In vitro Antibacterial activity of the Bark Extracts of *Anogeissus leiocarpus* on Some Bacterial Pathogens

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Abstract: The use of plant parts and extracts in traditional medicine has proved to be a good lead for the discovery of a potential plant derived antimicrobial agent. To this end we conducted *in vitro* screening of aqueous and alcoholic extracts of the bark of *Anogeissus leiocarpus* for antibacterial activity against clinical isolates of *Salmonella* Typhi*, Staphylococcus aureus, Pseudomonas aeruginosa, Shigella dysentriae, Klebsiella pnuemoniae* and *Streptococcus pyogenes* following standard microbiological procedure. The bark extracts showing inhibitory activity on the test bacteria were partially purified using solvent-solvent purification and the different fractions were tested for antibacterial activity. Results of phytochemical screening of the bark extracts of *A. leiocarpus* revealed the presence of saponins, alkaloids, phenols, tannins and glycosides. Antibacterial studies of the extracts revealed that the plant showed strong antibacterial activity against all the test organisms at 25 mg/ml concentration. The solvent- solvent purification of extracts revealed that ethyl acetate and butanol fractions contained the highest concentration of the bioactive component as revealed by the diameter of the zone of inhibition (11- 25 mm) but no inhibitory activity was observed with the chloroform fraction and residue. The minimum inhibitory concentration of the purified fractions ranges between 0.625 mg/ml (acetone extracts) for *Pseudomonas aeruginosa, Klebsiella pnuemoniae* and *Streptococcus pyogenes* to 2.5 mg/ml (aqueous extracts) for all test organisms. The minimum bactericidal concentration is in the range 0.625 mg/ml to 5 mg/ml. The findings showed that the extracts had appreciable in vitro activity against the test organisms and this plant is a potential source of leads for development of antibacterial agents.
Keywords: Antibacterial, Fractions, Susceptibility, *A. leiocarpus*,

INTRODUCTION

The recent years have seen a renewed interest in traditional medicine. This revival of interest in plant-Derived drugs is mainly due to the current widespread belief that "green medicine" is safe and more dependable than the costly synthetic drugs many of which have adverse side effects (Parekh and Chanda, 2006). The traditional use and therapeutic efficacy of many indigenous plants for managing microbial ailments is no longer in doubt. This is because they hold a very rich reservoir of phytoconstituents with potential chemotherapeutic value. Reports abound on the various bioactive phytoconstituents of plants which include tannins, alkaloids, terpenes and terpenoids, phenols, etc. These phytochemicals have been reported to have antibacterial, antifungal, antiparasitic, anticancer, anti-inflammatory activities (Sale *et al.,* 2007; Adamu *et al.,* 2017).

Many efforts have been made to discover new antimicrobial compounds from various kinds of sources such as microorganisms, animals and plants. The systematic screening of plants extracts used in folk medicine has proved to be a remarkable lead to the discovery of novel and effective bioactive compounds (Janovska *et al*., 2003). Despite the existence of potent antimicrobial and antifungal agents, resistant or multi-resistant strains are continuously appearing, imposing the need for a permanent search and development of new drugs (Silver, 1993).

Anogeissus leiocarpus (*Marke* in Hausa and *Tava* in Høba) is widely distributed and used in Nigeria for different purposes. The plant is a tropical tree that belongs to the family Combretaceae. It grows to height of between 28-39 m but typically it is 15-18 m in height with light green foliage (Dayok *et al.,* 2018). It has a dense crown and often drooping branches. The decoction and maceration of the stem bark of this plant has been used by traditional healers to treat various ailments such as anorexia, constipation, malaria, jaundice, fatigue, itching, eczema, psoriasis, carbuncles,

wounds, sores, boils, cysts and various forms of hepatitis and ulcers. Its extracts have also been used to treat helminthiasis, schistosomiasis, leprosy, diarrhea, amoebic dysentery. The stem is used as chewing sticks (Arbab, 2014; Mann *et al.*, 2014; Dayok *et al.,* 2018).

