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Antibacterial Potentials of the Ethanolic Extract of the Stem Bark of *Combretum micranthum* G. Don and Its Fractions

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Abstract

Ethanolic extract of the stem bark of *Combretum micranthum* and its fractions were evaluated for their antibacterial potentials against two Gram positive organisms (*Staphylococcus aureus* and *Bacillus subtilis*) and two Gram negative organisms (*Escherichia coli* and *Pseudomonas aeruginosa*)-all of both medical and pharmaceutical importance. The anti-bacterial potential was assesed using both the agar and tube dilution methods; Phytochemical and TLC analysis were also done to identify the content of the stem bark of the plant. Phytochemical screening results indicated the presence of flavonoids, tannin, saponins, anthraquinones and cardiac glycosides. The extract and its fractions exhibited potent antimicrobial activities against the test organisms with n- hexane fraction showing the mildest activity. The most pronounced activity was observed with the aqueous fractions and interestingly, against the Gram negative organisms. Results of the Minimum Inhibitory concentration (MIC) of the ethanolic extract and fractions range from 0.234375mg/ml-15mg/ml. The Minimum Bactericidal concentration (MBC) range from 0.9375mg/ml-30mg/ml confirming that the extracts are bactericidal and that their activity is concentration dependent. Thin Layer Chromatography (TLC) results showed a number of likely bioactive constituents which may have been responsible for the observed activities. Results are discussed in the context of the relevance of plant constituents in the control of organisms of health and pharmaceutical importance.

Keywords: ethanolic extract, *Combretum micranthum,* phytochemical, antimicrobial activity, bactericidal, thin layer chromatography

1. Introduction

Finding healing powers in plants is an ancient idea (Neeraj, 2010). All through history, plants have been of tremendous importance to medical and pharmaceutical studies. Ancient doctors were reported to methodically collect information about herbs and developed well-defined pharmacopoeias so that guidelines as well as how to treat varieties of ailments were available in writing (Stanbury, 2001). Today, higher plants are still believed to be sleeping giants of drug development and a virtually untapped reservoir of potentially useful source of drugs (Farnsworth & Morris, 1976).

Combretum micranthum belongs to the family *combrataceae*. It is commonly called Okan, Geza and Asaka by the Yoruba, Hausa and the Ibibio people of Nigeria. It will normally grow as a shrub, creeper or small tree usually 2-5m tall even though it may reach a height of 15m while the branches may reach a length of 20m. A plant of the sahel, sudan and guinea savannah forests, it quite commonly invades cultivated land and is always found on termite heaps. It is a browse plant for cattle and it is important feed in the dry season.

Combretum micranthum is used in West Africa for the treatment of different diseases (Irvine, 1961; Kerharo & Adam, 1974; Ake & Guinko, 1991). Roots and leaves infusion are used as a febrifuge or for vapour bath for fever or lubargo when hot. The leaves are diuretic and their infusion is used for treating fever especially when accompanied by vomiting, ordinary colic and to prevent vomiting generally. They are also used in treating diarrhoea, wounds and various skin diseases among many other medicinal uses to which various parts of this plant is mixed with.

The authors are interested in presenting results which show the good potentials of this plant against both Gram

positive and Gram negative organisms of medical and pharmaceutical importance especially those of Gram negative reaction.

2. Materials and Methods

2.1 Plant Collection and Authentication

The plant material (Stem bark of *Combretum micranthum*) was collected from Ikono Local Government area of Akwa Ibom State, Nigeria in September, 2010. It was authenticated in the Biological Science Department of University of Uyo, Nigeria.

2.2 Sample Preparation and Extraction

The plant material which was collected in the rainy season was slightly wet and was therefore, cut into small pieces and air dried. The pieces were further reduced into smaller size and then ground into powder using a mortar and pestle. 1kg of the powdered stem bark was marcerated with 70% (5L) ethanol at room temperature with intermittent shaking for 72 hours. After filtration, 3.6 litres of the filtrate obtained was concentrated using a water bath at temperature not exceeding 40 ^{o}C and the resulting extract stored in the refrigerator at 4^{o}C until needed.

2.3 Phytochemical Screening

This was done using standard methods as described by Trease and Evans (1989).

