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Antistaphylococcal Activity of N-Butanol and Aqueous Sub-Fractions of *Alchornea cordifolia* Leaves

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Authors' contributions

This work was carried out in collaboration between all authors. GOA designed the study, carried out the antibiotic and plant extract/fractions susceptibility testing, performed the statistical analysis, wrote the protocol, and wrote the first draft of the manuscript. BOO collected, purified and identified the test organisms. AM collected and extracted the plant leaves. YKEI and JAO managed the analyses of the study. All authors read and approved the final manuscript.

Research Article

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ABSTRACT

Aims: To carry out the antistaphylococcal activity of n-butanol and aqueous sub-fractions of *Alchornea cordifolia* (Schumach. And Thonn.) Müll. Arg. leaf extract against multidrug resistant *Staphylococcus aureus.*
Study Design: Characterization and antibiotic susceptibility determination of the test *S. aureus* isolates, extraction of *A. cordifolia* leaf, partitioning of the extract, Zones of inhibition and Minimum Inhibitory and Bactericidal Concentrations determination.
Place and Duration of Study: Department of Pharmaceutics and Pharmaceutical Microbiology, Ahmadu Bello University, Zaria, Nigeria. February 2010 to October 2011.
Methodology: *A. cordifolia* leaves were collected from Abuja, Nigeria. The activity of the ethanol extract, N-butanol (NSF) and aqueous (ASF) sub-fractions of the plant leaf against five clinical staphylococcal isolates and the standard Methicillin Resistant *S. aureus* (MRSA) ATCC 33591 were determined using agar-well diffusion and broth dilution methods. The antibiotic susceptibility pattern of the isolates was determined by the Kirby-

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Bauer-CLSI modified disc agar diffusion technique (DAD).
Results: The diameter zones of inhibition showed by ethanol extract against the test staphylococcal isolates ranged between 12 mm - 26 mm, while the diameter zones of inhibition observed from N-butanol sub-fraction and aqueous sub-fraction against the isolates were between 11 mm - 36.5 mm and 11 mm - 35 mm respectively. The diameter zones of inhibition of the sub-fractions against the standard MRSA ATCC 33591 ranged from 11 mm – 27.5 mm. The diameter zones of inhibition of the test antibiotics ranged from 10 mm to 23 mm. The Minimum Inhibitory Concentration (M. I. C.) and Minimum Bactericidal Concentration (M. B. C.) values produced by ethanol extract were higher than those of the sub-fractions. N-butanol sub-fraction produced the lowest M. I. C and M. B. C. values of 0.625 mg/ml – 1.25 mg/ml and 1.25 mg/ml – 2.5 mg/ml respectively. The M. I. C. and M. B. C. values of the N-butanol sub-fraction against the standard strain ATCC 33591 were 1.25 mg/ml and 2.5 mg/ml respectively.
Conclusion: The tested N-butanol and aqueous sub-fractions of *A. cordifolia* leaf were active against the *S. aureus* strains at low concentrations. The plant can be a possible candidate in the search for alternative antistaphylococcal agents.

Keywords: Antistaphylococcal; multidrug resistance; N-butanol; Alchornea cordifolia.

1. INTRODUCTION

Staphylococcus aureus is a species of bacterium which is human flora of the skin and noses of healthy people. Although it is usually harmless at these sites, it may occasionally get into the body (e. g. through breaks in the skin such as abrasions, cuts, wounds, surgical incisions or indwelling catheters) and cause infections [1, 2]. Infections caused by *S. aureus* range from mild (pimples or boils) to life-threatening ones such as infection of the bloodstream, heart valves, pneumonia, bones or joints.

Methicillin Resistant *S. aureus* (MRSA) is one of the greatly-feared strains of *S. aureus* which have become resistant to most antibiotics. MRSA strains are most often found associated with institutions such as hospitals, but are becoming increasingly prevalent in community-acquired infections. MRSA can cause more severe infections [3].