Among the people of Hong Local Government Area of Adamawa State, *A. leiocarpus* is used traditionally as a chewing stick, for wound and burn dressing as well as for relieving gastrointestinal disturbance and dysentery. As part of our investigation on the antibacterial properties of plants, the bioactivity screening of solvent-solvent purified fractions of *A. leiocarpus* Guill and Perr was undertaken on clinical isolates of *Staphylococcus aureus, Salmonella enterica* serovar Typhi*, Pseudomonas aeruginosa, Streptococcus pyogenes, Shigella dysentriae* and *Klebsiella pnuemoniae*.

MATERIALS AND METHODS

Collection of Plant Samples

The bark of *Anogeisus leocarpus* Guill and Perr, was obtained from the wild at Gashala, Hong Local Government Area of Adamawa State in the months of April /May. The plant was authenticated in the Department of Plant Science of Modibbo Adama University of Technology, Yola.

Preparation of Plant materials for Extraction

The stem bark samples of the plants were rinsed with clean water, cut into small pieces and then air dried on clean mats for 7 days at room temperature under shade. The dried samples were then pulverized using a mechanical grinder. The powdered sample was kept in an air tight container until required for further analysis.

Extraction Procedure

Fifty grams (50 g) of the prepared plant material was weighed into a 500 ml flask and 200 ml of water was added, then swirled and allowed to stand at room temperature for 72 hours with intermittent swirling at intervals of 12 h. After 72 h, the content was filtered using a whatman No. 1 filter paper and concentrated using a rotary evaporator and labelled aqueous extracts. A similar procedure was followed with ethanol (BDH Chemical poole UK) and acetone (BDH Chemical poole UK) and the concentrated extracts labeled as ethanol and acetone extracts respectively.

Phytochemical Screening

The extracts were screened for the presence of some phytochemicals namely; tannins, alkaloids, saponins, terpenes, phenols, glycosides as described by Trease and Evans, (1983) and Sofowora, (1993).

Test Organisms

Test organisms (*Staphylococcus aureus, Salmonella* Typhi*, Pseudomonas aeruginosa, Streptococcus pyogenes, Shigella dysentriae* and *Klebsiella pnuemoniae*) used for this study were isolated from clinical specimen obtained from the pathology Unit of some public hospitals in Yola. The isolates were duly authenticated using standard microbiological methods such as gram staining, catalase test, coagulase test, growth pattern on kligler iron agar, indole, methyl red and voges proskauer test as described by Chessbrough (2006)

Preparation of filter paper discs

Filter paper disc containing varying concentration of plant extracts was prepared as earlier described by Vineetha *et al*. (2015). Briefly an ordinary office hole punching machine was used to cut our filter discs of approximately 6 mm from whatman filter paper No. 1 The discs were then kept on flat surface and pressure applied on them to keep them straightened after which they were autoclaved at 121 ^{0} C for 30 minutes. The sterilized paper discs were then placed in a sterile petri dish 5 mm apart and then a pipette was used to transfer 0.02 ml (20 µl) directly on each paper disc since each 6 mm paper disc absorbs 0.02 ml taking precaution that the tip was in slight contact with the disc. The discs were allowed to dry in a clean incubator at 37 °C for 4 hours after which 30-35 discs were placed in small sterile air-tight labeled container with $CaCl_2$ (desiccant) at the bottom and labeled appropriately. The prepared filter paper

discs were then stored in the freezer until use.

Preparation of stock solution of plant extracts

To prepare concentration of the plant extracts, known weight of the extracts was dissolved in sterile DMSO to obtain working solution with concentrations of 100 mg/ml, 50 mg/ml and 25 mg/ml. The concentrations of extracts were expressed in mg/µl is given by $C_1V_1 = C_2V_2$ where C_1 = Concentration of stock solution, V_1=Volume of stock solution, C_2=Concentration of working solution and V_2=volume of working solution.

Antibacterial susceptibility testing

The agar disc diffusion method was used to determine the antibacterial activity of *A. leiocarpus*. The inoculum for each organism was standardized to 0.5 McFarland corresponding to 10^8 cells /ml after which a sterile Pasteur pipette was used to transfer 0.5 ml of the standardized inoculum onto the surface of Mueller Hinton agar plates and spread using a glass spreader. Filter paper discs impregnated with different concentrations (100 mg, 50 mg and 25 mg) of the crude and partially purified extracts were then placed equidistant to each other using sterile forceps on the Mueller Hinton agar plates seeded with each test organism. The plates were then incubated at 37 ^{0} C for 24 hours after which the mean diameter of the zone of inhibition of the extracts against each of the test organisms were measured in mm (Vineetha *et al*., 2015).

