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Assessment of Phytochemical Constituent, Antibacterial and Cytotoxic Activities of *A. laxiflora* (Ewe pepe) Extracts

O. J. Osabiya[1*], S. O. Bada[1], F. O. Ibitoye[1] and O. K. Aribisala[1]

[1]Department of Science Laboratory Technology, Rufus Giwa Polytechnic, P.M.B. 1019, Owo, Ondo State, Nigeria.

Authors' contributions

This work was carried out in collaboration between all authors. Author OJO designed the study and wrote the protocol. Author SOB reviewed the experimental design and all drafts of the manuscript. Author FOI performed the statistical analyses and wrote the first draft of the manuscript. Author OKA handled the laboratory work. All authors read and approved the final manuscript.

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ABSTRACT

Aim: This study was done to determine the phytochemical constituents, antibacterial and cytotoxic activities of leaf extracts of *A. laxiflora* ("ewe pepe").
Place and Duration of Study: Department of Science Laboratory Technology (Microbiology and Analytical Chemistry Laboratories), Rufus Giwa Polytechnic, Owo, Nigeria, between August 2016 and March 2017.
Methodology: The methods used include manual grinding of the air-dried leaves and solvent (aqueous and ethanol) percolation for 72 hrs. The resultant crude extracts were kept in sterile airtight bottles and stored in the refrigerator. Then, they were screened for phytochemical constituents. Furthermore, the plant extracts were screened for antibacterial activities against *Bacillus subtilis, Staphylococcus aureus, Escherichia coli, Enterococcus faecalis, Klebsiella pneumonia* and *Salmonella typhi.* In addition, the plant extracts were investigated for cytotoxic activity against *Artemia salina* nauplii.

**Corresponding author: E-mail: michaelococcus@gmail.com;*

Results: The results of the phytochemical analyses showed the presence of flavonoid, saponin, tannin, alkaloid and phenols in the aqueous and ethanol extracts of the plant. In the antibacterial activity screening, of the plant extracts showed a concentration dependent trend as higher activities was observed as the concentration gradient increased. *S. aureus* (19.33±0.58), *K. pneumoniae* (18.33±0.58) and *E. faecalis* (18.67±0.58) showed the highest susceptibility to the plant extracts while *S. typhi* showed the least susceptibility against the plant extracts. The lowest minimum inhibitory concentration (MIC) was found in *K. pneumoniae* (2.5 mg/ml) while *S. aureus, E. coli* and *E. faecalis* recorded an MIC of 5 mg/ml respectively. The ethanol extract of *A. laxiflora* was more active against the selected pathogens compared with aqueous extract. Moreover, in the cytotoxic assay, *A. salina* showed high mortality against the plant crude extracts. The LC_{50} value of less than 100 ppm/ml was obtained from the aqueous (41.01 ppm) and ethanol (8.91 ppm) extracts.
Conclusion: The results of this study revealed that the *A. laxiflora* leaf extracts contain important phytochemicals such as alkaloids, flavonoids, phenols, saponins and tannins which may be responsible for the antibacterial and cytotoxic effects exhibited by *A. laxiflora*t and new antimicrobial and anticancer agents may be developed from the plant.

Keywords: Phytochemical; antibacterial; cytotoxic; Alchornea laxiflora.

