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Original Research Article

Phytochemical and Antimicrobial Studies of Stored Dried Leaves of *Alchornea Cordifolia* (Shum &Thonn) Mull. Arg. (Euphorbiaceae)

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ABSRTACT

Background: Herbal medicines are promising remedies in the fight against antimicrobial resistance and the readily availability of target medicinal plants all year round is very essential. This study was aimed at assessing the antimicrobial properties of dried leaves of *Alchornea cordifolia* after being stored for five and a half years.Ethanol and hot water maceration, and soxhlet extraction of the stored dried leaves of *Alchornea cordifolia* were carried out followed by phytochemical screening. Antimicrobial test of the extracts against *Staphylococcus aureus, Escherichia coli, Pseudomonas aeruginosa,* and *Candida albicans* was carried out using agar cup plate and disc diffusion methods. Minimum inhibitory (MIC) and minimum bactericidal concentrations (MBC) of the plant extracts against the test organisms were conducted.Phytochemical screening revealed the presence of tannins, saponins and flavonoids. Both the aqueous and ethanol extracts were active against all the tested organisms: *S. aureus* was the most susceptible followed by *E. coli*, *Ps aeruginosa*, and *C. albicans*. The MIC values of the extracts against *C. albicans*, and *Ps. aeruginosa* were 20 mg/ml, 10 mg/ml and 10 mg/ml for hot water macerated extract, ethanol macerated extract and ethanol Soxhlet extract. The extracts of leaves of *A. cordifolia* were found to be active against the microorganism tested even after the leaves were stored for five and a half years. The activities observed were similar to that of fresh *A. cordifolia* leaves

Keywords: *Alchornea cordifolia*, antimicrobial properties, inhibitory concentration, bactericidal concentration

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Introduction

Alchornea cordifolia (Schumach and Thonn) Mull.Arg (Euphorbiaceae) is a perennial shrub or small tree, growing up to 8m high.[1] The common name of the plant is Christmas bush, and it's locally called ewe ipa (Yoruba), banbani (Hausa), Ubebe or ububo (Igbo) and uwonmen (Benin).[2] It is distributed throughout tropical Africa, from Senegal East to Kenya, Tanzania, South and Central Africa to Angola. It is also cultivated in the Democratic Republic of Congo for its medicinal use.[3]

Alchornea cordifolia leaves are used traditionally to treat wound cuts and sores. The leaves of leafy stems are used as infusion or chewed fresh for their sedative or antispasmodic activities, and to treat a variety of respiratory problems including sore throat, cough and bronchitis[3]. The leaves are also used for intestinal problems like gastric ulcers, diarrhea, amoebic dysentery. In Senegal, a leaf decoction is taken to treat tachycardia. In Nigeria, a decoction of bruised fruit is taken to prevent miscarriage. In veterinary medicine, a leaf decoction or root infusion is given to livestock to treat trypanosomiasis.[4]

The World Health Organization has described antimicrobial resistance as one of the top global public health and development threats.[5]

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It has affected countries in all regions and at all economic levels, and production of new synthetic antibiotic is expensive. The use of medicinal plants which are locally sourced, cheap, and easily accessible can be a safe and alternative means of combating this problem. In many populations of developing countries today herbal preparations are the first line of treatment. Eight percent of the world's population relies on medicinal plants for their primary health care.[6]

Alchornea cordifolia is a promising medicinal plant, in addition to its traditional uses in infections treatment, studies had been conducted severally to confirm these traditionally claimed uses as antibacterial;[2,7,8] Antifungal;[9] antidiarrheal, antiviral and antidiabetic properties.[10-12] *Alchornea cordifolia* and other medicinal plants are not cultivated everywhere and may be seasonal. For example, in Nigeria, *Alchornea cordifolia* is cultivated mostly in the southeastern part of Nigeria and scarcely in the Northern part, the only way the plant can be readily available in the Northern part of the country and to be available all year round is for it to be stored until when needed.

This study was aimed at determining the antimicrobial properties of stored leaves of *Alchornea cordifolia* to confirm if the antimicrobial activity can be maintained after five and a half years storage.

Materials and Methods

Sample collection and authentication

Sample of *Alchornea cordifolia* leaves were collected from University of Benin (UNIBEN), Benin City, Nigeria in December 2017. They were identified in the Department of Plant Biology and Biotechnology, UNIBEN and were taken to the herbarium of the Department of Biological Sciences, Ahmadu Bello University, where they were identified and a deposit of the plant was kept with Voucher No 401. The leaves were dried at room temperature and some of them were size reduced to coarse powder using mortar and pestle, while some of the

dried leaves were kept as much as possible in a suitable container and stored at room temperature.

