine significant differences ($p < 0.05$) between means by using SPSS statistical software package (SPSS, version 20.0).

RESULTS
Table 1 shows the results of phytochemical screening of ethanolic leaves extract of *Acacia nilotica*. The extract contains alkaloids, tannins, phenols, saponnins terpenoid, and steroids which are the secondary metabolites and are likely responsible for the antibacterial activity.

Table 1: Preliminary phytochemical screening of *Acacia nilotica* leaves extract

S/No	Phytochemical	Ethanolic extract
1.	Alkaloids	+++
2.	Saponins	+
3.	Tannins	++
4.	Phenols	+
5.	Flavonoids	ND
6.	Terpenoids	+++
7.	Steroids	+

Key
+ = slightly present, ++ = moderately present, +++ =significantly present, ND= not detected

Table 2 shows the antibacterial activity of ethanolic extract against *Staphylococcus aureus* and *Pseudomonas aeruginosa* ranging from (10.0 mm– 13.0 mm) and (7.7 mm – 12.7 mm) respectively. Standard drug used as control recorded zone of inhibition (13.3 mm) and (11.7 mm) on the test bacteria respectively.

Table 2: Antibacterial activity of *A. nilotica* leaves ethanolic extract on *S. aureus* and *P. aeruginosa*

Extract	Conc. mg/ml	Diameter zone of inhibition (mm)	
		S, aureus	*P. aeruginosa*
Ethanol	90	10.0 ± 1.00^a	7.7 ± 0.57^a
	120	12.7 ± 1.15^b	10.0 ± 1.00^b
	150	13.0 ± 1.00^b	12.7 ± 0.57^c
Control (Cipro.)	250	13.3 ± 1.15^b	11.7 ± 1.52^{bc}

The results were expressed as mean ± SD of triplicates. Values with different superscripts are significantly different when compared to control within a column. $P < 0.05$
The column chromatographic fractions obtained from *A. nilotica* leaves demonstrated antibacterial susceptibility only in fractions 2, 6, 7, 8 and 9 (Table 3).

Table 3: Antibacterial activity of Column Fractions of *Acacia nilotica* ethanolic leaf extract

S/No	Column Fraction 20 mg/ml	Diameter zone of inhibition (mm) *S. aureus*	*P. aeruginosa*
1.	Cf1 (1-5)	0.00	2.00
2.	Cf2 (6-15)	0.00	0.00
3.	Cf3 (16-18)	0.00	0.00
4.	Cf4 (19-23)	0.00	0.00
5.	Cf5 (24-29)	0.00	0.00
6.	Cf6 (30-37)	**12.0**	**9.00**
7.	Cf7 (38-47)	0.00	3.00
8.	Cf8 (48-57)	3.00	4.00
9.	Cf9 (58-72)	3.00	3.00
10.	Cf10 (73-74)	0.00	0.00

Key
0 = resistance, CF= combined fractions

Table 4 shows the minimum inhibitory concentration and minimum bactericidal concentration of the crude extract of *Acacia nilotica* Wild. leaves against *Streptococcus aureus* and *Pseudomonas aerugimosa*. The MIC of the extract was low 3.9 mg/ml indicating its effectiveness whereas on *P. aeruginosa* the MIC was 7.8 mg/ml. Minimum bactericidal concentrations were 7 mg/ml and 15 mg/ml on *S. aureus* and *P. aeruginosa* respectively.

Table 4: Minimum inhibitory concentration (MIC) and Minimum bactericidal concentration (MBC) of *A. nilotica* crude extract

Test Bacteria	Minimum Inhibitory Conc. Ethanol (mg/ml)	Minimum Bactericidal Conc. Ethanol mg/ml)
S. aureus	3.91	7.00
P. aeruginosa	7.81	15.00

DISCUSSION

The presence of bioactive compounds in the leaves extracts of *A. nilotica* is thought to be responsible for the antibacterial activity. Numerous investigations have proved that *Acacia nilotica* contains diverse classes of bioactive compounds such as tannins, alkaloids, terpenoids and flavonoids, which exhibit various pharmacological properties (Emam, 2010).

The phytochemical screening of the *A nilotica* leaves extract has shown that, the leaves contains alkaloids, tannins, phenols, terpenoids and steroids. This result is consistent with the findings by Muhammad *et al*. (2015), who reported similar compounds as in this study. But the result in other way contradicted with the result reported by Angelo, (2015), who reported that, tannins and flavonoids were not detected in ethanolic extract of *A. nilotica*; this might be attributed to different reagents and procedure employed in both studies. Several plants which contain alkaloids, tannins, glycosides have been shown to possess antimicrobial activity against number of microorganisms as it was investigated by Silver and Fermandes, (2010).

The antibacterial activity of Acacia extract was investigated by disc diffusion assay on MHA at different concentrations (90, 120 and 150 mg/ml). Positive control used in this study (Ciprofloxacin) shows significantly sized inhibition zones (13 mm) and (11 mm) against *S. aureus* and *P. aeruginosa* respectively. However, there were no significant differences between the ethanolic extract and the standard control used (Ciprofloxacin) at certain concentrations (p<0.05) three replicates. Negative control dimethyl-sulfoxide (DMSO) produced no observable inhibitory effect against any of the tested bacteria indicating that, the effect observed was as a result of the extract activity. The lowest concentration used (90 mg/ml) of Acacia inhibited the growth of both clinical isolates.

This is similar to the findings by Rosina *et al*. (2009) who reported the efficacy of *A. nilotica* extract against bacteria isolated from clinical samples. Similarly, Kavitha *et al*. (2013), reported the effectiveness of the plant against various multi drug resistant bacteria isolated from clinical samples. The ethanolic extract at150mg/ml shows bacterial growth inhibition (12.7 ± 0.57mm) and (13.0 ± 1.00mm) on *P. aeruginosa* and *S. aureus* respectively. The effectiveness reported in ethanolic extract may be due to its ability to extract a wide range of chemical compounds of the plant leaves..

Ethanolic extract was further subjected to column and thin layer chromatographic (TLC) techniques to obtain partially purified bioactive compounds. Out of the fractions obtained combined fraction (CF6) recorded the highest zone of inhibition (12 mm) and 9 mm) on *S. aureus* and *P. aeruginosa* respectively.

From our study, it was revealed that the solvent fractions demonstrated substantial bioactivity against tested bacteria at 20 mg/ml concentration. Of all the fractions, CF6 was reported to be most effective in showing inhibitory activity against both Gram-positive and Gram-negative bacteria, and the highest activity was shown against *S. aureus* giving the zone of inhibition of 12 mm. The least activity was shown on *P. aeruginosa* (9 mm). This is in accordance with the previous findings of Angela *et al*. (2016). In their work, fractions from *A. nilotica* leaves demonstrated antibacterial activity only in fractions 4, 5, and 6 on the tested bacteria.

The Gram-positive bacteria were more susceptible to the fractions, as compared to Gram-negative bacteria. Earlier findings also reported better antibacterial activity of *A. nilotica* extracts against gram positive cocci than gram negative bacilli, Sharma *et al*. (2014). This might be because of the difference in cell wall composition of Gram-negative and Gram-positive bacteria.

.Conclusion

The trend of using natural antimicrobials is becoming an attractive approach in the field of pharmaceuticals, because synthetic antimicrobials might be associated with various side effects. The results from this study suggest that the leaves of *Acacia nilotica* showed antibacterial activity against different bacterial species. They could be used as alternatives to common antimicrobial agents for treatment of bacterial infections. Further research is needed toward further isolation and characterization of bioactive compound present in the column fractions which could possibly be exploited for pharmaceutical use.

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