EFFECT OF HEAT ON THE ANTIMICROBIAL ACTIVITY OF *ALCHORNEA CORDIFOLIA* LEAF EXTRACTS.

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Abstract

Microbial load of the water, methanol, and ethyl acetate extracts of the leaf of Alchornea cordifolia was determined using standard microbiological methods. The water, methanol, ethyl acetate extracts of the leaf of Alchornea cordifolia were subjected to different forms of heat; dry heat, moist heat and sunlight. The leaf was also extracted using hot water- decoction. The antimicrobial activities of the extracts were then investigated against clinical isolates of Pseudomonas aeruginosa, Staphylococcus aureus, Escherichia coli, Candida albicans and the type organisms; Ps. aeruginosa ATCC10145, Staph. aureus ATCC 12600, E.coli ATCC 11775 and C.albicans ATCC 18804 using agar diffusion and broth dilution methods. The microbial load of the fresh leaf extracts ranged between 3.6 x 10^3 and 6.0 x 10^4 cfu/ml. The antimicrobial activity of the extracts subjected to moist heat increased more against the organisms especially Staph. aureus and no difference was observed against E.coli when compared with the extracts not subjected to any form of heat. There was a decrease in antimicrobial activity of the extracts exposed to sunlight and no antimicrobial activity was observed in the extracts subjected to dry heat. This study therefore provides a scientific basis for the traditional use of moist heat in the extraction of Alchornea cordifolia leaf.

Key words: Heat, Antimicrobial activity, *Alchornea cordifolia*.

INTRODUCTION

Herbal medicines have been the basis for many therapeutic drugs. Due to their ready availability and the fact that they are traditionally and relatively inexpensive, their cultivation, harvest and processing which are less costly and environmentally friendly, herbal preparations are still the first line of treatment for many of the world's population [1]. The main sources of medicine consisted of dry preparations obtained primarily from plants and vegetable. Natural products as crude plant extracts have been use for thousands of years and many of these ancient formulations have been recorded in ancient literatures [2]. Temperature is one of the major factors which can affect decomposition of herbal preparations. Increase in temperature leads to increase in the rate of chemical reaction in all active products. Deterioration of some thermolabile products can occur as a result of the increase in the rate of chemical reaction since they can only withstand certain range of temperature [3].

Alchornea cordifolia (Schum & Thonn.) Muel. Arg. belongs to the family Euphorbiaceae. It grows to a considerable height but always in a shrubby or scrambling habit. It is geographically distributed in secondary forests usually near water, moist or marshy places. It is called in Nigeria Ewe ipa by the Yorubas, Bambami (Hausa), Ububa (Ibo), Ukpaoromi (Ijaw), Uwonwen (Benin) and Mbom (Efik). It is known as Bulora in Senegal, Mand-Hira in Gambia, Bolontai in Sierra-Leone and Ayraba in Ghana. The plant has been used in traditional medicine to treat various ailments including microbial infections such as cough, diarrhea, skin ulcers, jaundice and malaria [4, 5, 6,]. The plant leaf is usually boiled by the traditional practitioners before dispensing out to patients, therefore there is need to investigate the effect of various types of heat on the antimicrobial activity of this plant leaf extracts.

MATERIALS AND METHODS

Collection of plant material:

The plant was collected from the side of a flowing stream in Idu-Abuja, Nigeria. The collection was made in October. The plant was identified in the herbarium of the National Institute for Pharmaceutical Research and Development, Idu-Abuja, Nigeria. A deposit of the plant specimen with the number NIPRD herbarium 4334 is in the institute for future reference.

Extraction of plant material

The leaves of *Alchornea cordifolia* (Muell. Arg.) were air dried and then reduced to coarse powder using wooden mortar and pestle. The water extract (WE) was prepared by macerating 300 gms of the leaf powder in 2 litres of water and left for 24 hours. The extract was filtered and the filtrate was freeze-dried. One hundred gram (100 g) of the fresh leaves were collected and boiled with distilled water in an aluminium pot over hot plate at 100^{o}C and cooled. This was repeated for three consecutive days in the morning and evening using 500ml of water. The leaf residue was removed and the extracts evaporated to dryness. Using soxhlet extractor, 1.0 kg of the powdered leaf were extracted successively and exhaustively each time with each of the various solvents starting from the less polar solvent to the more polar solvent. The solvents used were hexane, ethyl acetate and methanol respectively. After each extraction, the extract was concentrated, dried and weighed [7].

