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Original Research Paper

ANTIMICROBIAL PROPERTIES OF THE METHANOLIC EXTRACT OF THE LEAVES OF *NAUCLEA LATIFOLIA*

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ABSTRACT

Nauclea latifolia had been reported to have antimicrobial properties. The people of Ogidi in Idemili North Local Government Area of Anambra State, Nigeria, used it to treat wound infections. This study is therefore aimed at determining this claim to antimicrobial effect of this plant using the leaves of *Nauclea latifolia* which will also serve as criteria to recommend the Ethnopharmacological uses of the plant. The plant leaves were dried, powdered and extracted by cold maceration with methanol for 24 hours. This was concentrated using rotary evaporator. Phytochemical evaluation revealed the presence of alkaloids, tannins, sterols, glycosides and saponin in varying degrees. The antimicrobial activities (sensitivity test) of the crude extract and the standards (Augmentin; an antibacterial agent and ketoconazole; an antifungal agent) were evaluated against Gram positive and Gram negative bacteria and fungi using agar diffusion method at the concentration of 100 mg/ml for extract and 50 µg/ml for standards. The same method was used to evaluate the minimum inhibitory concentration but at different concentrations. Results show that methanolic extract of the leaves exhibited activity against *E.coli, S. dysenteriae, S. aureus, B. subtilis and A. niger,* with minimum inhibitory concentration (MIC) ranging from 3.35 mg/ml- 10.42 mg/ml. On *Pseudomonas aeruginosa,* the methanolic extract of leaves also show significant effectiveness when compared to the standard. The percent susceptibility test indicated that the effect of methanolic extract of leaves on *S. dysenteriae* is very high (98.4%). From the results obtained, it could therefore be concluded that the leaves of *Nauclea latifolia* possess broad spectrum antimicrobial activities. *Nauclea latifolia* may be useful in the formulation of antimicrobial agent that could be used for the treatment of microbial infections of different origins.

Keywords: *Nauclea latifolia*, Ketoconazole, Antimicrobial activity, Minimum Inhibitory Concentration (MIC), Augmentin.

INTRODUCTION

Nauclea latifolia Smith (family: Rubiaceae) is a straggling, evergreen, multi-stemmed shrub or small tree native to tropical Africa and Asia. It grows up to an altitude of 200 m. It is widespread in the humid tropical rainforest zone or in savannah woodlands of West and Central Africa. It grows rarely over 20ft high, bole crooked; or a larger tree over 100ft high and 8ft girth, in closed

forest. The plant has rough bark, leaves are 7 by 4-5 inches and are glabrous obovate. Flower head is up to 2mm in diameter, sweet scented and sought by bees. Three other related species *Nauclea pobeguini, N. diderichii,* and *N. vanderguchtii* are forest trees. *N. Diderichii* is planted in Omo forest reserve, Nigeria. In the folk medicine, the species *N. diderichii* and *N. orientalis* are used in the same way as *N. latifolia. Nauclea latifolia* has an open canopy and terminal spherical head lined cymes of white flowers. The flowers are joined with their calyces. The fruit is syncarp, up to 3 inches in diameter. The tree is flowering from April to June. The fruits are ripening from July to September. Baboons eat them and disperse the seeds. Livestock eat shoots and leaves. The fruits are edible too.

PLANT TAXONOMY

Family: RUBIACEAE
Genus: *Nauclea*
Species: *latifolia*
Accepted name: *Nauclea latifolia* Sm.
Synonyms: *Sarcocephalus latifolius* (Sm.) E.A.Bruce
Common names: English; African peach, Pin cushion tree, Guinea peach.
Igbo; Ubulinu.
French; Scille maritime, oignon marine.
Hausa; Tafashiya, tafiyaigia.