Extraction Procedure
Maceration method
A hundred grams (100 g) of the powdered leaves were weighed on an electronic balance and transferred into a beaker, 1 litre of hot distilled water was added to the powder and the mixture was shaken occasionally for 6 hours and then left for 72 hours. The extract was then filtered and evaporated to dryness in a water bath at 60ºC. The dried extract was then weighed and stored properly in tightly fitted bottles. The same procedure was repeated using ethanol 70% as solvent. The percentage yield of the extracts was calculated.

Soxhlet apparatus
Powdered leaves, 100 g was weighed and transferred into a Soxhlet apparatus and 96% ethanol was used for the extraction. This extraction was done at 60°C temperature with continuous reflux for 16 hours. The extract was thereafter evaporated on a water bath and the percentage yield was calculated.

Phytochemical Screening
The extracts were subjected to phytochemical screening to test for saponins, tanins, flavonoids, carbohydrates, alkaloids and cardiac glycosides according to the procedures described by Trease and Evans.[13]

Purification of the organisms
The organisms: *Pseudomonas aeruginosa, Staphylococcus aureus, Escherichia coli,* and *Candida albicans* were collected from the Department of Pharmaceutical Microbiology laboratory, Ahmadu Bello University, Zaria. They were purified by sub-culturing them on their individual selective media. *Pseudomonas aeruginosa* was grown on cetrimide agar, *Staphylococcus aureus* on mannitol salt agar, *E. coli* on Eosin methylene blue agar and *Candida albicans* on Sabouraud dextrose agar.

Antimicrobial activity testing of Alchornea cordilfolia stored dried leaves extracts
Cup plate method
Single-strength molten sterile nutrient agar (20 ml) was poured into sterile petri dishes and allowed to set. The sterile media plates were flooded with 2 ml of standardized inoculum (equivalent to 0.5 Mc Farland 1.5×10^6 CFU/mL) and the excess was drained off. A sterile cork borer (4 mm) was used to bore seven equidistant cups into the agar plates. One drop of sterile molten agar was used to seal the bottom of the bored hole, so that the extract would not sip beneath the agar. The seven various concentrations of the extracts (20 mg/ml, 10 mg/ml, 5 mg/ml, 2.5 mg/ml, 1.25 mg/ml, 0.625 mg/ml and 0.3125 mg) were prepared by serial dilution using sterile distilled water; 0.1 ml of the different concentrations of the extracts were added into the bored holes. One hour pre-diffusion time was allowed. The plates were incubated at 37ºC for 18 hours. The zones of inhibition were measured in millimeters. A negative control was prepared which contained only the sterile nutrient agar to show that the nutrient agar used was not contaminated.

Disc method
Sterile nutrient agar (20 ml) each was poured into sterile petri dishes and allowed to set. The sterile media was flooded with 2 ml standardized inoculum (equivalent to 0.5 Mc Farland 1.5×10^6 CFU/mL) and the excess was drained off. Discs were made from filter paper and were sterilized. The seven various concentrations of the extracts (20 mg/ml, 10 mg/ml, 5 mg/ml, 2.5 mg/ml, 1.25 mg/ml, 0.625 mg/ml and 0.3125 mg) were prepared by serial dilution using sterile distilled water. The sterilized filter papers were soaked in the various concentrations of the extracts, they were then removed from the extracts and the excess was drained off. The different filter papers with the various concentrations were placed on the inoculated plate equidistant to each other. One hour pre-diffusion time was allowed. The plates were then incubated at 37 ºC for 18 hours. The zone of inhibition was measured in millimeters. Gentamicin 10 µg, and ofloxacin 30 µg were used as standard controls.

Determination of Minimum Inhibitory Concentration (MIC)
The method described by Akinpelu and Kolawole,[14] was used with little modification to determine the MIC of the plant extracts against the test isolates. A two-fold dilution of the extracts was prepared, 2ml aliquots of the different concentrations (40 mg/ml, 20 mg/ml, 10 mg/ml, 5 mg/ml, 2.5 mg/ml) of the extracts were added to 18 ml of pre-sterilised molten nutrient agar medium for bacterial test isolates and sabouraoud dextrose agar medium for the fungal isolate. The mixtures were poured into sterile petri dishes and allowed to set. The dry surface of the media were later streaked with standardized 18-hour-old culture. The inoculated bacterial plates were incubated at 37 ºC for 72 hours while the inoculated fungal plates were incubated at 25 ºC for 96 hours. The plates were thereafter observed for the presence or absence of growth. The MIC was considered as the lowest concentration which inhibited the growth of the respective organisms.