Determination of the microbial load of the extracts:

A 10%$^{w}/_{v}$ of each of the extracts was prepared with sterile normal saline. One milliliter (1 ml) of the solution was withdrawn into a sterile test tube containing 9 ml sterile normal saline, the mixture was properly agitated and 1 ml was withdrawn into another test tube containing 9 ml normal saline. This procedure was continued to obtain the following dilution, 1: 10, 1:100, 1:10,000 and 1:1,000,000. From the dilution, 0.1 ml was spread over the surface of already prepared sterile nutrient agar plate by the use of sterile glass spreader. A pre-diffusion time of 1 hour was allowed after which the plates were incubated at 37oC for 24 hours. Each dilution was plated out in duplicate. The colonies were counted and the number of viable organisms per ml of the extracts was calculated.

Determination of the effect of heat:

Part of the extracts, except the hot water extract, was placed in hot air oven for 30mins at 100^{o}C, while some were autoclaved at 121^{o}C for 15mins. Some of the extracts were kept in transparent bottles on glass window (for sunlight) for six months.

Susceptibility testing:

Overnight broth cultures were diluted appropriately using McFarland scale (0.5 McFarland which is about10^{6} cfu/ml). The molten sterile nutrient agar (20ml) was poured into sterile petri dish and allowed to set. The sterile nutrient agar 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 about six 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 extract will not sip beneath the agar. 0.1ml of the different concentrations (20 mg/ml – 1.25 mg/ml) of the extracts was added to fill the bored holes. One hour pre-diffusion time was allowed, after which the plates were incubated at 37^{o}C for 18 hours. The zones of inhibition were then measured in millimeter. Control plates were prepared and incubated appropriately.

Determination of the Minimum Inhibitory Concentrations (M.I.C.) and Minimum Bactericidal Concentrations (M.B.C) of the Extracts:

Eight tubes of 2.5 ml nutrient broth were arranged in rows. The first tube contained double strength broth. To the first was added 2.5 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. Two drops of standardized inoculum was added to the each tube. 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 37^{o}C 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 prevent growth was streaked on sterile nutrient agar plates containing inactivating agents 3% v/v Tween 80 incubated at 37^{o}C 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 [8].

RESULTS

The result showed the presence of both gram positive rods and cocci as bacterial contaminant in the water, methanol and ethyl acetate extracts.

The level of contamination was between 6.0 x 10^4 and 3.6 x10^3 cfu/ml. The extracts subjected to moist heat had increase antimicrobial activity against the test organisms when compared with the fresh extracts. The increase was more pronounced against *Staphylococcus aureus*. Decrease in antimicrobial activity was observed in the extracts exposed to sunlight. The extracts subjected to hot air oven showed no antimicrobial activity. There was no significant difference between the antimicrobial activity of the fresh extracts, the autoclaved extracts and the hot water extracts at P>0.05. While there was significant difference between the fresh extracts and the extracts exposed to sunlight at P< 0.05 (Tables 1 – 4).

Table 1: The susceptibility of the organisms to the different concentrations (mg/ml) of fresh extracts.