1. INTRODUCTION

In recent years, drug resistance to human pathogenic bacteria and fungi has been commonly reported from all over the world. Therefore, the increasing prevalence of multidrug resistance strains of bacteria and the recent appearance of strains with reduced susceptibility to antibiotics raises the specter of untreatable bacterial infections and adds urgency to the search for new infection-fighting strategies [1]. Infectious diseases are caused by pathogenic microorganisms, such as bacteria, viruses, parasites or fungi and are the second leading cause of death worldwide. Human infections, particularly those involving the skin and mucosal surfaces constitute a serious problem, especially in tropical and subtropical developing countries [2]. Methicillin – resistant *Staphylococcus aureus* (MRSA), *Staphylococcus aureus, Escherichia coli, Pseudomonas aeruginosa, and Candida albicans* are reported be the most frequent skin pathogens [3]. Moreover, emergence of multiple drug resistant strains of microorganisms due to indiscriminate use of antibiotics to treat infectious diseases has generated interest in herbal medicine and as antimicrobials against pathogenic microorganisms. Antimicrobial potential of different medicinal plants is being extensively studied all over the world [4], but only a few studies have been carried out in systematic manner. However, in the absence of any scientific proof of their effectiveness the validity of these remedies remain questionable as their use is locally restricted.

Investigations into the chemical and biological activities of plants during the past two centuries have yielded compounds for the development of modern synthetic organic compounds and the emergence of medicinal chemistry as a major route for the discovery of novel and more effective therapeutic agents [5]. Medicinal uses of plants range from the administration of the roots, barks, stems, leaves and seeds to the use of extracts and decoctions from the plants [6]. About one-fourth of all medicines we use, come from rainforest plants [7] and many scientific researches have been carried out on these plants as well as their secondary metabolites of medicinal importance; alkaloids, flavonoids, terpenes etc, have been reported, pure compounds have also been isolated and characterised [8,9].

Cytotoxicity is the quality of being toxic to cells. Cell exposed to a cytotoxic compound can respond in a number of ways. Cytotoxicity can cause damage to cell membrane. The cells may undergo necrosis, in which they lose membrane integrity and die rapidly as a result of cell lysis; They can stop growing and dividing or they can activate a genetic program of controlled cell death, termed apoptosis. Cytotoxicity assays are used widely in drug discovery research to help predict which compounds might have safety concerns in humans before significant time and expense are incurred in their development [10].

Alchornea laxiflora which is commonly called ‘pepe’ belongs to Euphorbiaceae family. It is an evergreen shrub up to six metre in height. It is from Northern and Southern Nigeria and West Cameroon. It is also widely spread in central, eastern and Southern tropical Africa. The stems, especially the branches are used in Nigeria as

chew-sticks and its leaves are used to preserve kolanut in Nigeria [11]. The decoctions of the leaves of *A. laxiflora* are also used in the management of inflammatory and infectious diseases [12]. The antimicrobial activities of the agents from leaves of *A. laxiflora* have been demonstrated to show marked antimicrobial activities that were of significant differences among other *Alchornea* varieties [11] and related species, however, there is a dearth of information on the cytotoxic potential of this plant. Therefore this study was designed to determine phytochemical component, *in vitro* antibacterial and cytotoxic activities of the leaf of this plant.

2. MATERIALS AND METHODS

2.1 Collection, Identification and Extraction of the Extracts

Fresh leaves of *A. laxiflora* were harvested from uncultivated farmland located in Ifon, Ondo State, South-Western Nigeria in August, 2016. The plant was then authenticated at the Herbarium section of the Department of Forest Resources Technology and a voucher specimen (XAL32024) was deposited (in the same Department) Rufus Giwa polytechnic, Owo. The authenticated plant materials were washed and cleaned thoroughly with tap water and then air-dried under shade. The dried samples were then ground into coarse powder with the aid of a mechanical grinder and were stored in clean air-tight containers, and kept in a cool, dry place until required for use.

One hundred gram (100 g) of the powdered sample was soaked in 300 ml of different solvents (ethanol and water) for 72 hr with intermittent stirring using sterile spatula. The plant extracts were then filtered through Whatman No 1. filter paper into bijou bottles and then dried using rotary evaporator at a temperature of 50℃ to yield crude extracts [13]. Different concentrations of the extracts were prepared by diluting 0.10 g, 0.20 g, 0.30 g, 0.40 g and 0.50 g of the extracts in 100 ml of 0.01% Tween-20 to obtain concentrations of 10 mg/ml, 20 mg/ml, 30 mg/ml, 40 mg/ml and 50 mg/ml respectively [14].