Alchornea cordifolia (Schumach and Thonn) Müll. Arg. (Euphorbiaceae) is distributed in secondary forests usually near water, moist or marshy places. It grows to a considerable height of about 4 m tall but is always of a shrubby or scrambling habit [4]. The leaf extracts have been reportedly used as alternative remedies in various African countries such as Senegal in the treatment of venereal diseases, conjunctivitis, bronchitis, cough [5, 6]; Zaire in the treatment of urinary tract infection, infected wound, diarrhoea, cough, [7, 8]; in Sierra Leone it was used for diarrhoea [9] and in the Southern Nigeria for gonorrhoea and cough [10, 11]. Some of the bacterial infections mentioned above have been associated with *S. aureus*. Preliminary work has been done in other places like Ghana [12] and Southern Nigeria [13, 14, 15, 16, 17] but the objective of this work is to investigate the antibacterial activity of N-butanol (NSF) and aqueous (ASF) sub-fractions of ethanol extract of *A. cordifolia* leaf found in the Northern Nigeria against multidrug resistant *S. aureus* isolates including MRSA.

2. MATERIALS AND METHODS

2.1 Collection, Identification and Preparation of Plant Leaf

A. cordifolia leaves were collected from Abuja, Nigeria, from February 2010 – June 2010. They were authenticated in the herbarium of the Department of Biological Sciences, Ahmadu Bello University, Zaria, Nigeria, where a voucher with specimen number 401 was kept for future reference. The leaves were washed with distilled water and air-dried under shade and then reduced to powder using mortar and pestle. They were subsequently grounded into fine powder using electric grinder.

2.2 Extraction and Fractionation of the Plant Leaf

Using the Soxhlet extractor 440 g of the powdered leaves were extracted with 1,550 ml of 70% ethanol at room temperature until all the extractable components was exhausted (yield 52%). The extract was concentrated and kept in a dessicator. Fifteen gram (15 g) of the ethanol extract (EE) was partitioned between N-butanol and distilled water resulting in the NSF (yield 49.6%) and the ASF (yield 61.3%). These fractions were evaporated under reduced pressure at a temperature below 50ºC, yielding dry residue, considered as the fractions. The fractions were stored in a dessicator until required.

2.3 Antistaphylococcal Activity

2.3.1 Purification of organisms

The clinical isolates of *S. aureus* were collected from the Department of Pharmaceutics and Pharmaceutical Microbiology, Ahmadu Bello University, Zaria, Nigeria. The staphylococcal isolates were purified and characterized by established microbiological methods. The developed colonies were then streaked on the mannitol salt agar and incubated at 37ºC for 18 hours. Catalase, coagulase and DNAse tests were carried out to confirm the isolates. The bacterial isolates were kept on agar slants at 4ºC until needed.

2.3.2 Preparation of inoculums

Eighteen-hour broth culture of the *S. aureus* was standardized according to Clinical Laboratory Standards Institute [18] by diluting in sterile normal saline until the turbidity matched that of McFarland standard of 0.5 which is approximately 1.5×10^8 CFU/ml for the inoculation of agar plates.

2.3.3 Antibiotic susceptibility testing

The susceptibility pattern of the test isolates to 15 µg erythromycin, 30 µg tetracycline, 30 µg cloxacilin, 10 µg gentamicin, 25 µg cotrimoxazole, 30 µg chloramphenicol, 30 µg amoxillin-clavulanic acid, 25 µg amoxicillin (Abtek) and 30 µg teicoplanin (Oxoid, England) were determined using Kirby-Bauer-CLSI modified disc agar diffusion technique (DAD) [19]. One millilitre (1.0 ml) of standardised overnight culture of each isolate (containing 1.5×10^8 CFU/ml) was used to flood the surface of Mueller-Hinton Agar (MHA) plates and excess drained off and dried while the petri dish lid was in place. The standard antibiotic discs were then aseptically placed at reasonable equidistance on the inoculated MHA plates and allowed to stand for 1 h. The plates (prepared in duplicates for each isolate) were then

incubated at 37ºC for 18 h [20]. The diameter of the zones of inhibition produced by each antibiotic disc was measured and recorded.