Determination of minimum inhibitory concentration (MIC)

A stock solution of 100 mg/ml was prepared by dissolving 1000 mg of extract in 10 ml of DMSO. The stock solution was then serially diluted in double fold using nutrient broth pre seeded with test organism adjusted to 0.5 McFarland to obtain extract of the following concentrations 100 mg/ml, 50 mg/ml, 25 mg/ml, 12.5 mg/ml, 6.25 mg/ml and 3.125 mg/ml. A set of test tubes containing broth only and DMSO were used as controls. All the tubes were then incubated at 37 ^{o} C for 18-24 hours. After the period of incubation, the tube containing the least concentration of extract showing no visible sign of growth was taken as the minimum inhibitory concentration (Vinothkumar, *et al.*, 2010).

Determination of minimum bactericidal concentration (MBC) of the extracts against the tested organisms

A Pasteur pipette was used to transfer a 0.1 ml of broth culture from the test tubes in the MIC determination that did not show any visible sign of growth, and inoculated onto sterile nutrient agar plates by the spread plate method. The plates were then incubated at 37 ^{o} C for 24 hours. The least concentration that did not show any growth of test organisms was considered as the MBC.

Solvent-Solvent Purification and Antibacterial activity of A. *leiocarpus* extracts

About 500 mg of each of the three extracts of *A. leiocarpus* was dissolved in 10 ml of water in a separating funnel and was mixed thoroughly by gentle swirling. A measuring cylinder was used to add 30 mls of hexane into the separating funnel containing the aqueous solution and was shaken vigorously to mix. The mixture was then allowed to settle for 5 minutes to form the organic layer and the aqueous layer. The lower organic layer was separated from the aqueous layer using a separating funnel to give hexane layer (hexane layer A) and mother liquor (aqueous layer). To the aqueous layer 30 ml of hexane was added again and was shaken vigorously. The two layers were separated as above and the hexane layer (Hexane layer B) was added to the hexane layer A. The hexane was dried to give fraction A. The aqueous phase was further extracted with 30 ml of ethyl acetate twice as with hexane and the ethyl acetate soluble fraction was concentrated by allowing the solvent to evaporate to dryness to get the fraction B. Furthermore, 30 ml of butanol was added to the aqueous phase twice and the butanol fraction was concentrated as with ethyl acetate fraction to give fraction C. The aqueous phase was further fractionated with

chloroform following the same procedure in order to get fraction D. The aqueous phase after extraction with chloroform was then concentrated to get fraction E. The antibacterial activity, MIC and MBC of the various fractions were determined as described above except that for the MIC and MBC the concentrations of used were between 20 mg/ml to 0.625 mg/ml and only for fractions showing activity.

pH and Temperature stability

The pH and temperature stability of the extracts were also determined using the method of Sammuelson *et al.,* (1985). To determine the effect of pH on the antibacterial activity of the extracts, 50 mg/ml concentration of the extracts was adjusted to pH between 6.0 and 8.0 using a pH meter (Labtech digital pH meter).

To determine the thermal stability of the bioactive component, 50 mg concentration of the extract was subjected to heat treatment in a water bath at 60 ^{0}C and 100 ^{0} C for 1 hour after which the antibacterial activity of each of the treated and non-treated extracts were determined as described previously

Determination of shelf life of extract

For determination of shelf life, antibacterial activity of the extracts of *A. leiocarpus* was determined after storage for a period of 10 months following the method described earlier.