Test Organisms	Zones of Inhibition (mm)														
	Water Extract					Methanol Extract					Ethyl Acetate Extract				
	20	10	5	2.5	1.25	20	10	5	2.5	1.25	20	10	5	2.5	1.25
Ps. aeruginosa	26± 0.5	23± 0.5	20± 0.0	17± 0.5	13± 0.0	30± 0.0	28± 0.5	23± 0.5	22± 0.5	20± 0.1	30± 1.1	29± 0.2	22± 0.5	18± 0.0	12± 0.0
Ps. aeruginosa ATCC 10145	15± 0.0	11± 0.0	00	00	00	22± 1.1	18± 0.1	15± 0.1	00	00	23± 0.4	20± 0.5	19± 0.0	13± 0.0	00
Staph. aureus	26± 0.0	24± 0.5	22± 0.1	18± 0.0	12± 0.1	28± 0.2	25± 0.2	21± 0.5	15± 1.1	13± 0.4	33± 0.0	30± 0.0	25± 0.4	18± 0.1	15± 0.1
Staph. aureus ATCC 12600	25± 0.2	21± 0.5	19± 0.1	16± 0.2	13± 0.5	25± 0.0	22± 0.2	19± 0.0	15± 0.5	13± 0.0	26± 0.2	23± 0.2	19± 0.2	16± 0.1	10± 0.5
E. coli	12± 0.5	00	00	00	00	15± 0.3	12± 0.5	00	00	00	30± 0.2	28± 0.2	24± 0.0	19± 0.2	17± 0.1
E.coli ATCC 11775	23± 0.0	18± 0.0	16± 0.5	00	00	27± 0.5	21± 0.1	15± 1.1	00	00	22± 0.0	20± 0.5	19± 0.2	16± 0.1	10± 0.2
C. albicans	13± 0.1	11± 0.2	00	00	00	12± 1.1	11± 0.0	10± 0.1	00	00	15± 0.4	13± 0.1	11± 0.2	00	00
C. albicans ATCC 18804	16± 0.5	14± 0.0	00	00	00	20± 0.4	17± 0.3	15± 0.3	12± 0.4	00	20± 0.1	18± 0.0	13± 0.0	10± 0.1	00

The results are expressed as mean ± standard deviation

Table 2: The susceptibility of the organisms to the hot water extracts (decoction).

Test Organisms	Zones of Inhibition (mm)				
	20 mg/ml	10 mg/ml	5mg/ml	2.5 mg/ml	1.25mg/ml
Ps. aeruginosa	28± 0.2	25±0.2	24±1.1	21±0.2	16±1.1
Ps. aeruginosa ATCC 10145	18± 0.5	14±0.1	11±0.0	00	00
Staph. aureus	30±0.1	27±0.0	25±0.1	20±0.5	18±0.0
Staph. aureus ATCC 12600	29±0.0	25±0.5	23±0.2	20±0.4	16±0.2
E. coli	15±0.0	10±0.0	00	00	00
E.coli ATCC 11775	24±0.2	19±0.1	17±0.0	00	00
C. albicans	16±0.5	15±0.0	00	00	00
C. albicans ATCC 18804	19±0.0	15±0.0	00	00	00

The results are expressed as mean ± standard deviation

Table 3:The susceptibility of the organisms to the different concentrations (mg/ml) of autoclaved extracts.

Test Organisms	Zones of Inhibition (mm)														
	Water Extract					Methanol Extract					Ethyl Acetate Extract				
	20	10	5	2.5	1.25	20	10	5	2.5	1.25	20	10	5	2.5	1.25
Ps. aeruginosa	27± 0.0	25± 0.0	22± 0.2	19± 0.0	14± 0.1	31± 0.5	26± 0.2	24± 0.0	24± 0.0	20± 0.1	31± 0.0	30± 0.1	24± 0.0	20± 0.2	13± 0.5
Ps. aeruginosa ATCC 10145	18± 0.2	13± 0.2	10± 0.1	00	00	24± 0.2	17± 0.0	17± 0.1	12± 0.4	00	25± 0.0	22± 0.0	20± 0.0	15± 0.2	11± 1.1
Staph. aureus	30± 0.5	28± 0.5	26± 0.2	22± 0.5	15± 0.5	31± 0.2	29± 0.4	24± 1.0	19± 0.2	15± 0.2	36± 0.2	34± 0.1	30± 0.0	22± 1.1	19± 0.2
Staph. aureus ATCC 12600	28± 0.2	25± 0.1	23± 0.5	19± 0.5	15± 0.0	29± 0.2	26± 0.1	22± 0.3	19± 0.4	14± 0.1	31± 0.0	28± 0.0	23± 0.4	20± 0.2	13± 0.3
E. coli	13± 0.1	00	00	00	00	15± 0.0	12± 0.5	00	00	00	30± 0.2	28± 0.0	24± 0.1	20± 0.4	17± 0.0
E.coli ATCC 11775	23± 0.0	18± 1.1	17± 0.1	00	00	26± 0.1	21± 0.0	16± 0.0	00	00	23± 0.5	20± 0.5	19± 0.0	16± 0.2	10± 0.2
C. albicans	14± 0.0	13± 0.5	00	00	00	14± 0.2	11± 0.3	10± 0.5	00	00	17± 0.2	15± 0.4	13± 0.3	00	00
C. albicans ATCC 18804	17± 0.0	15± 0.2	00	00	00	22± 1.0	16± 0.2	15± 0.1	11± 0.0	00	21± 0.5	20± 0.0	15± 0.2	12± 0.0	00