2.3.4 Testing for the antistaphylococcal activities of the extract and fractions

Molten sterile MHA (20 ml) was poured into sterile petri dish and allowed to set. The sterile MHA plates were flooded with 1.0 ml of the standardized inoculum and the excess was drained off. A sterile cork borer (No. 4) was used to bore equidistant cups into the agar plate. One drop of the molten agar was used to seal the bottom of the bored hole, so that the test agent will not sip beneath the agar. The EE and the NSF were dissolved in 10% dimethylsulfoxide (DMSO) while the ASF was dissolved in sterile distilled water. 0.1ml of the different concentrations (20.0, 10.0, 5.0, 2.5, 1.25 and 0.625 mg/ml) of the extract/fraction was added to fill the bored holes. Negative control was prepared by putting 0.2 ml of pure solvent (ethanol or n-butanol) in one of bored hole and aqueous solution of Gentamicin in another bored hole which served as positive control. One hour pre-diffusion time was allowed, after which the plates were incubated at 37ºC for 18 h. The zones of inhibition were then measured in millimeter. The above method was carried out in triplicates and the mean of the triplicate results was taken [21].

2.3.5 Minimum Inhibitory Concentration (M. I. C.) and Minimum Bactericidal Concentration (M. B. C.)

Eight tubes of 2.5 ml sterile Mueller Hinton broth were arranged in rows. The first tube contained double strength broth. To the first tube was added 2.5 ml of 20 mg/ml of the extract/fraction and thoroughly but gently mixed, 2.5 ml of the mixture was withdrawn and to the second tube and mixed properly, this dilution was continued serially to the last tube, after mixing, 2.5 ml was withdrawn from the last tube and discarded. Following the above procedure steps, the two-fold dilution row ranging from 20 mg/ml – 0.625 mg/ml was prepared. Two drops of standardised inoculums were added to each tube so that the final concentration of 3.8×10^5 CFU/ml was reached. Three controls were set up to show the sterility of the media, the extract/fractions and to ascertain the growth promoting property of the media. The tubes were incubated at 37ºC for 18 hours. The lowest concentration of the extract/fraction in the test tubes that showed no growth was considered as the M. I. C. of the extract/fraction against the test bacteria. After incubation a loopful from the tubes containing the least concentration of the extract/fraction which prevented growth was streaked on sterile nutrient agar plates containing inactivating agents 3% v/v Tween 80 incubated at 37ºC for 24 hours. The least concentration of the extract/fraction in the test agar plates that showed no growth was considered as the M. B. C. of the extract/fraction against the test bacteria [22].

2.4 Statistical Analysis

Results were expressed as mean ± standard deviation. The data was analysed using Student's t-test. $P < 0.05$ was considered significant and $P > 0.05$ not significant.

3. RESULTS

The EE showed zones of inhibition at the concentrations of 5.0 mg/ml - 20.0 mg/ml (Table 1). The activity of the EE is concentration dependent, the lower the concentration the lower the activity. SA 4 was more susceptible to the extract than MRSA ATCC 33591.

Table 1. Susceptibility of the test isolates to EE of *A. cordifolia* leaf

Test isolates	Zones of inhibition (mm) at following concentrations (mg/ml).					
	20.0	**10.0**	**5.0**	**2.5**	**1.25**	**0.625**
MRSA ATCC 33591	20 ± 1.0	18 ± 0.3	13 ± 0.7	NI	NI	NI
SA 1	22 ± 0.6	17 ± 0.7	14 ± 0.5	NI	NI	NI
SA 2	24 ± 0.0	18 ± 0.5	14 ± 0.1	NI	NI	NI
SA 3	19 ± 0.5	17± 0.3	15 ± 0.4	NI	NI	NI
SA 4	26 ± 0.3	20 ± 0.6	14 ± 1.0	NI	NI	NI
SA 5	16 ± 1.1	14 ± 1.0	12 ± 0.3	NI	NI	NI

SA – Staphylococcus aureus, NI – No Inhibition, The results are expressed as mean ± standard deviation,

The diameter zones of inhibition of NSF ranged between 11.0 mm - 36.5 mm. The least concentration of NSF tested (0.625 mg/ml) inhibited the growth of the test isolates except SA 5. NSF produced larger zones against the test isolates SA 2, SA 3, SA 4 at 0.625 mg/ml – 20.0 mg/ml and SA 5 at 1.25 mg/ml - 20 mg/ml. The fraction inhibited the growth of MRSA ATCC 33591 even at 0.625 mg/ml (Table 2).