2.4 Fractionation of Extract

The extract (20g) was dissolved in ethanol and further partitioned with 100mls of chloroform 100mls of n-hexane and 100mls of water. Phases obtained were concentrated to dryness by allowing it to evaporate on the laboratory bench.

2.5 Test Organisms

The organisms used for this study were

Staphylococcus aureus (NCTC6571)

Bacillus subtilis (NCTC8853)

Pseudomonas aeruginosa (ATCC2787853)

Escherichia coli (10418)

They were obtained from the Pharmaceutical Microbiology laboratory of the Faculty of Pharmacy, University of Uyo-Nigeria.

2.6 Antibacterial Assay

2.6.1 Innoculum Standardization

Standard bacterial cultures were prepared by sub culturing a loopful of each of the bacteria into sterile nutrient broth and incubated at 37 ^{o}C for 16 hours. The suspensions were adjusted to a turbidity of 10^6 colony forming units (cfu)/ml which is equal to 0.5 Mcfarland standard using visual comparison.

2.6.2 Bacterial Susceptibility Test

The agar well diffusion method modified for its suitability (Sudhakar, Rau, Roa, & Rajo, 2006) was used. The media used was prepared according to manufacturer's instructions and asceptically poured into sterile Petri dishes and allowed to solidify. An overnight culture of each of the test organisms adjusted to a turbidity of 10^6 using the the 0.5 Mcfarland standard was introduced into each dish. A 4mm sterile cork borer was used to bore holes equidistant from each other on the plates. Using a sterile pipette, different concentrations of the extract and fractions were introduced into the wells. I mg/ml of ampiclox was introduced into one of the wells and used as a control. The plates were allowed to stand for one hour before incubation to allow for the diffusion of the agent into the media. They were then incubated for 24 hours at 37 ^{o}C. The diameter of the zones of inhibition was then measured to the nearest millimetre.

2.6.3 Determination of Minimum Inhibitory Concentration

The MIC was determined using the tube dilution method (Baron & Fingold, 1990). A two- fold serial dilution of the extract and fractions was carried out asceptically to give varying concentrations ranging from 30mg/ml-0.23435mg/ml. Each of the dilutions was inoculated with 0.1ml of the standardized inoculum and the tubes were incubated at 37^{0}C for 24 hours and microbial growth observed as turbidity in tubes was looked out for. The lowest concentration that showed no growth was considered the MIC. The whole procedure was replicated

using Ampiclox and Zosyn at concentrations ranging from 0.5mg/ml-0.0004mg/ml. A positive control tube containing broth with organisms without plant extract and a negative control tube containing extract without organisms were also incubated along.

2.6.4 Determination of Minimum Bacterial Concentration

From the MIC tubes which showed no growth, 0.1ml aliquots were taken and transferred unto antibacterial free nutrient agar plates. The plates were incubated at 37 ^{o}C for 24 hours. The lowest concentration that showed no growth was recorded as the MBC.

2.6.5 Chromatographic Studies

The extract and the ethanol fractions were subjected to thin layer chromatographic studies to determine the component of the extract responsible for the observed bioactivity. The method employed was a modified form of that reported by Udobi et al. (2010) with methanol 100% and Methanol: Ammonia (200:3) as solvent systems.

3. Results

Yields from the partitioning of crude ethanolic extract were n-hexane (1.03g), chloroform (1.0g), ethanol (0.01g) and aqueous residue (8.85g).

Tables 1-8 containing the results obtained are presented in the appendix.

Table 1. Types of compounds identified in the phytochemical screening of the ethanol extract

Components	Presence/Absence (+)/(-)
Alkaloid	-
Saponins	+
Flavonoids	+
Tannins	+
Phlobatanins	_
Anthraquinones	+
Cardiac glycosides	+
Terpenes	+

Table 2. Inhibition zones (mm) of the ethanol extract of *Combretum micranthum* against test organisms

Extract	Conc. (mg/ml)	*B. subtilis*	*S. aureus*	*E. coli*	*P. aeruginosa*
Ethanol	80	17	21	24	26
	40	14	17	22	21
	20	11	11	16	11
	10	4	10	15	9
	5	2	8	13	8
	2.5	No Inhibition	6	12	7
	1.25	No Inhibition	5	10	6
Ampiclox	1	11	26	25	No Inhibition