2.2 Test Microorganisms

The bacteria used in the research were six clinical isolates (*Bacillus subtilis, Staphylococcus aureus, Escherichia coli, Enterococcus faecalis, Klebsiella pneumoniae, Salmonella typhi*) gotten from Federal Medical Center, Owo, Nigeria in October, 2016.

2.3 Qualitative Phytochemical Screening

The extracts of the different plant parts were subjected to qualitative phytochemical analysis for the presence of tannins, saponin, flavonoids, alkaloids and phenol were carried out on the extracts using standard procedures as described by [15,16].

2.3.1 Test for tannins

1 ml of extract was boiled in 20 ml of water in a test and then filtered. A few drops of 0.1% ferric chloride was added and observed green or a blue – black coloration which confirmed the presence of tannin.

2.3.2 Test for saponin

About 5 ml of the extract was boiled in 20 ml of distilled water in a water bath and filtered. 10 ml of the filtrate was mixed with 5 ml of distilled water and shaken vigorously for a stable persistent froth. The frothing was mixed with 3 drops of olive oil and shaken vigorously, then observed for the formation of emulsion which confirmed a positive presence of saponins.

2.3.3 Test for flavonoids

A 3 ml portion of 1% Aluminum chloride solution was added to 5 ml of each extract. A yellow coloration was observed indicating the presence of flavonoids. 5 ml of dilute ammonia solution were added to the above mixture followed by addition of concentrated H_2SO_4. A yellow coloration disappeared on standing. The yellow coloration which disappeared on standing indicating a positive test for flavonoids.

2.3.4 Test for alkaloids

A 1 ml portion of the extract was stirred with 5 ml of 1% aqueous HCl on a steam bath and filtered while hot. Distilled water was added to the residue and 1 ml of the filtrate was treated with a few drops of either Mayer's reagent (Potassium mercuric iodide- solution gave a positive test for alkaloids.

2.3.5 Test for phenol

A 5 ml portion of the extract was pipetted into a 30 ml test tube, and then 10 ml of distilled water

was added to it. 2 ml of ammonium hydroxide solution and 5 ml of concentrated amyl alcohol were also added and left to react for 30 min.The development of bluish-green colour was taken as a positive presence of phenol.

2.4 *In vitro* Antibacterial Susceptibility Test

The extracts obtained from the test plants were screened against the test bacteria by agar well diffusion method [17]. A 25 ml aliquot of Mueller-Hinton agar (MHA, Lab Oratorios Britania, Argentina) was poured into each Petri plate. When the agar solidified, the pathogenic test organisms were inoculated on the surface the plates (1×10^6 *cfu*/ml) using a sterile glass spreader and allowed to sink properly. Subsequently, the surface of the agar was punched with 6 mm diameter cork borer into wells and a portion of 50 µl of each of the extract concentrations was filled into the wells. Control wells containing the same volume of 30% Dimethyl sulphoxide (DMSO) served as negative control, while Chloramphenicol (50 µg) was used as positive control for the plates respectively and the plates were incubated at 37℃ for 24 h. Each experiment was carried out in triplicate and the diameter of the zones of inhibition was then measured in millimeters.

2.4.1 Minimum inhibitory concentration (MIC)

The MIC of the plants extracts were determined by double dilution broth methods of Ghosh et al. [18]. Twofold serial dilutions of the extracts were prepared in Mueller-Hilton broth to achieve a decreasing concentrations ranging from the least concentration that produced clear zone of inhibition (10 mg/ml to 0.156 mg/ml). All tubes with the controls were labeled accordingly. Each dilution was seeded with 1 ml of standardized inoculums (1.0×10^6 cfu/ml) and incubated at 37℃ for 24 hr. A tube containing only seeded broth (i.e. without plant extract) was used as the positive control while the un-inoculated tube was used as negative control. The lowest concentration of each extract that showed a clear of inhibition was when compared with the controls was considered as the MIC.