RESULTS

Aqueous and alcoholic extracts from the bark of *A. leiocarpus* gave different yields for each of the solvents used. The aqueous, ethanol and acetone extracts yielded 6.0 g, 5.0 g and 2.9 g respectively. The pH values of the extracts of the bark of *A. leiocarpus* was 3.8. Result of phytochemical screening of extracts of *A. leiocarpus* showed the presence of tannins, alkaloids, terpenoids, cardiac glycosides and saponin. The antibacterial activity of the crude ethanolic extracts of the bark of *A. leiocarpus* showed the highest inhibitory activity against all the test organisms at 100 mg/ml with diameter of zone of inhibition ranging from 17 mm for *Streptococcus pyogenes* to 26 mm for *Pseudomonas aeruginosa.* This was followed by the acetone extracts at the same concentration with a zone of inhibition of 17 mm for *Salmonella* Typhi and 22 mm for *Staphylococcus aureus* and *Pseudomonas aeruginosa*. All the extracts revealed varying inhibitory activities at 25 mg extract concentration (Table 1). The MIC values of the crude bark extracts against the test organisms was in the range 3.125 mg to 6.5 mg/ml concentration whereas the MBC values of the extracts of was in the range of 3.125-6.25 mg/ml (Table 2).

Solvent-solvent purification of the bark extracts of *A. leiocarpus* produced four fractions namely hexane fraction (A), ethyl acetate fraction (B), butanol fraction (C), chloroform fraction (D) and residue (E). The pH values for fractions A, B and C of the extracts were in the range of 3.8-4.7 but the values for fraction D and residue E were in the range of 5.5-6.3 respectively.

Results of antibacterial susceptibility screening revealed that the ethyl acetate fraction (B) and butanol fraction (C) showed significant antibacterial activity against the test organisms compared to the hexane fraction (A) whereas chloroform fraction (D) and residue (E) showed no measurable zone of inhibition against the test organisms (Table 3). The MIC and MBC values of fraction B and fraction C of ethanol, acetone and aqueous extracts were in the range of 0.625- 2.5 mg/ml (Table 4)

The antibacterial activity of the extracts of the plant on test organisms was reduced when treated at 100 ^{o}C for 1 hour and when treated at pH 7.0 and the activity was lost at pH 8.0 (Table 5). After preservation for ten months, result of antibacterial studies indicates that the antibacterial activity against the test organisms did not reduce appreciably (Table 6).

Table 1. Results of antibacterial activity of crude extracts of A. *leiocarpus* against **test organisms.**

	S. aureus	*S. pyogenes*	*S.* Typhi	*S. dysentriae*	*K. pneumoniae*	*P. aeruginosa*
A100	21	18	15	20	18	22
A50	17	14	12	17	16	17
A25	13	12	11	14	11	15
E100	23	22	17	20	20	23
E50	19	20	14	16	17	20
E25	10	15	12	14	13	19
C100	25	17	23	25	21	26
C50	20	15	19	21	18	20
C25	13	11	14	17	14	16

KEY: 100, 50 and 25 are concentrations in mg/ml
A, E and C represents Aqueous, Acetone and ethanol extracts of plant

Table 2: Minimum inhibitory concentration (mg/ml) and minimum bactericidal concentration (mg/ml) of extracts *A. leiocarpus*

	AQE		EE		AE	
Test Organisms	MIC	MBC	MIC	MBC	MIC	MBC
S. aureus	6.25	6.25	3.125	6.25	3.125	3.125
S. pyogenes	12.5	25	6.25	6.25	6.25	6.25
S. Typhi	6.25	12.5	6.25	6.25	6.25	6.25
S. dysentriae	3.125	6.25	6.25	6.25	3.125	3.125
K. pneumonia	3.125	6.25	3.125	6.25	3.125	3.125
P. aeruginosa	6.25	12.5	3.125	6.25	3.125	6.25

AQE – Aqueous extracts; EE – Ethanol Extracts; AE- Acetone Extracts; MIC→ minimum inhibitory concentration; MBC→ minimum bactericidal concentration.

Table 3: Results of antibacterial activity of different fractions of *A. leiocarpus* (25 mg) against the test organisms.

Extract Fractions	Diameter of zone (mm)					
Aqueous	*S. aureus*	*S. pyogenes*	*S.* Typhi	S. *dysentriae*	*K. pneumoniae*	*P. aeruginosa*
Hexane	-	6	7	7	9	-
Ethyl Acetate	13	14	15	15	12	14
Butanol	13	12	15	13	14	16
Chloroform	-	-	-	-	-	-
Residue	-	-	-	-	-	-
Ethanol						
Hexane	6	-	-	12	-	-
Ethyl Acetate	12	11	14	14	18	23
Butanol	15	12	19	13	15	21
Chloroform	-	-	-	-	-	-
Residue	-	-	-	-	-	-
Acetone						
Hexane	7	-	15	-	-	12
Ethyl Acetate	19	22	16	16	25	20
Butanol	12	20	14	15	22	20
Chloroform	-	-	-	-	-	-
Residue	-	-	-	-	-	-

