JASEM ISSN 1119-8362

Full-text Available Online at www.ajol.info and www.bioline.org.br/ja

J. Appl. Sci. Environ. Manage. *March*, 2015
Vol. 19 (1) 303 - 308

In vitro assessment of the antimicrobial activities of leaf and stem extracts of *Alchornea cordifolia*

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***KEY WORDS*:** *Alchornea cordifolia*, plant extracts, antimicrobial, bacteria, fungi.

ABSTRACT: *In vitro* assessment of the antimicrobial effect of leaf and stem extracts of a commonly used plant – *Alchornea cordifolia* was carried out on 10 bacteria and 5 fungi. Five solvents– chloroform, methanol, ethanol, petroleum ether and water were used for extraction. Chloroform extract from leaf and stem resulted in the highest zone of inhibition against the test bacteria (33 – 56mm). Ethanol extracts showed inhibition zone ranging between 10 – 14mm; methanol extract showed inhibition zone of 13 – 21mm only for the leaf extract. Petroleum ether extract of only the stem resulted in inhibition of the test bacteria (10 – 15mm). Leaf and stem water extracts did not result in any zone of inhibition. Only chloroform extracts from both leaf and stem resulted in fungal growth inhibition ranging from 22 – 60mm (leaf) and 14 – 40mm (stem). *Staphylococcus haemolyticus* was most sensitive to leaf chloroform extracts (50mm), followed by *E. coli* (49mm); while *Samonella* sp. was the most sensitive to stem extracts (56mm). *Rhizopus sp*. was the most sensitive to both chloroform leaf extracts (60mm) and stem extracts (40mm) out of the 5 fungi screened. The highest minimum inhibition concentration (MIC) of 0.125mg/ml was obtained from the leaf chloroform extract on *Pseudomonas sp.* The implication of the findings was discussed.

http://dx.doi.org/10.4314/jasem.v19i2.18

Introduction

The global need for alternative prevention and treatment options and products for diseases that are safe, effective and economical comes from the rise in disease incidence (Tichy and Novak, 1998). Despite several chemical therapeutic agents being commercially available, these chemicals can alter oral microflora and have undesirable side-effects such as vomiting, diarrhea, tooth staining (Badria and Zidan, 2004) and some have been said to be carcinogenic. Hence, the search for alternative products continues and natural phytochemicals isolated from plants used in traditional medicine are considered as good alternatives to synthetic chemicals (Prabu *et al*., 2006). Many substances synthesized by plants have been reported to be useful for the maintenance of health in humans and animals (Lai and Roy, 2004). Many of these substances are secondary metabolites of which at least 12,000 of them have been isolated from different parts of plants including *Alchornea cordifolia*.

A.cordifolia is used in the treatment of bacterial, fungal, parasitic and inflammatory disorders (Mavar-Manga *et al*., 2004). The parts mostly used for medicine are the leaves, stem and roots (Odugbemi, 2006). The roots are widely used externally and internally to treat different ailments such as fever, rheumatism, tooth-ache, cough and sore (Odugbemi, 2006). *A. cordifolia* is used locally by the people of Wilberforce Island, Nigeria to stop bleeding from wounds when the leaves are squeezed and placed on the wound (Odugbemi, 2006). A mixture of the leaves and native chalk is used to stop miscarriage (Macfoy and Sama, 1990). The twig stops stomach ache and diarrhea when chewed while leaf extracts are used to treat athletes' foot (Macfoy and Sama, 1990).

Secondary metabolites which constitute an important source of pharmaceutical drugs have been reported to be present in the leaves of *Alchornea cordifolia* (Osadebe and Okoye, 2003). In this report we investigated the antimicrobial effects of leaf and stem extracts of *Alchornea cordifolia* on some microorganisms associated with common diseases in the tropics.

MATERIALS AND METHODS

Sample collection: Fresh leaves and barks of *Alchornea cordifolia* were collected from the Bowen University campus, Iwo, Osun State.

Extraction of plant materials: The fresh leaves collected were sun dried for three weeks and pulverised using a blender. The extracting solvents were chloroform, ethanol, methanol, petroleum ether and sterile distilled water. Forty grams of the powdered mass were soaked in 180ml of each solvent in a tightly closed container under aseptic conditions for 5 days. The filtrate was collected and a water bath was used to expel the solvents so that the extract appeared pasty (Owoseni and Ogunnusi, 2006).