Table 2. Susceptibility of the test isolates to NSF of *A. cordifolia* leaf

Test isolates	Zones of inhibition (mm) at following concentrations (mg/ml).					
	20.0	**10.0**	**5.0**	**2.5**	**1.25**	**0.625**
MRSA ATCC 33591	27.5 ± 0.5	24 ± 0.4	20.5 ± 0.7	17.5 ± 0.3	14 ± 1.4	11 ± 0.0
SA 1	26.5 ± 1.0	24.5 ± 1.0	19 ± 1.4	17 ± 0.0	16 ± 0.0	13 ± 0.0
SA 2	28 ± 1.0	26.5 ± 0.4	24 ± 1.4	20 ± 0.7	15 ± 0.2	13 ± 0.2
SA 3	30 ± 0.0	28.5 ± 1.0	25 ± 0.0	17.5 ± 0.5	14 ± 0.0	13 ± 0.0
SA 4	36.5 ± 1.0	33 ±1.4	30 ± 0.0	24 ± 0.4	20 ± 0.0	18 ± 0.4
SA 5	30 ± 0.0	27± 0.0	25 ± 0.5	18 ± 0.0	12 ± 1.4	NI

SA – Staphylococcus aureus, NI – No Inhibition, The results are expressed as mean ± standard deviation

Table 3 showed that the diameter zones of inhibition showed by ASF of test plant leaf were lower than that of the NSF fraction. SA 4 was more susceptible to the aqueous fraction at all the test concentrations while SA 1 was the least susceptible. The MRSA ATCC 33591 was also susceptible at 0.625 mg/ml to the aqueous fraction (Table 3).

Table 4 showed the M. I. C. and M. B. C. values of the EE, NSF and ASF of *A. cordifolia* leaf. The fractions produced lower M. I. C. and M. B. C. values than the extract.

Using the Clinical Laboratory Standard Institute [23] antibiotics zones of inhibition break point for *S. aureus*, the results in Table 5, was interpreted as sensitive, intermediate and resistant. All the test isolates (100%) were resistant to cloxacillin and amoxycillin while all of them (100%) were sensitive to Gentamicin and Cotrimoxazole. Teicoplanin was effective against 80% of the test isolates.

Table 3. Susceptibility of the test isolates to ASF of *A. cordifolia* leaf

Test isolates	Zones of inhibition (mm) at following concentrations (mg/ml).					
	20.0	**10.0**	**5.0**	**2.5**	**1.25**	**0.625**
MRSA ATCC 33591	23 ± 0.2	21 ± 0.1	20 ± 0.0	18 ± 0.0	13 ± 0.0	11 ± 0.0
SA 1	19 ± 0.3	17 ± 0.4	17 ± 0.3	15 ± 0.0	12 ± 0.0	NI
SA 2	25 ± 0.2	23 ± 1.1	22 ± 0.0	18 ± 0.0	15 ± 0.0	NI
SA 3	27 ± 0.3	21± 0.5	18 ± 0.0	15 ± 0.4	12 ± 0.1	NI
SA 4	35± 0.1	32± 1.6	26 ± 1.4	20 ± 1.0	15 ± 0.7	13 ± 0.0
SA 5	27 ± 1.5	25 ± 0.4	20 ± 0.1	15 ± 0.2	12 ± 0.4	NI

SA – Staphylococcus aureus, NI – No Inhibition, The results are expressed as mean ± standard deviation

Table 4. M. I. C. and M. B. C. of EE, NSF and ASF fraction of *A. cordifolia* leaf against test staphylococcal isolates