Determination of Minimum Bactericidal Concentration (MBC)
The method described by Olorundare *et al,*[15] was used with little modification. From the MIC test, samples were taken from the plates with no visible growth and sub-cultured onto freshly prepared nutrient agar plates. The plates were incubated for up to 72 hours at 37ºC. The MBC was the minimum concentration of the extracts that showed no bacterial growth on the fresh agar medium.

Data processing and analysis
The inhibition diameters are presented as mean ± standard deviation.

Results and Discussion

The percentage yield of the extracts was calculated as A/B x 100
A = weight of extract
B = weight of powdered plant part
The percentage yield of the ethanol Soxhlet extract was the highest (20.48%), followed by the ethanol macerated extract (18.58%), and the lowest yield was the hot water macerated extract (13.22%). The exhaustive extraction of the components of the leaves by the Soxhlet apparatus might be responsible for the highest yield obtained using the apparatus. The difference in the percentage yield of ethanol and hot water macerated extracts might be due to solvent polarity.
The result of the phytochemical screening showed that *Alchornea cordifolia* leaves contained flavonoid, tannins and saponin (Table 1). According to Trease and Evans,[16] the antimicrobial activities of the plant extract depend basically on the active constituents such as tannins, flavonoids, saponins, and alkaloids. Tannin possesses astringent properties which act by forming protective layer of coagulated protein.[17] The antibacterial activity of tannin is expressed through its ability to pass through the bacterial cell wall up to the internal membrane, thereby interfering with the metabolism of the cell, leading to cell destruction.[17,18]
Saponin possesses antimicrobial activities by reducing the surface tension resulting in increased permeability of cells which results in the discharge of intracellular compounds and causes the death of the organism.[19,20] Flavonoids (also known as polyphenolic compounds) possess antimicrobial activity by inhibiting nucleic acid synthesis, cytoplasmic membrane function, and energy metabolism.[20] They have also been found to reduce adhesion and biofilm formation.[21-23]
Flavonoids, tannins, saponins, alkaloids and phenols had been isolated from previous studies on phytochemical screening of *A. cordifolia* leaves.[2,24]
All the extracts showed zones of inhibition against all the organisms. It was observed that the activities increased with an increase in concentration (Tables 2 - 4). The antimicrobial activities of the extracts of the stored *A. cordifolia* leaves were compared with that of hexane extract of fresh leaves of *A. cordifolia*. The result obtained by Adeshina *et al*,[2] was presented in Table 5. The result obtained from the antimicrobial test of the ethanol and water extracts of the stored leaves of *Alchornea cordifolia* showed antimicrobial activity against all the test organisms. The extracts showed the highest zone of inhibition

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against *Staphylococcus aureus*, followed by *Escherichia coli*, and *Pseudomonas aeruginosa*. The least susceptible organism was *Candida albicans*. This implies that the leaves of stored *Alchornea cordifolia* had the highest activity against *S. aureus* and least activity against *C. albicans.* The ethanol extracts showed higher activity than the water extract. The antimicrobial activities observed with the extracts of stored leaves *Alchornea cordifolia* in this study were closely related to that of fresh leaves of the plant that was reported by Adeshina *et al*.[2]

The MIC values of HWM extract for *S. aureus* and *E. coli* was 10 mg/ml and for *C. albicans* and *Ps aeruginosa* was 20 mg/ml. *S. aureus* had MBC values of 20 mg/ml for both HMW extract and ETM extract, and 10 mg/ml for ETS extract (Table 6). The Minimum Inhibitory Concentration against *S. aureus* was the lowest while that of *C. albicans* and *P. aeruginosa* were the highest which implied that *S. aureus* was the most inhibited organism and *C. albicans* and *Ps. aeruginosa* were the least inhibited.

There were minimum bactericidal concentration values for *Ps. aeruginosa*, *E.coli* and *S. aureus* (Table 7)*;* this implied that the stored leaves of *Alchornea cordifolia* had bactericidal action against these three organisms. This MIC and MBC reported in this study were similar to previous studies on MIC and MBC of extracts of fresh leaves of *Alchornea cordifolia* sampled in Ebonyi and Bauchi States in Nigeria against the same organisms.[25,26]

It was observed that the zones of inhibition of the cup plate method were higher than that of the disc method. This was because the extract in the cup plate method was in direct contact with the organisms while part of the extracts in the disc method had been absorbed by the filter paper.