Test Organisms: Ten bacteria and five fungi were used as test organisms. These were 4 Gram positive bacteria which included *Staphylococcus aureus*, *Bacillus* sp., *Staphylococcus haemolyticus* and *Micrococcus* sp. and 6 Gram negative bacteria which included *Escherichia coli*, *Pseudomonas* sp., *Proteus* sp., *Citrobacter* sp., *Klebsiella* sp., and *Salmonella* sp. The fungi tested were *Candida* sp., *Aspergillus niger*, *Aspergillus flavus*, *Rhizopus* sp. and *Penicillium* sp. These microorganisms were obtained from the microbial collections of the Microbiology unit, Department of Biological Sciences, Bowen University, Iwo, Nigeria. The bacteria isolates were sub-cultured on nutrient agar slants and incubated at 37°C for 24 hours while the fungal isolates were sub-cultured on potato dextrose agar and incubated at 25°C for 72 hours.

Test for antibacterial activity of extracts: The antibacterial assay was carried out using the agar well diffusion method (Irobi *et al*., 1996). The inoculum suspensions at 0.5Mcfarland ($10^5 – 10^8$ cells/ml) were tested against the effect of the crude extract at the concentration of 1mg/ml. Sterile cotton swabs were used to transfer inoculums from the standardized suspensions to the entire surface of Mueller-Hinton agar. A 5mm cork borer was used to bore wells on the already inoculated agar by pushing the cork borer into the agar and removing the agar plugs (Owoseni and Ajayi, 2010). The different extracts were dropped into the wells to fullness and properly labelled. The preparations were left to diffuse for 1 hour on the work bench and the plates were then incubated at 37°C for 24 hours without inverting them. The zones of inhibition around the wells were measured in millimeters. Antibiotic sensitivity test kits were used as control. All experiments were carried out in duplicate.

Test for anti-fungal activity of extracts: The fungal isolates were grown on Sabouraud dextrose agar at 25°C until they produced spores. The fungal spores were harvested by pouring sterile distilled water on the surface of the plate. The water was then poured into a sterile Petri dish. Spore suspension (1ml) was evenly spread on the surface of Sabouraud dextrose agar (SDA) plate using sterile glass. The plate was left for some minutes in order to allow fungal spores to penetrate into the agar. Wells were bored into the agar media using sterile 5mm cork borer. The different extracts were dropped into the wells to fullness at the concentration of 1mg/ml. The plates were left on the work bench for 1 hour in order to allow for the diffusion of the extracts into the agar. The plates were incubated for 48 hours at 25°C without inversion. The zones of inhibition were measured in millimeters. The effect of the extracts on the fungal isolates was compared with miconazole at a concentration of 25mg/ml (Owoseni *et al*., 2010). All experiments were carried out in duplicate.

Determination of Minimum Inhibitory Concentration (MIC) and Minimum Bactericidal Concentration (MBC): The MIC and *MBC* of the chloroform extract against different pathogenic bacteria were determined by the tube-dilution method. This was determined by adding 1ml of 1, 0.5, 0.25, 0.125, and 0.0625 mg/ml of the active chloroform extract into test tubes containing 9ml sterile nutrient broth. The organisms that showed susceptibility to the different solvent extracts were introduced into the broths containing different concentrations of each extract. The tubes were then incubated for 24 hours at 37°C. The MIC was taken as the lowest concentration of the extracts that did not permit any visible growth (CLSI, 2012). The tubes that showed no turbidity in the MIC test were taken and a loop-full from each tube was streaked on Mueller Hinton agar. The plates were incubated for 24 hours at 37°C and the absence/presence of growth was observed. The lowest concentration of the extracts that showed no growth was recorded as the MBC (CLSI, 2012).

Control experiments: The Standard antibiotic chloramphenicol was used as a positive control. Discs impregnated with 30µg chloramphenicol (Fondoz laboratories) was placed on inoculated Mueller-Hinton agar plates and incubated for 24 hours at 37°C. Zones of inhibition were measured in millimetres. (Olajuyigbe and Awoniyi, 2005). Miconazole (25mg/ml) was used as a positive control for the fungal isolates. The extracting solvents, chloroform, petroleum ether, methanol, ethanol and water were used as negative control.

RESULTS AND DISCUSSION

The antibacterial effect of each solvent extract of the leaf is shown in Table 1. The chloroform extract was the most active of all the extracts tested. The highest zone of inhibition was 50mm against *Staphylococcus haemolyticus*, 49mm against *E.coli* and the lowest was 33mm against *Proteus* sp. The ethanol leaf extract gave a zone of inhibition of 14mm against *Staphylococcus aureus*, followed by 12mm against *Bacillus* sp. and 10mm against *Klebsiella* sp. The methanol leaf extract was also active with the highest zone of inhibition being 21mm against the Gram positive bacterium *Staphylococcus aureus. Klebsiella* sp. and *Bacillus* sp. had a zone of inhibition of 13mm each. The distilled water and petroleum ether leaf extract were not active against any of the test bacteria.