TRADITIONAL USES OF *Nauclea latifolia*

Nearly all plant parts are useful in treatment of diseases. Infusions and decoctions of the stem bark and leaves of *Nauclea latifolia* are used for the treatment of stomach pain, constipation, fever, and diarrhea. In kano (Nigeria) the plant is used as a chewing stick and as a remedy against stomach ache and tuberculosis (Deeni & Hussain 1991). In Ivory Coast infusions and decoctions from stems and roots of *N. latifolia* are used against malaria by traditional healers (Benoil-vical, Valentin, cournal et al. 1998). In Kinshasa, extracts and preparations together with other plants are applied against diarrhea (Tona, Kambu, Ngimbi et al. 2000). Abbiw (1990) stated that root infusion is used in Sudan for the treatment of gonorrhoea and also the roots and leaves are used in Ghana for treating sores. In Nigerian local medicine, the fruits are sometimes used in the treatment of piles and dysentery. Because of its reported antimalarial activity, the plant has been known as 'Africa cinchona' or 'Africa quinine' (Abbiw 1990). Gidado, Ameh, & Atawodi (2004), demonstrated that aqueous extract of the leaves of *N. latifolia* possess hypoglycaemic activity in alloxan-induced diabetic rats. The plant is also used in hypertension (Akabue and Mittal, 1982), gastrointestinal tract disorders (Maduabunyi,1995), sleeping sickness (Kerharo, 1974), and prolong menstrual flow (Elujoba, 1995).

MATERIALS AND METHODS

MATERIALS

STANDARD DRUGS

Amoxicillin/ potassium clavulanate (Augmentin$^{(R)}$) , Ketoconazole; (Korlyns).

MICROORGANISMS

- Gram positive bacteria; *Staphylococcus aereus* and *Bacillus subtilis*
- Gram negative bacteria*; Escherichia coli, Pseudomonas aeruginosa* and *Shigella dysentariae.*
- Fungi; *Candida albicans* and *Aspergillus niger*.

REAGENTS/ CHEMICALS

Methanol , Dragendorff's reagent, Hager's reagent, Concentrated sulphuric acid (H_2SO_4) (BDH Chemicals), concentrated hydrochloric acid, ferric chloride hexahydrate, copper sulphate pentahydrate ,sodium tartarate, sodium hydroxide,

BIOLOGICAL MEDIA

Nutrient Agar (Fluka), Nutrient Broth (Fluka), Saubaraud dextrose agar (Biochemika)

EQUIPMENT

Electrical weighing balance (OHAUS Model 2610), Laboratory Incubator (Sanfa-Moodel No DNP-9022A), wooden mortar and pestle, laboratory oven (Surgienfield Instrument, England), digital water bath (Sanfa-Model No

DK420), autoclave (Health team instrument, England), Refrigerator (LG), Bunsen burner.

APPARATUS/GLASS WARES

Petri-dishes, Pasteur pipettes, swab sticks, calibrated syringes, measuring cylinders, beakers, test tubes, reagents bottles, stirrer, inoculating loop, cork borer.

METHODS

PLANT COLLECTION AND IDENTIFICATION AND PREPARATION

The fresh leaves of Nauclea Latifolia were collected from a bush at Ogidi, Idemili North Local Govt. Area Anambra State, Nigeria in November 2010. The plant was identified by Dr Ezugwu of Department of Pharmacognosy, Faculty of Pharmaceutical Sciences, University of Nigeria,Nsukka. The leaves were dried under shade for seven days. The dried materials were powdered using locally made mortar and pestle, weighed in an electronic weighing balance and stored in an air-tight container.

EXTRACTION PROCEDURE:

500gm of the pulverised powder of the leaves of *Nauclea latifolia* were macerated in 1500ml (1.5L) of methanol (AnalaR grade) respectively, in about 3L capacity glass ware and were made air-tight and left for two days with intermittent shaking. The mixture was filtered using Whatman's filter paper No. 1 to obtain a solution devoid of solids. The methanol extracts were evaporated using rotary evaporator to get dried concentrate.

PHYTOCHEMICAL SCREENING

Qualitative assay for the presence of secondary plant metabolites were carried out on methanolic extracts of leave *of Nauclea latifolia* using the standard procedures (Harborne 1991), (Trease and Evans, 1989).