Test isolates	M. I. C. (mg/ml)			M. B. C. (mg/ml)		
	EE	**NSF**	**ASF**	**EE**	**NSF**	**ASF**
MRSA ATCC 33591	10.0	1.25	2.5	20.0	2.5	5.0
SA 1	5.0	1.25	2.5	10.0	2.5	5.0
SA 2	5.0	1.25	1.25	10.0	2.5	2.5
SA 3	5.0	1.25	2.5	10.0	2.5	5.0
SA 4	5.0	0.625	1.25	10.0	1.25	2.5
SA 5	10.0	2.5	2.5	20.0	2.5	5.0

SA – Staphylococcus aureus, EE – Ethanol Extract, NSF – N-butanol Sub-Fraction, ASF – Aqueous Sub-Fraction

4. DISCUSSION

4.1 Antistaphylococcal Activity of the EE, NSF and ASF

Generally, the EE of *A. cordif*olia leaf and its derived fractions; NSF and ASF, showed antistaphylococcal activity against the entire test *S. aureus* isolates and the type MRSA. This result agreed with that of the work carried out in the Southern Nigeria by Igbeneghu et al., [16] who reported that the aqueous-ethanol extract of *A. cordifolia* leaf possess antistaphylococcal activity. The methanol and ethyl acetate extracts and their derived fractions, of the leaves of *A. cordifolia* found in the Northern Nigeria have been reported to inhibit the growth of *S. aureus* [24, 25]. Also the aqueous extracts of *A. cordifolia* showed the highest levels of antibacterial activity against MRSA according to the work done in Ghana by Pesewu et al., [12]. The antibacterial activity of the extracts of *A. cordifolia* leaf can be due to the presence of chemical constituents such as gallic, elagic acid, protocatechic acid, tannins, saponin, phenolic acids, alkaloids, flavonoids, quercetin, hyperin, guaijaverin and anthocyanidine glycosides [11, 26, 27, 28, 29, 30, 31].

Table 5. Antibiotic susceptibility pattern of the test staphylococcal isolates

Test isolates	Zones of inhibition (mm) and CLSI interpretive criteria								
	ERY	**TET**	**CXC**	**GTM**	**COT**	**CHL**	**AMC**	**AMX**	**TEC**
SA 1	21±0.7(I)	NI (R)	NI(R)	22±0.0(S)	23±1.5(S)	NI (R)	17±1.4(R)	10±0.0(R)	15±0.7(S)
SA 2	22±0.7(I)	NI (R)	NI(R)	22±1.4(S)	23±0.7(S)	NI(R)	19±0.5(R)	11±0.0(R)	16±0.6(S)
SA 3	20±0.0(I)	15±1.0(I)	NI(R)	21±2.0(S)	22±0.0(S)	20±1.5(S)	16±0.6(R)	12±0.0(R)	18±0.7(S)
SA 4	15±0.0(I)	13±1.4(R)	NI(R)	23±0.7(S)	22±1.0(S)	NI(R)	NI(R)	NI(R)	19±2.0(S)
SA 5	NI(R)	NI(R)	NI(R)	23±0.0(S)	22±0.7(S)	20±1.4(S)	19±0.7(R)	12±0.0(R)	13±0.0(I)

ERY – 15 µg Erythromicin, TET – 30 µg Tetracycline, CXC - 30 µg Cloxacilin GTM - 10 µg Gentamicin, COT – 25 µg Cotrimoxazole, CHL – 30 µg Chloramphenicol, AMC – 30 µg Amoxicillin-Clavulanic acid, AMX – 25 µg Amoxicillin, TEC – 30 µg Teicoplanin, SA – Staphylococcus aureus, S – Sensitive, I – Intermediate, R –Resistant.

However, the diameter zones of inhibition observed from the EE was significantly smaller at $P<0.05$ than the zones of inhibition observed from NSF and ASF. This suggests that there is the possibility of the presence of more of the chemical constituents in the fractions and that the substances are in a purer form. Moreover, crude extracts may contain inactive substances which may also antagonize the antibacterial actions of one another [32]. However, on review of the literature there appears to be no such investigations reported on the antistaphylococcal activity of N-butanol and aqueous sub-fractions of the ethanol extract of *A. cordifolia* leaf and therefore, the findings here are the first report of the antistaphylococcal activity of these fractions of the ethanol extract of *A. cordifolia* leaf.