Comparing the results obtained from extracts of fresh dried leaves of *Alchornea cordifolia* with those from dried stored leaves, the results were similar. This therefore means that leaves can be stored for later use especially when the leaves are out of season or in a locality where the plant is not available.

Table 1: Result of Phytochemical analysis of the stored extracts of *Alchornea cordifolia* leaves

Phytochemicals	HMW	ETM	ETS
Saponin	+	+	+
Tannins	+	+	+
Flavonoid	+	+	+
Carbohydrate	-	-	-
Alkaloid	-	-	-
Glycoside	-	-	-

Key: HMW = hot water macerated extract + = present
ETM = Ethanol macerated extract - = absent
ETS = Ethanol Soxhlet extract

Table 2: Zone of inhibition of hot water macerated extract of stored *Alchornea cordifolia* leaves using cup plate and disc methods

Concentration	Cup plate method (mm)				Disc method (mm)			
	S. aureus	*C. albicans*	*E.coli*	*P. aeruginosa*	*S. aureus*	*C. albicans*	*E.coli*	*P. aeruginosa*
20 mg/ml	17.0 ±1.4	14.4 ± 1.4	16.5 ± 0.7	15.0 ± 1.4	15.0 ± 0.0	13.5 ± 0.7	14.5 ± 0.7	14.0 ± 0.0
10 mg/ml	14.0 ± 0.0	10.0 ± 0.0	13.0 ± 1.4	13.0 ± 0.0	13.0 ± 0.0	10.0 ± 0.0	12.0 ± 1.4	12.0 ± 1.4
5 mg/ml	NA	NA	NA	NA	NA	NA	NA	NA
2.5 mg/ml	NA	NA	NA	NA	NA	NA	NA	NA
1.25 mg/ml	NA	NA	NA	NA	NA	NA	NA	NA
0.625 mg/ml	NA	NA	NA	NA	NA	NA	NA	NA
0.3125 mg/ml	NA	NA	NA	NA	NA	NA	NA	NA
Gentamicin 10 µg	28.0	17.0	24.0	26.0	28.0	17.0	24.0	26.0
Ofloxacin 10 µg	30.0	15.0	19.0	30.0	30.0	15.0	19.0	30.0
Water	NA	NA	NA	NA	NA	NA	NA	NA
Normal saline	NA	NA	NA	NA	NA	NA	NA	NA

NA = No activity

Table 3: Zone of inhibition of ethanol macerated extract of stored *Alchornea cordifolia* leaves using cup plate and disc methods

Concentration	Cup plate method (mm)				Disc method (mm)			
	S. aureus	*C. albicans*	*E.coli*	*P. aeruginosa*	*S. aureus*	*C. albicans*	*E.coli*	*P. aeruginosa*
20 mg/ml	18.0± 1.4	14.5± 2.1	17.0± 0.0	17.0± 1.4	17.0± 1.4	14.0± 1.4	15.0± 0.0	14.5± 2.2
10 mg/ml	15.0± 1.4	11.0± 0.0	13.5± 0.7	13.5± 0.7	13.5± 0.7	11.0± 0.0	12.5± 0.7	12.0± 0.0
5 mg/ml	NA	NA	NA	NA	NA	NA	NA	NA
2.5 mg/ml	NA	NA	NA	NA	NA	NA	NA	NA
1.25 mg/ml	NA	NA	NA	NA	NA	NA	NA	NA
0.625 mg/ml	NA	NA	NA	NA	NA	NA	NA	NA
0.3125 mg/ml	NA	NA	NA	NA	NA	NA	NA	NA
Gentamicin 10 µg	28.0	17.0	24.0	26.0	28.0	17.0	24.0	26.0
Ofloxacin 10 µg	30.0	15.0	19.0	30.0	30.0	15.0	19.0	30.0
Ethanol	NA	NA	NA	NA	8.0	7.0	8.0	6.0
Normal saline	NA	NA	NA	NA	NA	NA	NA	NA

NA = No activity

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Table 4: Zone of inhibition of ethanol Soxhlet macerated extract of stored *Alchornea cordifolia* leaves using cup plate and disc methods