2.5 Determination of Cytotoxic Effect of Plant Extracts

The brine shrimp (*Artemia salina*) lethality bioassay was carried out according to the method described by [19]. Brine shrimp eggs were hatched in artificial sea water prepared by dissolving 38 g of salt in 1 liter of distilled water, filtered and put in shallow rectangular dish. A plastic divider with several holes of 2 mm size was clamped in the dish to make two equal compartments. Brine shrimp eggs were placed in one side of the compartment while the other compartment was illuminated. After 48 h of illumination, phototrophic nauplii (Brine shrimp larvae) were collected by using pipette from the lightened side. Samples were then prepared by dissolving 20 mg each of the extracts in 2 mls of DMSO from where further diluted concentrations of 1000, 100, 10 and 1 ppm were prepared. A 4ml portion of the artificial sea water was added into each test tube and 20 shrimps were transferred into it. This was followed by the addition of 1 ml of each of the test extracts and of previously prepared concentrations and maintained under illumination at room temperature. Survivors were counted with the aid of magnifying glass after 24 h. The percentage mortality was calculated using Abbot's formula and the LC_{50} was also determined [20,21].

2.6 Data Analysis

Data were presented as mean ± standard error (SE). Significance difference between different groups was tested using two-way analysis of variance (ANOVA) and treatment means were compared with Duncan's New Multiple Range Test (DNMRT) using SSPS window 7 version17.0 software. The significance was determined at the level of $p \leq 0.05$.

3. RESULTS AND DISCUSSION

3.1 Phytochemical Constituents of *Alchornea laxiflora* Leaf

The result of the phytochemical screening of the aqueous and ethanol extracts of *Alchornea laxiflora* leaf revealed the presence of tannins, alkaloid, saponin, flavonoid and phenol in varying proportion as revealed in their reactions (Table 1). These phytochemicals have been reported to be present in the members of the family Euphorbiaceae which may be responsible for their medicinal properties. Also, earlier study by Osuntokun and Olajubu [22] reported these phytochemicals in *A. laxiflora*. The presence of phenols confirms the antimicrobial property of the plant since phenol and its compounds are extensively used as disinfectants and it is the

standard for bactericides [23]. Flavonoids are reportedly active as anti-spasmodic, antifungal and antibacterial agent. This may explain why the plant is usually used as remedy for spasmodic bronchitis and diarrhea [24]. Moreover, the presence of tannins, alkaloids and saponins suggests that the plant may possess a wide spectrum of antimicrobial property as these plant chemicals are known for their synergy in their activities against several microbial agents [25].

Table 1. Phytochemical composition of *Alcornea laxiflora*

Phytochemical	Ethanol extract	Aqueous extract
Tannin	+	+
Alkaloid	+	+
Saponin	+++	++
Flavonoid	++	++
Phenol	+	+

Key: + = Present in trace amount, ++ = Present in moderate amount, +++ = Present in abundance

3.2 Antibacterial Activities of *Alchornea laxiflora* Leaf Extracts

The results of the antibacterial activities of the plant extracts showed a concentration dependent trend as higher activities was observed as the concentration gradient increased. The extracts exhibited different degrees of antimicrobial activity against tested bacteria. However, at the highest concentration used (50 mg/ml), the extracts showed comparable activities with the pure commercial antibiotic (chloramphenicol) used as positive control. Especially the ethanol extract of the plant leaf which showed higher antibacterial activities than aqueous extract. This might be due to the differences in the concentration of the phytocompounds of various secondary metabolites present in the extracts as well as the extracting ability of the solvents. This corroborates the observations of Abo and Ashidi [26]. *S. aureus* (19.33±0.58), *K. pneumoniae* (18.33±0.58) and *E. faecalis* (18.67±0.58) showed the highest susceptibility to the plant extracts while *S. typhi* showed the least susceptibility against the plant extracts. These observations have been made earlier by Osuntokun [27] who reported low antibacterial activities of *A. laxiflora* against *S. typhi* and *S. paratyphi.* The demonstration of antimicrobial activity against both Gram positive and Gram negative bacteria may be indicative of the broad spectrum antibiotic compounds in the extracts of the plant and could support its folkloric use in Tradomedicine in Southwestern Nigeria.