TESTS FOR ALKALOIDS

Being bases, a 100mg of the crude extract extracts was boiled for about 2 minutes with 5ml of 2M HCl on a steam bath. The mixtures were filtered and to 1ml of filterate was added 2 drops of Dragendorf's reagent (bismuth potassium iodine solution), (brick red colouration test). To another 1ml of each filtrate, 2 drops of Hagers (saturated solution of picric acid) reagent was also added. The test tubes were observed for coloured precipitates.

TESTS FOR TANNINS

Preparation of ferric chloride test solution;

Ferric chloride hexahydrate....7.50g.

Concentrated hydrochloric acid....1.0ml.

Water to. ....100mls.

A few drops of ferric chloride was added to the test tubes containing about 2ml of extract. The test tubes ware observed for greenish-black precipitates.

Another 2ml each of the extracts was diluted to 10ml and few drops of lead acetate solution added. A reddish colour indicates the presence of tannins.

TEST FOR SAPONINS

Frothing test; 5ml of the extract was diluted with 10ml of distilled water; the solutions were then vigorously shaken and observed on standing to obtain persistent of foam.

TEST FOR STEROLS

Preparation of Liebermann-Burchard's reagent**;**

Acetic anhydride....1.0g.

Concentrated sulphuric acid.....2.0g.

Chloroform. ....30ml.

Liebermann Burchard's reagent was added the sample and was observed for bluish green colour reaction.

TESTS FOR GLYCOSIDES

Preparation of Fehling's solution;

Copper sulphate pentahydrate....3.46g.

Sodium potassium tatarate....17.3g.

Sodium hydroxide.....6.0g.

Water to ....100ml.

5ml of the extract was mixed separately with 2ml of Fehling's solution (freshly prepared) and then boiled in water bath for 15 minutes. The sample was observed for a brick red precipitate which indicates the presence of reducing sugars and is also an indirect determinant of presence of glycosides.

A 3ml of dilute sulphuric acid was added to the mixture (above) and was boiled for another 15min. and cooled. The sample was observed for an increase in the amount of precipitate previously formed. Theoretically, more precipitate indirectly confirms the presence of glycosides since the reducing sugars are obtained from the hydrolysis of glycosides present.

ANTIMICROBIAL ACTIVITY SCREENING

PREPARATION OF INOCULUMS

The bacteria used were *Shigella dysenteriae, Pseudomonas aeruginosa, Escherichia coli, Bacillus subtilis and Staphylococcus aureus.* The fungi used were *Aspergillus niger and Candida albicans. All* microorganisms were isolated from clinical specimens obtained from the Medical Microbiology department of Madonna University Elele, Rivers State Nigeria. The test organisms were separately prepared by subculturing the pure isolates in nutrient agar and incubated at 37^{o}C for 24 hours for bacteria and in sabauraud agar for 72 hours for fungi. Two loopfuls of the microbial culture were collected using sterilized (heat fixed) inoculating loop into 10mls nutrient broth contained in sterilized universal bottles and then incubated at 37^{o} C overnight for subsequent use. 0.2ml of the overnight cultures of different organisms were then diluted with 20ml nutrient broth to give 1 in 100 dilution equivalent to 1x 10^{6}cfu/ml which were then used for the study.

STERILISATION OF WORKING MATERIALS

Petri dishes and pipettes, were washed with detergents, rinsed with distilled water and wrapped with aluminium foil before they were sterilised in hot air oven at a temperature of about 100^{0}C for about one hour. The laboratory benches were cleaned with 70% alcohol before and after each experiment.

The tests carried out were;

- Sensitivity test.
- Minimum inhibitory concentration (MIC).
- Minimum bactericidal concentration (MBC)

PREPARATION OF THE CULTURE MEDIA

Culture media are commercially available in the dehydrated form. The nutrient agar medium was prepared by suspending 28g of the nutrient agar in one litre of distilled water. The suspension was then dissolved completely. It was then sterilised by autoclaving at 121^{o}C for 15 minutes.