Concentration	Cup plate method (mm)				Disc method (mm)			
	S. aureus	***C. albicans***	***E.coli***	***P. aeruginosa***	***S. aureus***	***C. albicans***	***E.coli***	***P. aeruginosa***
20 mg/ml	19.5 ± 2.1	14.5 ± 0.7	18.0 ± 1.4	17.0 ± 1.4	17.5 ± 0.7	14.0 ± 0.0	16.0 ± 0.0	14.5 ± 2.2
10 mg/ml	16.0 ± 1.4	12.0 ± 0.0	14.5 ± 2.2	14.0 ± 2.8	14.5 ± 0.7	11.0 ± 0.0	13.5 ± 0.0	12.0 ± 0.0
5 mg/ml	NA	NA	NA	NA	NA	NA	NA	NA
2.5 mg/ml	NA	NA	NA	NA	NA	NA	NA	NA
1.25 mg/ml	NA	NA	NA	NA	NA	NA	NA	NA
0.625 mg/ml	NA	NA	NA	NA	NA	NA	NA	NA
0.3125 mg/ml	NA	NA	NA	NA	NA	NA	NA	NA
Gentamicin 10 µg	28.0	17.0	24.0	26.0	28.0	17.0	24.0	26.0
Ofloxacin 10 µg	30.0	15.0	19.0	30.0	30.0	15.0	19.0	30.0
Ethanol	8.0	7.0	7.0	7.0	8.0	7.0	7.0	7.0
Normal saline	NA	NA	NA	NA	NA	NA	NA	NA

NA = No activity

Table 5: Zone of inhibition of hexane extract of fresh leaves of *A. cordifolia*

Test bacteria	Zone of inhibition (mm)					
	20mg/ml	**10mg/ml**	**5mg/ml**	**2.5mg/ml**	**1.25mg/ml**	**GTM**
Ps. aeruginosa	13 ± 0.2	11 ± 0.1	NI	NI	NI	31 ± 0.0
Ps. aeruginosa ATCC 10145	11 ± 0.0	NI	NI	NI	NI	32 ± 0.2
S. aureus	23 ± 0.0	19 ± 0.1	18 ± 0.2	14 ± 0.2	NI	23 ± 0.0
S. aureus ATCC 12600	22 ± 0.2	17 ± 0.0	15 ± 0.1	12 ± 0.2	NI	25 ± 0.1
E. coli	11 ± 0.2	NI	NI	NI	NI	20 ± 0.2
E. coli ATCC 11775	16 ± 0.1	14 ± 0.3	11 ± 0.1	NI	NI	22 ± 0.0
Proteus sp	15 ± 0.2	13 ± 0.0	11 ± 0.1	NI	NI	21 ± 0.0

GTM = Gentamicin 2µg; NI = no inhibition

Conclusion

The extracts of the stored leaves of *Alchornea cordifolia* possessed antimicrobial activities against, *S. aureus, E. coli, Ps. aeruginosa* and *C. albicans*. The stored dried leaves had similar activity with the fresh leaves. Therefore it can be said that the leaves of *Alchornea cordifolia* retained their activity even after being stored for five and a half years. This implies that they can be used for infection treatment after being stored for a long period.

Conflict of Interest

The authors declare no conflict of interest.

Authors' Declaration

The authors hereby declare that the work presented in this article is original and that any liability for claims relating to the content of this article will be borne by them.

Table 6: Minimum inhibitory concentration (MIC) of extracts of stored *Alchornea cordifolia* leaves

Test Organism	Minimum Inhibitory Concentration (mg/ml)		
	HWM	**ETM**	**ETS**
S. aureus	10	10	5
C. albicans	20	10	10
E. coli	10	10	10
P. aeruginosa	20	10	10

KEY: HMW = Hot Water Macerated Water
ETM = Ethanol Macerated Extract
ETS = Ethanol Soxhlet Extract

Table 7: Minimum bactericidal concentration (MBC) of extracts of stored *Alchornea cordifolia* leaves

Extract	Parameters	*S. aureus*	*E. coli*	*Ps. aeruginosa*
Hot water macerated extract	MIC (mg/ml)	10	10	20
	MBC (mg/ml)	20	10	20
	Activity Power = MBC/MIC	2 Bactericidal	1 Bactericidal	1 Bactericidal
Ethanol Macerated Extract	MIC (mg/ml)	10	10	10
	MBC (mg/ml)	20	20	20
		2	2	2

	Activity Power = MBC/MIC	Bactericidal	Bactericidal	Bactericidal
Ethanol Soxhlet Extract	MIC (mg/ml)	5	10	10
	MBC (mg/ml)	10	20	20
	Activity Power = MBC/MIC	2 Bactericidal	2 Bactericidal	2 Bactericidal

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