KEY: - = no zone of inhibition, A =hexane fraction; B =Ethyl acetate fraction;C =butanol fraction; D =chloroform fraction; E =residue

Table 4 : Minimum inhibitory concentration (mg) and minimum bactericidal concentration (mg) of fractions *A. leiocarpus*

		S. aureus	*S. pyogenes*	*S.* Typhi	*S. dysentriae*	*K. pneumoniae*	*P. aeruginosa*
Aqueous Extract	MIC	2.5	2.5	2.5	2.5	2.5	2.5
FrB	MBC	5.0	5.0	2.5	2.5	5.0	5.0
FrC	MIC	2.5	2.5	2.5	2.5	2.5	2.5
	MBC	5.0	5.0	5.0	2.5	5.0	2.5
Ethanol Extract							
FrB	MIC	1.25	2.5	1.25	1.25	1.25	1.25
	MBC	2.5	2.5	1.25	2. 5	1.25	2.5
FrC	MIC	1.25	2.5	1.25	1.25	1.25	1.25
	MBC	1.25	5.0	1.25	1.25	1.25	1.25
Acetone Extract							
FrB	MIC	0.625	1.25	1.25	1.25	1.25	1.25
	MBC	0.625	1.25	1.25	2.5	1.25	1.25
FrC	MIC	1.25	0.625	1.25	1.25	0.625	0.625
	MBC	1.25	1.25	1.25	1.25	0.625	1.25

Key
FrB → Ethyl Acetate Fraction
FrC→ Butanol Fraction
MIC → Minimum inhibitory concentration
MBC → Minimum Bactericidal concentration

Table 5: Effect of pH and Temperature (^{o}C) treatment on the antibacterial activity of A. *leiocarpus.*

	pH Treatment of Extracts				Treatment ^{0}C	
Test Organism	**UTE**	**6.0**	**7.0**	**8.0**	**60**	**100**
S. aureus	+++	++	+	±	++	++
S. pyogenes	++	++	+	±	++	++
S. Typhi	+++	++	+	±	++	++
S. dysentriae	+++	++	+	±	+++	+++
K. pneumonia	+++	++	+	±	+++	++
P. aeruginosa	+++	++	+	±	+++	+++

Key: UTE → Not treated extract, → Zone of inhibition ≤ 6.0 mm. → Zone of inhibition 7-10 mm. ++ → Zone of inhibition 11-15 mm, +++ → Zone of inhibition 16-20 mm.

Table 6: Results of antibacterial activity of extracts of *A. leiocarpus* (25 mg/ml) after 10 months of preservation.

	Aqueous		Ethanol	
	A	B	A	B
S. aureus	++	++	+	+
S. pyogenes	++	+	++	++
S. Typhi	++	++	++	++
S. dysentriae	++	+	++	++
K. pneumonia	++	+	++	++
P. aeruginosa	++	++	++	++

A → Original extract, →Extract preserved for 10 months, + → Zone of inhibition 7- 10 mm . ++ → Zone of inhibition 11-15 mm, ++ + → Zone of inhibition 16-20 mm.

DISCUSSION

The use of medicinal plants is indigenous and synonymous with the African tradition. Among the Høba people in Hong local government area of Adamawa State, Nigeria, a great variety of plants are used to treat and prevent diseases. In this study, extracts from the bark of *A. leiocarpus* were tested for antibacterial activity against clinical bacterial isolates. The extracts of the bark of this plant showed varying degrees of inhibitory activity against all test organisms employed in this study as evidenced by differences in diameter of zone of inhibition this finding is also in agreement to previous work by Mann *et al.* (2010). These difference can be attributed to each solvent's ability to extract particular bioactive molecules than the other. It can also be attributed to the initial population density of the organisms, their growth rate and rate of diffusion of the antimicrobials (Li *et al*., 2017). Also, Prescott *et al*. (2002) reported that effect of an agent varies with target species. *S. aureus, Lactobacillus spp, S. typhii and Bacillus spp* are more susceptible at low concentration of the antimicrobial agent used than *E. coli* or *P. aeruginosa*. This could therefore explain differences in susceptibility demonstrated by the different isolates.