SENSITIVITY TEST

The method used here is agar diffusion method. 20ml of nutrient agar was melted in water bath at 100^{O}C for thirty minutes after which they were stabilised at 45^{0}C for 15 minutes. Each molten agar inoculated with 0.2ml (containing 10^{6}CFU) of a 24-hour culture of the test organisms. The dishes were rotated to homogenize with the microorganism. The seeded agar were poured into separate sterile petri-dishes and allowed to set. Using a sterile cork borer, four cups were bored in the set agar. The plates were turned upside down, divided into four and cups were labelled appropriately. The extract was reconstituted by dissolving 1gm of each in 1ml of water. Two cups in each petri dish were filled with 100mg equivalent of the methanolic extracts from the leaves of *Nauclea latifolia.* The remaining cups were filled with 25µg/ml equivalent of standard drug. The extracts from each part of the plant were tested against five bacteria (*Escherichiaa Coli, Pseudomonas auriginosa, Neisserria gonorrhoea, Staphylococcus aureus, Bacillus subtilis and Shigella dysenteriae*). 25µg/ml of amoxicillin/potassium clavulanate solution was used as comparative standard drug for the bacteria.

Sabouraud dextrose agar was used as the culture medium for the fungi and the above procedure repeated. The extract was tested against two fungi

(*Aspergillus niger and Candida albicans*). The comparative drug used was ketoconazole (25µg/ml).

The plates were allowed to stand for one hour to allow adequate diffusion of the extracts and the drugs. The plates seeded with bacteria were then incubated at 37^0C for 24 hours and other plates seeded with fungi were incubated at 28^0C for 5days. The zones of inhibition were measured in millimetre (mm) and the average found and recorded (Collin et al. 1995).

DETERMINATION OF MINIMUM INHIBITORY CONCENTRATION (MIC)

The back of the agar plate was divided into 5 parts. 0.2 ml of a standard suspension of each microorganism was placed into 2 separate petri dishes for each microorganism. The prepared sterile molten agar was poured into each of the plates and mixed by rotating each plate to homogenise the microorganism. The agar was allowed to set on a flat surface for 10min.

A cup was made up on the agar using a sterile cork borer of 9mm in diameter at the centre of each section. The cups were labelled to indicate the concentration of the extract to be introduced into each cup. After boring the cups the extract was introduced into the corresponding cups and allowed for diffusion for about 15 minutes and incubated at 37^oC for 24 hours. The fungi dishes were incubated at 28^oC for four days. The zones of inhibition were measured and the mean recorded. The inhibition distance was determined by subtracting the diameter of the cup (9mm) from the zone of inhibition. A graph of the square of the corresponding mean inhibition distance was plotted against the log concentration of the extracts and of the drugs. A straight line of best fit was drawn and extrapolated to the log concentration axis. The resultant intercept was recorded as the log minimum inhibitory concentration (MIC) against that organism. The antilog of this is the minimum inhibitory concentration.

STATISTICAL ANALYSIS

The results was expressed as means ± standard error of mean (S.E.M). Percentage susceptibility of the extract was evaluated using the standards as 100%. The significance difference between the mean values were measured using analysis of variance (ANOVA) at $P<0.05$.

Secondary Metabolites Present in the Extracts

Table 1: Secondary plant metabolites found in methanolic extract of *Nauclea latifolia*

Secondary Metabolites	ME
Alkaloids	++
Glycoside	++
Saponins	++
Sterols	+
Tanins	++

KEY: ME = methanolic extract.++ = presence of the compound.- = compound not detected.+ = trace amount of compound.

SENSITIVITY TEST RESULT

The results showed that the clinical isolates were all susceptible to methanolic extract of the leaves.

Table 2: Sensitivity results of the extract and standards

	Mean inhibition zone diameter						
Extracts	P.A	E.C	S.A	S.D	B.S	C.A	A.N
ME / L	11.9±0.4	19.2±0.9	20.1±1.1	18.7±1.7	19.3±0.3	21.1±1.0	11.7±0.3
AUG.	15.0±1.0	28.3±1.1	25.4±0.4	19.0±1.2	27.2±0.8	-	
KET.	-	-	-	-	-	27.1±0.05	18.0±0.8

KEY:

P.A = *Pseudomonas aeruginosa.* *E.C.* = *Escherichia coli.* *S. A.* = *Staphylococcus aureus* *S.D.* = *Shigella dysenteriae* *B. S.* = *Bacillus subtilis.* *C. A.* = *Candida albicans* *A.N.* = *Aspergillus niger.* AUG. = augmentin^{R} KET. = ketoconazole. L = leaves ME. = methanolic extract – no activity

PERCENT SUSCEPTIBILITY TEST

Table 3: Result of percent susceptibility of the extract and standards

Microorganisms	methanolic extract	standards	
	L	AUG	KET.
P. aeruginosa	79	100	-
E. coli	67	100	-
S. aereus	79	100	-
S. dysentariae	98.4	100	-
B. subtilis	70.9	100	-
C. albicans	77.9	-	100
A. niger	65	-	100

KEY – No activity AUG. = augmentin^{R} KET. = ketoconazole L=leaves

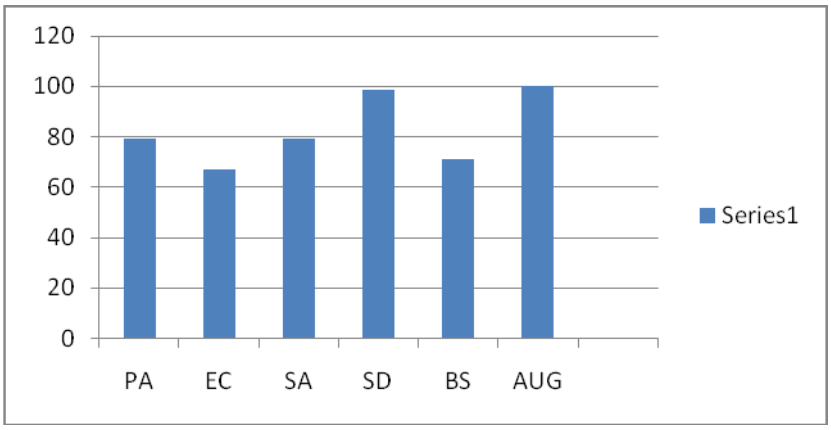

Figure 1: Bar chart of % susceptibility of methanol extract for bacteria

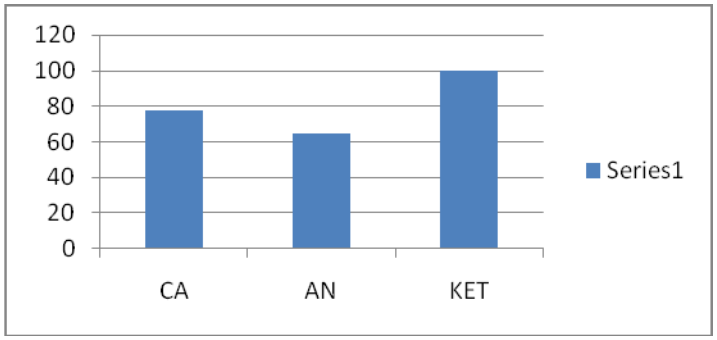

Figure 2: Bar chart of % susceptibility of methanol extract for fungi

Table 4: Result of Inhibition Zone Diameter (mm) of methanolic extract of leaves of *Nauclea latifolia* at different concentrations (mg/ml).

Organisms	Mean Inhibition Zone Diameters (mm) at different concentration (mg/ml) of the extract.				
	Concentration (mg/ml)				
	200.0	100.0	50.0	25.0	12.5
P.aeruginosa	13.4	12.3	10.0	8.3	-
E. coli	20.5	18.6	17.5	14.5	10.5
S. aereus	22.5	18.9	15.5	12.0	8.5
S. dysentariae	21.5	17.8	16.2	13.5	13.1
B. subtilis	23.0	20.1	15.5	11.0	10.5
C. albicans	25.7	20.0	17	14.6	7.2
A. niger	12.4	9.3	8.6	8.0	7.1

Table 5: Result of Inhibition Zone Diameter squared (mm^2) and Log Concentration of Methanolic extract of leaves of *Nauclea latifolia*