Table 1: *In vitro* antibacterial activity of the extracts of *Alchornea cordifolia* leaves.

Test bacteria	**Zone of inhibition (mm)**					
	Chloroform	Methanol	Ethanol	Water	Petroleum ether	Chloramphenicol (30µg)
***Bacillus* sp**	45	13	12	-	-	17
S. aureus	41	21	14	-	-	18
S. heamolyticus	50	-	-	-	-	25
***Micrococcus* sp**	47	-	-	-	-	24
***Salmonella* sp**	42	-	-	-	-	13
***Klebsiella* sp**	35	13	10	-	-	15
***Pseudomonas* sp**	37	-	-	-	-	-
***Proteus* sp**	33	-	-	-	-	10
E. coli	49	-	-	-	-	15
***Citrobacter* sp**	36	-	-	-	-	24

Table 2 shows the results of the antibacterial activity of *Alchornea cordifolia* stem extracts on test microorganisms. The test bacteria were totally resistant to the distilled water and methanol extracts. The ethanol extract was only active against *Staphylococcus aureus* 12mm. The petroleum ether extract was active on *Micrococcus* sp 15mm and *Proteus* sp 10mm. The chloroform extract was most active with the highest zone of inhibition of 56mm against *Salmonella* sp. The lowest was 35mm against *Citrobacter* sp. Tables 1 and 2 shows the antibiotic activity of 30µg chloramphenicol on the test bacteria. Chloramphenicol was used as the positive control and zone of inhibition was measured in millimetres. *Staphylococcus heamolyticus* was the most susceptible with the diameter of zone of inhibition of 25mm while *Pseudomonas* sp. was resistant to the antibiotic. The extracting solvents were used as negative control.

Table 2: *In vitro* antibacterial activity of the extracts of *Alchornea cordifolia* stem.

Test bacteria	**Zone of inhibition (mm)**					
	Chloroform	Methanol	ethanol	Water	Petroleum ether	Chloramphenicol (30µg)
***Bacillus* sp**	37	-	-	-	-	17
S. aureus	52	-	12	-	-	18
S. heamolyticus	52	-	-	-	-	25
***Micrococcus* sp**	51	-	-	-	15	24
***Salmonella* sp**	56	-	-	-	-	13
***Klebsiella* sp**	40	-	-	-	-	15
***Pseudomonas* sp**	38	-	-	-	-	-
***Proteus* sp**	45	-	-	-	10	10
E. coli	46	-	-	-	-	15
***Citrobacter* sp**	35	-	-	-	-	24

Tables 3 and 4 show the antifungal activity of the leaf and stem extracts of *A. cordifolia.* The chloroform extracts of *A. cordifolia* leaf was the only one that showed anti-fungal properties against the five test fungi. The chloroform leaf extract showed the highest zone of inhibition of 60mm against *Rhizopus* sp and a minimum zone of inhibition of 22mm against *Penicillium* sp. while methanol, ethanol, distilled water, petroleum ether leaf extract were not active against the test fungi. The chloroform stem extract had the highest zone of inhibition of 40mm against *Rhizopus* sp. and a lowest zone of inhibition of 14mm against *Penicillium* sp. while methanol, ethanol, and distilled water stem extract were not active against the test fungi. Petroleum ether stem extract had zone of 30mm against *Candida* sp.

JASEM ISSN 1119-8362

Full-text Available Online at www.ajol.info and www.bioline.org.br/ja

J. Appl. Sci. Environ. Manage. *March*, 2015
Vol. 19 (1) 303 - 308

Table 3: *In vitro* antifungal activity of the extracts of *Alchornea cordifolia* leaf

Test Fungi	Zone of inhibition (mm)					
	Chloroform	Methanol	Ethanol	Water	Petroleum ether	Miconazole
Rhizopus sp.	60	-	-	-	-	15
flavus	30	-	-	-	-	14
niger	45	-	-	-	-	16
Candida sp.	38	-	-	-	-	15
Penicillium sp.	22	-	-	-	-	15

Table 4: *In vitro* antifungal activity of the extracts of *Alchornea cordifolia* stem