N-butanol fraction was more active than the ASF as a significant difference at $P<0.05$ between the zones of inhibition showed by NSF and the ASF was observed. This means that more of the active substances were concentrated in the NSF than in the ASF. N-butanol fraction of the aqueous extract of *Solidago chilensis* was reported to be more active against *S. aureus* than the aqueous fraction of the same extract [33]. Ozcelik et al. [34] also reported the antibacterial activity of the N-butanol fraction of *Cirsium hypoleucum* against *S. aureus* to be higher than that of the aqueous fraction of the plant.

The sub-fractions of the EE of *A. cordifolia* leaf showed high antistaphylococcal activity with M. I. C. and M. B. C. values in the range of 0.625 mg/ml – 2.5 mg/ml and 1.25 mg/ml – 5.0 mg/ml against the tested *S. aureus* isolates and MRSA respectively. The antibacterial activity of these sub-fractions from the EE is different from the results of Pesewu et al. [12] and Igbeneghu et al. [16] who reported that aqueous extracts of *A. cordifolia* showed the highest levels of antibacterial activity overall with M. I. C's against MRSA in the range of 1.6 - 3.1 mg/ml and M. B. C's in the range of 6.3 - 12.5 mg/ml and that aqueous-ethanol extract of *A. cordifolia* leaf showed M. I. C. values of 3.5 mg/ml – 12.6 mg/ml against tested *S. aureus* isolates respectively. The low M. I. C. and M. B. C. values of the fractions especially the NSF confirm high activity of the fractions at low concentrations. High activity of antimicrobial agent at low concentration is very essential for chemotherapeutic purposes because of their toxicity to the patient's system [25].

The antibacterial susceptibility showed by the test *S. aureus* isolates including MRSA to NSF and ASF, is encouraging due to the fact that one of the biggest challenges in treating staphylococcal infections is that many strains of the *S. aureus* bacteria have developed resistance against a number of different known antibiotics including the newer ones and are increasingly becoming more difficult to treat so there is the need for search for new antibiotic probably from plant. The first-line treatment for serious invasive infections due to MRSA is currently glycopeptide antibiotics (vancomycin and teicoplanin). Vancomycin Resistant *S. aureus* (VRSA) is a strain of *S. aureus* that has become resistant to the glycopeptides. The first case of *S. aureus* truly resistant to glycopeptide antibiotics was reported in 2002 [35]. It has also been reported that few MRSA resistant to vancomycin and/or teicoplanin have been found in the USA and although there is concern that they may become more common [3].

On comparing the activity of the NSF and ASF of *A. cordifolia* with those of the standard antibiotics especially Gentamicin and Cotrimoxazole which were active against the test isolates, there is the possibility of using these plant fractions as anti staphylococcal agents. Patel et al. [36] reported that about 75% of the populations of Africa still rely on traditional healing practices and medicinal plants for their daily health care needs. Over the years, the World Health Organization [37] advocated that countries should interact with traditional medicine with a view to identifying and exploiting aspects that provide safe and effective remedies for ailments of both microbial and non-microbial origins [38]. The availability of

plants and that they are inexpensive are also advantages that can be exploited. The acute toxicity studies which revealed the intraperitoneal LD_{50} of 1131.4 mg/kg and 800 mg/kg of the ethanol extract of *A. cordifolia* leaves in mice, which fall within the practically non-toxic range according to the classification by Loomis [39] have been reported by Osadebe and Okoye [40] and Igbeneghu et al. [16] respectively. This indicates that the test sub-fractions especially the NSF of the EE of A. *cordifolia* leaves could be safe for potential alternative treatment of staphylococcal infections.

5. CONCLUSION

Ethanol extract and its derived sub-fractions, NSF and ASF, showed antibacterial activity against multidrug resistant *S. aureus* including MRSA. The traditional use of *A. cordifolia* leaf found in the Northern Nigeria in the treatment of staphylococcus related infections has been justified by this study. The result presented in this work indicates that further isolation of active compound from the NSF and ASF which can be used as effective anti-staphylococcal products in future should be carried out.

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

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