Organisms	Square of Mean Inhibition Zone Diameter (mm^2) of methanolic extract				
	Log concentration (200mg/ml-12.5mg/ml)				
	2.30103	2.00000	1.69897	1.39794	1.09691
P. aeruginosa	179.59	151.29	100.00	68.89	0
E. coli	420.25	345.96	299.29	210.25	106.09
S. aereus	506.25	357.21	240.25	144	72.25
S. dysentariae	462.25	316.84	262.44	182.25	171.61
B.subtilis	529	404.01	240.25	121	110.25
C. albicans	660.49	400	289	213.16	51.84
A. niger	153.76	86.49	73.96	64	50.41

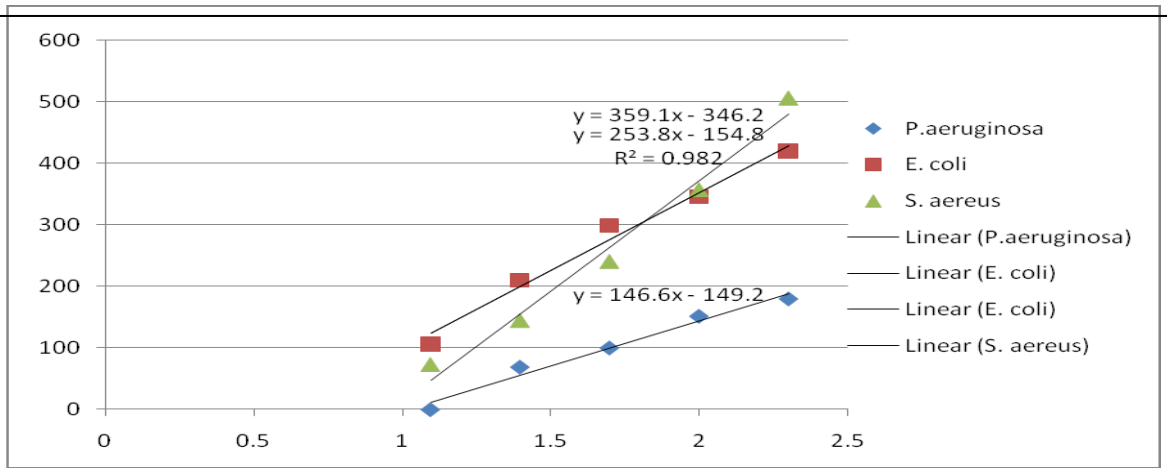

Figure 3: Methanol extract of leaves: Plot of square of inhibition zone diameter (IZD^2) (mm) against log concentration of methanolic extract of the leaves of *Nauclea latifolia*.

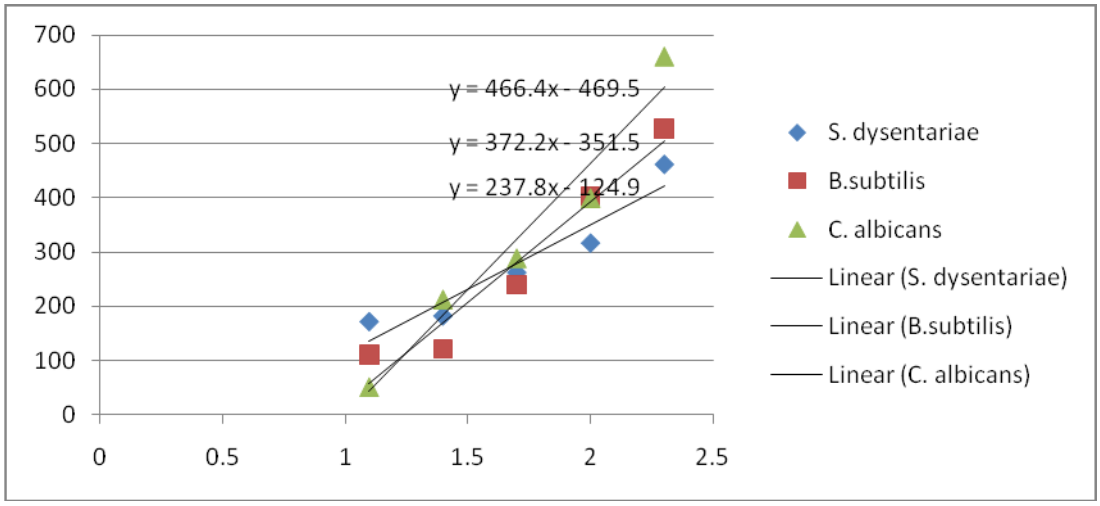

Figure 4: Plot of square of inhibition zone diameter (mm^2) against Log Concentration of methanolic extract of the leaves of *Nauclea latifolia*.