A striking observation from this research is the susceptibility of the versatile pathogen *Staphylococcus aureus* which is a major nosocomial pathogen with low intrinsic susceptibility to antimicrobial agents and very high ability to acquire resistance was significantly inhibited by the leaf extracts of the plant with zone of inhibition that are comparable to the positive controls and could be further exploited for chemotherapeutic agents that could be used against infections caused by multiple antibiotic resistant strains that are very common in Nigeria. The minimum inhibitory concentration (MIC) is the highest dilution or least concentration of the extracts that inhibit growth of organisms. It is an important diagnostic because it helps in confirming resistance of microorganisms to antimicrobial agents. The lowest MIC was found in *K. pneumoniae* (2.5 mg/ml), while *S. aureus, E. coli* and *E. faecalis* recorded an MIC of 5mg/ml. This suggests that potent antimicrobials may be developed from the plant.

3.3 Cytotoxic Activities of *Alchornea laxiflora* Leaf Extracts

In other to study the toxicity of the plant's leaf extracts, brine shrimp lethality bioassay was employed which was based on the ability of tested samples to kill laboratory cultured brine shrimp (*Artemia salina*) nauplii. The assay is considered a useful tool for preliminary assessment of toxicity since the brine shrimp is highly sensitive to a variety of chemical substances and it represents a rapid in-exposure and simple bioassay for testing plant extracts bioactivity which in most cases correlates reasonably well with cytotoxic and anti-tumour properties [28].

Percentage lethality of brine shrimp at four different concentrations (1, 10, 100, 1000 ppm/ml) of *Alchonea laxiflora* leaf extracts showed higher mortality percentage with the increase in concentrations of the extracts. This observation is in line with the reports of Pisuthanan [29]. The LC_{50} value of less than 100 ppm/ml was obtained from the aqueous (41.01 ppm) and ethanol (8.91 ppm) extracts. All the LC_{50} values higher than 1000ppm according to are not significant while those within the range of 0-100 ppm/ml are considered to be very toxic [30].

Table 2. Antimicrobial activity of aqueous extract of *Alchornea laxiflora* leaf on selected pathogens (Zones of inhibition in mm)

Conc. (mg/ml) organisms	10	20	30	40	50	DMSO (30%)	Chl (100 µg/ml)
B. subtilis	NI	NI	6.67±0.58^{a}	9.33±0.58^{b}	10.00±0.00^{cd}	NI	11.00±0.00^{d}
S. aureus	3.67±0.58^{a}	6.67±0.58^{b}	10.67±0.58^{c}	13.00±0.00^{d}	13.00±0.00^{d}	NI	11.33 ±0.58^{c}
E. coli	2.67±0.58^{a}	5.33±0.58^{b}	7.67±0.58^{c}	10.67±0.58^{d}	13.00±0.00^{e}	NI	13.67±0.33^{e}
E. faecalis	7.67±0.58^{a}	11.33±0.58^{b}	14.00±0.00^{c}	17.33±0.58^{d}	18.67±0.58^{e}	NI	11.33±0.33^{b}
K. pneumoniae	NI	5.67±0.58^{a}	9.33±0.58^{b}	11.67±0.58^{c}	13.67±0.58^{d}	NI	10.00±0.00^{b}
S. typhi	NI	NI	NI	6.33±0.58^{d}	8.33±0.58^{e}	NI	13.33±1.00^{c}