4. Discussion

The ethanolic extract of *Combretum micranthum* and its fractions exhibited significant antimicrobial activity against some of the microorganisms studied. This supports the use of the plant in traditional medicine. The phytochemical screening revealed the presence of bioactive compounds to which have been imputed the antimicrobial activity observed in this study. The results obtained showed the presence of bioactive compounds like saponin, flavonoids, tannins, anthraquinones, cardiac glycosides and terpenes (Table 1). All of them known to have antibacterial properties. The presence of tannins in the extract may explain the use of the plant for

treating diarrhoea, haemorrhages swellings and other diseases. (Tschehe, 1971; Lamikanra, Ogundaini, & Ogungbamila, 1990). Phytochemical testing also reveals the absence of alkaloids. This differs from the results of Bassene et al. (1986). However, it is known that the plant composition will depend on the geographical origin (Roa & Rout, 2003).

Table 3. Zone of inhibition (mm) of bacterial activity of distinct fractions of the ethanol extract of C*ombretum micranthum* against test organisms

Concn(mg/ml)	Ethanol	Chloroform	Aqueous	n-hexane	Organism
80	17	16	20	7	*B. subtilis*
	21	19	25	8	*S.aureus*
	24	16	28	15	*E. coli*
	26	13	30	11	*P. aeruginosa*
40	14	14	18	5	*B. subtilis*
	17	13	21	6	*S.aureus*
	22	11	25	11	*E. coli*
	21	11	25	7	*P. aeruginosa*
20	11	12	16	3	*B. subtilis*
	11	11	19	4	*S.aureus*
	16	9	21	8	*E. coli*
	9	8	15	5	*P. aeruginosa*
10	4	10	12	NZ	*B. subtilis*
	10	9	16	NZ	*S.aureus*
	15	7	19	6	*E. coli*
	9	7	13	NZ	*P. aeruginosa*
5	12	8	9	NZ	*B. subtilis*
	8	7	14	NZ	*S.aureus*
	13	6	17	5	*E. coli*
	8	6	11	NZ	*P. aeruginosa*
2.5	NZ	6	6	NZ	*B. subtilis*
	6	5	12	NZ	*S.aureus*
	12	5	15	NZ	*E. coli*
	7	NZ	10	NZ	*P. aeruginosa*
1.25	NZ	NZ	NZ	NZ	*B. subtilis*
	5	NZ	10	NZ	*S.aureus*
	10	NZ	13	NZ	*E. coli*
	6	NZ	9	NZ	*P. aeruginosa*
Ampiclox	B. subtilis	S. aureus	E. Coli	P. aeruginosa	
(1mg/ml)	11	26	25	NZ	*B. subtilis*
	11	30	27	NZ	*S.aureus*
	19	32	30	NZ	*E. coli*
	8	25	28	NZ	*P. aeruginosa*

NZ-No zone of Inhibition.

Results obtained showed that the extract and its fractions had broad antibacterial spectrum activity and that the effects were concentration dependent (Table 3). The ethanol extract and its aqueous fraction than they were more active than n-hexane and chloroform fractions. This may be related with the polarity of the active compounds.

It was of interest to note the fact that the inhibition was higher for Gram negative than for Gram positive organisms (Table 3) while *Pseudomonas aeruginosa* used as a test organism was found to be resistant to the

reference drug "Ampiclox" (Table 3).

Table 4. Results showing the minimum inhibitory concentration MIC (mg/ml) of the ethanol extract of *Combretum micranthum* on the growth of test organisms

Isolates	Concentration of extract (mg/ml)							
	15	7.5	3.75	1.8	0.94	0.47	0.23	0.12
B. subtilis	-	-	-	+	+	+	+	+
S. aureus	-	-	-	-	-	+	+	+
E. coli	-	-	-	-	-	-	-	+
P. aeruginosa	-	-	-	-	-	-	+	+

Key: + = Growth; - = No growth.