The high inhibitory activity of the aqueous and organic extracts of *A. leiocarpus* provides a scientific basis for the use of this plant by traditional healers for the management of infectious diseases such as dysentery, typhoid fever, pneumonia, wounds and burns in Hong LGA of Adamawa State. Furthermore, Mann *et al*. (2010) reported that the methanollic extracts of *A. leiocarpus* exhibited antimicrobial activity against *Staphylococcus aureus, Lactobacillus spp, Bacillus spp* and *Salmonella typhii* which is in agreement with findings from our study. This potent antibacterial property of extracts of *A. leiocarpus* is not surprising since to keep out potential invaders, plants produce a wide range of selective antibacterial, antifungal and antiviral compounds either in a constitutive or an inducible manner (Cowan, 1999, Grayer and Harbone, 1994). It may be concluded that the presence bioactive phytochemical constituents (tannins, saponins, alkaloids) in respective plants extracts is responsible for the inhibitory action of the extracts of *A. leiocarpus* against the test organisms. The minimum inhibitory concentration and minimum bactericidal concentrations of crude extracts of *A. leiocarpus* against the test organisms were in the range of 3.125-25.00 mg/ml and 3.125-50.00 mg/ml respectively. These values are lower than the 200 mg/ml – 400 mg/ml reported for *Streptococcus mutans* and *Staphylococcus aureus* reported by Dayok *et al*. (2018) for ethanolic and aqueous extracts of *A. leiocarpus* in Jos, Plateau state. It is worthy of note that the MIC values of acetone and ethanol extracts of *A. leiocarpus* were low and further verifies the fact that extracts of *A. leiocarpus* exhibits strong antibacterial activity against the test organisms. The minimum bactericidal concentration for crude extracts of *A. leiocarpus* against *Pseudomonas aeruginosa* reported by *Dayok et al*. (2018) is 400 mg/ml which is higher than values obtained from this study although basically the worked with crude leaf extracts which may contain less bioactive compounds than the bark.

Solvent-solvent purification involves the separation of compounds with different polarities and since the active components will be expected to be soluble in either one or another solvent, this technique is meant to purify the crude extract by removing undesirable components. In this study, although hexane extracted the major amount of dry weight, this fraction showed the lowest antibacterial activity against the test organisms (diameter of zone 6-12 mm at 25 mg/ml concentration) compared to ethyl acetate fraction (diameter of zone 11-25 mm) and butanol fraction (diameter of zone 12-20 mm) at 25 mg/ml concentration. This finding is however at variance with the work of Kubmarawa *et. al.* (2002) who reported that the hexane fraction of *Commiphora*

kerstingii was most effective against *S. aureus, P. aeruginosa* and *S.* Typhi. This difference may be because of extraction of some inactive components like waxes and oils present in the extracts by hexane. The extracts were heat stable but were labile at alkaline pH (Table 3). This is in agreement with Molan (1992) who reported that many natural antimicrobials including honey are active against animal pathogens at acidic pH. However, since it has been reported that besides the production of antibacterial compounds, plants have been reported to act either directly on the microorganism or indirectly on the immune system (Samie *et al.,* 2005). If compounds that stimulate the immune system are present in these extracts and are not labile at alkaline pH and high temperature, they will give good therapeutic results.

The antimicrobial activity of the extracts of *A. leiocarpus* remained unchanged after 10 months of storage in the refrigerator. This reveals the fact that these extracts may be kept in dried condition for a long time for medicinal purposes. Das *et al*., (1998) have reported that the taxol content of *Taxus baccata* and the potency of its extracts stored at room temperature for 1 year decreased by 30-40% and 70-80% respectively while storage in a freezer and out of direct sunlight produced no adverse deterioration. This result implies that the bioactive compound present in the extract is stable at room temperature over the 10 months period of storage.

CONCLUSION

The findings from this study lends credence to the use of plants as medicines for the management of infectious such as those investigated in this study. This study on antibacterial effects of fractions of *A. leiocarpus* has shown that this plant holds a rich reserve of potent antimicrobial agents that could be exploited for drug development.

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