Test Fungi	Zone of inhibition (mm)					
	Chloroform	Methanol	Ethanol	Water	Petroleum ether	Miconazole
Rhizopus sp.	40	-	-	-	-	15
flavus	24	-	-	-	-	14
niger	27	-	-	-	-	16
Candida sp.	22	-	-	-	-	15
Penicillium sp.	14	-	-	-	-	15

The result of the MIC of the chloroform leaf extract revealed that *Pseudomonas* sp. had the highest MIC with a value of 0.125mg/ml (Table 5). The highest MBC was shown by *Pseudomonas* sp. with a value of 0.0625mg/ml. The results of the MIC of the chloroform stem extract revealed that *Bacillus* sp. and *Staphylococcus heamolyticus* were moderately sensitive to the stem extract with MIC value of 1mg/ml. *Staphylococcus aureus* and *Pseudomonas* sp. showed the highest MBC to the stem extract with a value of 0.0625mg/ml.

Table 5: Minimum Inhibitory Concentration (MIC) and Minimum Bactericidal Concentration (MBC) of chloroform extracts of *Alchornea cordifolia* leaf and stem

Test bacteria	MIC		MBC	
	Chloroform leaf Extract (mg/ml)	Chloroform stem extract (mg/ml)	Chloroform leaf extract (mg/ml)	Chloroform stem extract (mg/ml)
Proteus sp	0.5	0.5	0.25	0.25
Salmonella sp	0.5	0.5	0.25	0.25
Citrobacter sp	0.5	0.5	0.25	0.25
Micrococcus sp	0.5	0.25	0.25	0.125
E. coli	0.25	0.5	0.125	0.25
Klebsiella sp	0.25	0.5	0.125	0.25
Pseudomonas sp	0.125	0.125	0.625	0.625
S. aureus	0.25	0.125	0.125	0.625
Bacillus sp	0.5	1	0.25	0.5
S. heamolyticus	0.25	1	0.125	0.5

The antimicrobial evaluation of chloroform extract of the leaf and stem of *Alchornea cordifolia* on the selected bacteria and fungi revealed the antimicrobial efficacy of the leaf and stem of this plant. The observed inhibitory properties of the extract against these pathogenic bacteria and fungi indicate that *Alchornea cordifolia* possesses a broad spectrum of antimicrobial activity. Several plants that are rich in tannins have been shown to possess antibacterial activities against a number of microorganisms (El-Mahmood, 2009). Tannins have been reported to hinder the development of micro-organisms by their ability to precipitate and inactivate microbial adhesion enzymes and cell envelope proteins (Chung et al., 2006). The antimicrobial activity of flavonoids is due to their ability to complex with extracellular and soluble protein and to complex with bacterial cell wall; thereby disrupting their membrane integrity (Chung et al., 2006). All the extracts showed more antibacterial activity against the Gram-positive bacteria than the Gram-negative bacteria. The observation that plant derived compounds often show considerable activity against Gram-positive bacteria than Gram-negative species has been made by Nostro et al. (2000). This led to the hypothesis that; plants produce compounds that can be effective antimicrobials if they find their way into the cell of the pathogen (Nostro et al., 2000).

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Earlier work carried out on the phytochemical screening of the methanolic extract of *Alchornea cordifolia* by Adeshina et al. (2007) showed the presence of the secondary metabolite: tannins, saponins, alkaloids, and phenols. The antimicrobial properties of plants have been linked to the presence of these secondary metabolites (Rojas et al., 2006; Nikitina et al., 2007; Udobi et al., 2008; Rafael et al., 2009; Adeshina et al., 2010). This can explain the broad spectrum of antimicrobial activity showed by the methanolic extract against Gram positive, Gram negative bacteria and the yeast *Candida albicans.*

The low activity of the chloroform extracts against *Proteus* sp and *Klebsiella* sp (Gram negative bacteria) was not unexpected since resistance of Gram negative bacteria to most antibacterial agents is well documented (Adeshina et al., 2010). *Salmonella* sp which is the most sensitive of the test organisms to the chloroform extract has been implicated in the aetiology of many ailments (Adeyemo et al., 1994; Nedolisa, 1998; Ebie et al., 2001). With the appreciable levels of inhibition exhibited by the extract of *A. cordifolia* against the test organisms, it is obvious that the plant is a potential source of novel antimicrobial drugs. The results of this work showed that most of the chloroform extracts from *A. cordifolia* effectively inhibited the growth of pathogenic bacteria and fungi. The findings from this study show that *A. cordifolia* has prospects and purification of the extracts would maximize its potentials.

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