The results are expressed as mean ± standard deviation

Table 4: The susceptibility of the organisms to the different concentrations (mg/ml) of extracts exposed to sunlight.

Test Organisms	Zones of Inhibition (mm)														
	Water Extract					Methanol Extract					Ethyl Acetate Extract				
	20	10	5	2.5	1.25	20	10	5	2.5	1.25	20	10	5	2.5	1.25
Ps. aeruginosa	16± 0.5	15± 0.1	12± 0.0	00	00	20± 0.0	19± 0.1	18± 0.5	12± 0.5	00	22± 0.0	17± 0.0	16± 0.4	13± 0.5	11± 0.2
Ps. aeruginosa ATCC 10145	11± 0.0	10± 0.0	00	00	00	16± 0.3	13± 0.1	11± 0.0	00	00	15± 0.4	14± 0.5	12± 0.2	00	00
Staph. aureus	18± 0.5	17± 0.5	15± 0.5	12± 0.2	00	21± 0.2	16± 0.0	13± 0.2	00	00	25± 0.2	20± 0.1	14± 0.5	12± 0.2	11± 0.0
Staph. aureus ATCC 12600	18± 0.2	15± 0.4	13± 0.2	00	00	19± 0.0	17± 0.0	10± 0.0	00	00	19± 0.1	15± 0.0	13± 0.4	11± 0.0	00
E. coli	10± 0.0	00	00	00	00	12± 0.0	10± 0.1	00	00	00	21± 0.2	18± 0.5	16± 0.0	13± 0.4	10± 0.0
E.coli ATCC 11775	14± 0.1	12± 0.0	10± 0.0	00	00	20± 0.4	14± 0.1	10± 0.0	00	00	15± 0.4	13± 0.4	11± 0.2	00	00
C. albicans	11± 0.0	00	00	00	00	10± 0.0	00	00	00	00	12± 0.4	11± 0.1	00	00	00
C. albicans ATCC 18804	12± 0.0	10± 0.0	00	00	00	15± 0.2	12± 0.2	10± 0.2	00	00	17± 0.2	14± 0.2	11± 0.1	00	00

The results are expressed as mean ± standard deviation

The Minimum Inhibitory Concentration (M. I. C) values of the extracts ranged between 20 mg/ml - 0.625 mg/ml while the values of Minimum Bactericidal/Fungicidal Concentration was 20 mg/ml – 1.25 mg/ml (Tables 5 – 6).

Table 5: The M.I.C. of the fresh extracts and extracts subjected to various types of heat

Test Organisms	M.I.C. (mg/ml)											
	Fresh Extracts			Autoclaved Extracts			Hot water Extracts			Extracts exposed to Sunlight		
	WE	ME	EAE	WE	ME	EAE	WE	ME	EAE	WE	ME	EAE
Ps. aeruginosa	5	5	1.25	2.5	5	1.25	2.5	5	1.25	NA	20	20
Ps. aeruginosa ATCC 10145	10	5	2.5	5	5	2.5	5	5	2.5	NA	20	20
Staph. aureus	1.25	1.25	0.625	1.25	0.625	0.625	1.25	1.25	0.625	20	20	10
Staph. aureus ATCC 12600	5	5	1.25	2.5	1.25	1.25	2.5	2.5	1.25	20	20	20
E. coli	20	20	1.25	20	20	1.25	20	10	1.25	NA	NA	NA
E. coli ATCC 11775	5	5	1.25	5	5	1.25	5	5	1.25	20	20	20
C. albicans	20	20	5	10	10	2.5	10	10	5	NA	NA	NA
C. albicans ATCC18804	10	10	5	5	5	2.5	5	5	2.5	NA	NA	20