Values are Mean±S.E.M (mm), Values followed by different alphabet along the rows are significantly different at P =.05, NI = No inhibition, Chl = Chloramphenicol, B.S = Bacillus subtilis, S.A = Staphylococcus aureus, E.C = Escherichia coli, E.F = Enterococcus faecalis, K.P = Klebsiella pneumoniae, S.T = Salmonella typhi

Table 3. Antimicrobial activity of ethanol extract of *Alchornea laxiflora* leaf on selected pathogens (Zones of inhibition in mm)

Conc. (mg/ml) organisms	10	20	30	40	50	DMSO (30%)	Chl (100 µg/ml)
B. subtilis	4.00±0.00^{a}	7.33±0.58^{b}	9.67±0.58^{c}	11.00±0.00^{c}	12.67±0.58^{d}	NI	11.00±0.00^{c}
S. aureus	6.00±0.00^{a}	11.67±0.58^{b}	14.33±0.58^{c}	17.33±0.58^{d}	19.33±0.58^{e}	NI	11.67±0.57^{b}
E. coli	6.67±0.58^{a}	10.33±0.58^{b}	13.67±0.58^{c}	15.33±0.58^{d}	17.67±0.58^{e}	NI	13.67±0.33^{c}
E. faecalis	5.67±0.58^{a}	9.33±0.58^{b}	11.67±0.58^{c}	14.33±0.58^{d}	15.00±0.00^{d}	NI	11.33±0.33^{c}
K. pneumoniae	7.67±0.58^{a}	12.33±0.58^{c}	15.00±0.00^{d}	15.00±0.00^{d}	18.33±0.58^{e}	NI	10.00±0.00^{c}
S. typhi	NI	NI	5.00±1.00^{a}	8.67±0.58^{b}	12.67±0.58^{c}	NI	12.33±0.58^{a}

Values are Mean±S.E.M (mm), Values followed by different alphabet along the rows are significantly different at P = .05, NI = no inhibition, Chl = Chloramphenicol, B.S = Bacillus subtilis, S.A = Staphylococcus aureus, E.C = Escherichia coli, E.F = Enterococcus faecalis, K.P = Klebsiella pneumoniae, S.T = Salmonella typhi

Table 4. Minimum inhibitory concentration of *Alchornea laxiflora* extracts (mg/ml)

Organism	Aqueous	Ethanol
B. subtilis	25	10
S. aureus	10	5
E. coli	10	5
E. faecalis	2.5	5
K. pneumoniae	12.5	2.5
S. typhi	40	30

Table 5. Percentage mortality of brine shrimps at different concentrations of the extracts of *Alchornea laxiflora*

Dosage (ppm)	Initial larvae	Aqueous			Ethanol		
		No. of survivors	No. of deaths	% mortality	No. of survivors	No. of deaths	% mortality
Leaf							
1000	20	0	20.00 ± 1.15^{d}	100	0	20.00 ± 0.01^{c}	100
100	20	3	17.33 ± 2.07^{c}	85	1	19.00 ± 0.02^{c}	95
10	20	13	9.33 ± 2.00^{b}	35	5	15.67 ± 0.07^{b}	75
1	20	19	1.33 ± 1.15^{a}	5	12	11.33 ± 0.00^{a}	40
LC_{50}				**41.01**			**8.91**

Values followed by different superscripts across each column are significantly different at P=0.05

The lethality concentration (LC_{50}) of the plant extracts were found to be within the range considered to be very toxic [31]. Some brine shrimps results that are already available [32] provide circumstantial evidence that plant extracts with LC_{50} values of 20 mg/ml have a likelihood of yielding anticancer compounds.

4. CONCLUSION

From the phytochemical screening results of this study, the *A. laxiflora* leaf extracts contain important phytochemicals such as alkaloids, flavonoids, phenols, saponins and tannins. It may be suggested that the presence of these phytochemicals may be responsible for the antimicrobial and cytotoxic effects exhibited by the plant and new antimicrobial and anticancer agents may be developed from the plant. However, further studies are needed to ascertain its toxicity to humans.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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