Table 5. Showing results of minimum Inhibitory concentration mg/ml of distinct fractions of the ethanol extract of *Combretum micranthum* against tested organisms

Fraction	Concentration of Extract (mg/ml)											
	30	15	7.5	3.75	1.88	0.94	0.47	0.23	0.11	0.06	0.03	
Aqueous		-	-	-	-	-	+	+	+	+	+	*B. subtilis*
		-	-	-	-	-	-	+	+	+	+	*S.aureus*
		-	-	-	-	-	-	-	+	+	+	*E. coli*
		-	-	-	-	-	-	+	+			*P. aeruginosa*
n- hexane	-	-	+	+	+	+	+	+				*B. subtilis*
	-	-	-	+	+	+	+	+				*S.aureus*
	-	-	-	+	+	+	+	+				*E. coli*
	-	-	+									*P. aeruginosa*
Chloroform		-	-	-	-	+	+	+	+	+	+	*B. subtilis*
		-	-	-	-	+	+	+	+	+	+	*S.aureus*
		-	-	-	-	+	+	+	+	+	+	*E. coli*
		-	-	+	+	+	+	+	+	+	+	*P. aeruginosa*

Table 6. Minimum inhibitory concentration of Ampiclox (Ampicillin and cloxacillin) against test organisms

Concentration of drug (ug/ml)	Isolates			
	B. subtilis	*S. aureus*	*E.coli*	*P. aeruginosa*
1000.00				+
500.00	-	-	-	+
250.00	-	-	-	+
125.00	-	-	-	+
62.00	-	-	-	+
31.00	-	-	-	+
15.63	-	-	-	+
7.81	-	-	-	+
3.90	+	-	-	+
1.95	+	-	+	+
0.97	+	+	+	+
0.48	+	+	+	+

Key: + = Growth; - = No growth.

MIC results obtained for the aqueous fraction show a range from 234ug/ml (*E.coli*) to 937ug/ml (*B. subtilis*). This shows that the lowest concentration of the aqueous extract used in the test inhibited the Gram negative organism E. coli more than it inhibited the Gram positive *B.subtilis*. This is important because Gram negative organisms are known to be resistant to a great deal of plant extract possibly because of their known double membrane barrier. The MIC values of the standard drug was observed to be significantly lower than that of the extract and fractions most likely because the standard drug is in a purer form than the fractions

It is possible to obtain compounds responsible for biological activity in a pure form beginning with their separation using a chromatographic technique. The bioactive compounds in the extracts and fractions studied were successfully separated using Thin Lay chromatography (Table 8). We are interested in performing further studies of these compounds to determine the role of each of them in the antibacterial activity of the extract.

Table 7. Minimum bacterial concentration (mg/ml) of fractions of ethanolic extract of *Combretum micranthum* against test organisms

	Concentration of extract (mg/ml)								
	30	15	7.5	3.75	1.875	0.94	0.47	0.23	Test org.
Aqueous		-	-	-	-	+	+	+	*B. subtilis*
fraction		-	-	-	-	-	-	+	*S.aureus*
		-	-	-	-	+	+	+	*E. coli*
		-	-	-	-	-	-	+	*P. aeruginosa*
n-hexane	-	+	+	+					*B. subtilis*
fraction	-	-	+	+					*S.aureus*
	-	-	+	+					*E. coli*
	-	+	+	+					*P. aeruginosa*
Chloroform		-	-	-	+	+			*B. subtilis*
fraction		-	-	-	+	+			*S.aureus*
		-	-	-	+	+			*E. coli*
		-	+	+	+	+			*P. aeruginosa*

+ = Growth; - = No growth.

Table 8. Showing Rf values of separated components of the ethanolic extract of combretum micranthum and its aqueous fractions

	RF values	
Extract	100% MeOH	Meth(200): Ammonia(3)
A1	0.08	0.06
A2	0.21	0.19
A3	0.44	0.65
A4	0.57	0.84
B1	0.08	0.05
B2	0.21	0.07
B3	0.44	0.07
	0.57	

Key: A = aqueous fraction; B = Ethanolic extract.

5. Conclusion

The study has validated the ethnomedicinal use of this plant as a panacea for microbial infections, the content of the extract from the phytochemical analysis confirmed the reason for antimicrobial activities of the plant against both gram positive and Gram negative bacteria. This plant is recommended for such use only when its non toxicity is confirmed through further work.

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