Key: NA = No activity

WE = Water Extract ME = Methanol Extract EAE = Ethyl Acetate Extract

Table 6: The M.B.C. of the fresh extracts and extracts subjected to various types of heat

Test Organisms	M.B.C. (mg/ml)											
	Fresh Extracts			Autoclaved Extracts			Hot water Extracts			Extracts exposed to Sunlight		
	WE	ME	EAE	WE	ME	EAE	WE	ME	EAE	WE	ME	EAE
Ps. aeruginosa	10	10	2.5	5	10	2.5	5	10	2.5	NA	NA	NA
Ps. aeruginosa ATCC 10145	20	10	5	10	10	5	10	10	5	NA	NA	NA
Staph. aureus	2.5	2.5	1.25	2.5	1.25	1.25	2.5	2.5	1.25	NA	NA	20
Staph. aureus ATCC 12600	10	10	2.5	5	2.5	2.5	5	5	2.5	NA	NA	20
E. coli	20	20	1.25	NA	NA	2.5	NA	20	2.5	NA	NA	NA
E. coli ATCC 11775	10	10	2.5	10	10	2.5	10	10	2.5	NA	20	20
C. albicans	NA	NA	10	20	20	5	20	20	10	NA	NA	NA
C. albicans ATCC18804	20	20	10	10	10	5	10	10	5	NA	NA	NA

Key: NA = No activity

WE = Water Extract ME = Methanol Extract EAE = Ethyl Acetate Extract

DISCUSSION

The results of the antimicrobial activity of the extracts showed that the extracts subjected to autoclave were more active than the fresh water, methanol and ethyl acetate extracts of *Alchornea cordifolia. Bacillus* sp. and *Staphylococcus aureus* were isolated from the fresh extracts. Microorganisms can produce functionally active protein and enzymes which can be responsible for inactivating some of the antimicrobial principles of the plant or involve in conformational changes that will prove to be protective against the attacking antimicrobial constituents. Therefore it is possible that the use of autoclave eradicated these bacteria, thus enhanced the antimicrobial activity of the extracts. The decoction extracts of *A. cordifolia* also exhibited high antimicrobial activity against the organisms. This result is similar to the findings of Bolaji *et al*., [9]; Doughari *et al*., [10]; El-Mahmood *et al*., [11];

El-Mahmood, [12], who reported that decoction of different plant extracts showed high antimicrobial activity. This finding lends credence to the practice of the herbalist of making decoction of the plant for use. The hot water extracts of *A.cordifolia* have been reportedly used for many infections such as veneral disease, conjunctivitis, dermatoses and gonorrhoea [13, 14]**.** Hot water has the ability to extract more active constituents from plants than cold water due to the heat that is involved. The high antimicrobial activity expressed by the hot water extract can also be due to the presence of metallic ions in the distilled water which are known to form complexes with macro-molecules such as saponins and tannins, thereby reducing their bioavailability and hence activity [9]. The antimicrobial activities of the extracts exposed to sunlight against the test organisms reduced drastically when compared with the activity of the fresh extracts. Natural sunlight lies in the wavelength range 290 – 780 nm of which only the higher energy (U.V.) range (290 – 320 nm) cause photodegradation [15]. *Alchornea cordifolia* leaf extract was found to contain tannins, saponins, flavonoids, alkaloids, phenols, glycosides [16]. Many of these compounds fall into phenolic group and phenols have been reported to undergo oxidation upon exposure to light [3]. Conjugated double bonds present in the compound can combine with free radicals produced by sunlight to also cause degradation.

Dry heat completely destroyed the antimicrobial activity of the plant extracts. The use of dry heat is destructive and this lead to the complete loss of the antimicrobial activity. In conclusion, moist heat increase the antimicrobial activity of the water, methanol and ethyl acetate extracts against the test organisms. Heat generated from sunlight reduced the antimicrobial activity of the extracts. The heat from dry air oven completely destroyed the antimicrobial activity of the plant extracts. Therefore, it can be suggested that extracts could be subjected to moist heat either by autoclave or boiling, before use for the treatment of infection especially staphylococcal infections, as these methods can take care of extracting more active ingredients and also sterilize the extracts.

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