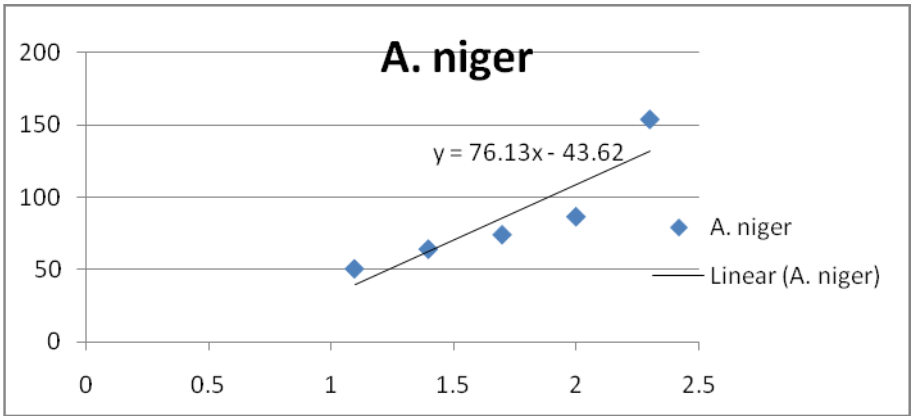

Figure 5: Plot of square of inhibition zone diameter (mm^2) against Log Concentration of methanolic extract of the leaves of *Nauclea latifolia*.

Table 6: Results of log conc. (mg/ml) of the extracts of leaves of *Nauclea latifolia* and the standards (µg/ml)

Test Organisms.	ME/L [(mg/ml]	AUG. [---- (µg/ml)----]	KET.
P. aeruginosa	1.018	0.697	-
E. coli	0.609	0.260	-
S. aereus	0.964	0.160	-
S. dysentariae	0.525	0.186	-
B. subtilis	0.944	0.380	-
C. albicans	1.006	-	0.087
A. niger	0.573	-	0.722

Key;Aug. = augmentin^R ket. = ketoconazole. L = leaves ME. = methanolic extract – no activity

The log of concentrations were extrapolated from the x-axis of the graph and then converted to antilogarithm which gave the minimum inhibitory concentration (MIC) of each of the clinical isolates in mg/ml for the extracts and µg/ml for the standards.

Table 7: Results of MIC (antilog.) of the extract and standards

Test	ME/L	AUG.	KET.

Organisms.	[(Mg/ml) -]	[---- (µg/ml) --]	
P. aeruginosa	10.42	4.98	-
E. coli	4.07	1.82	-
S. aereus	9.20	1.45	-
S. dysentariae	3.35	1.53	-
B. subtilis	8.80	2.40	-
C. albicans	10.14	-	1.22
A. niger	3.74	-	5.23

Key; Aug. = augmentin^{R} ket. = ketoconazole. L = leaves ME. = methanolic extract – no activity

RESULT

The phytochemical analysis revealed the presence of tannins, saponins, alkaloids, sterols and glycosides in varying degrees. These secondary metabolites have been reported to exhibit varied biochemical and pharmacological effects in animals and microorganisms when ingested (Trease and Evans 2008). The high content of saponins and tannins could be the basis for its antimicrobial activity which has been associated with antimicrobial effects. Tannins act by coagulating the cell wall proteins (Trease and Evans, 1983), while saponin causes the lysis of the bacterial cells (Robinson, 1975). Thus, this may therefore explain the demonstration of antimicrobial activity by the methanollic extract of leaves of *Nauclea latifolia*.

De and Ifeoma (2002), reported that the phytochemical components also offer plants themselves protection against infection by pathogenic microorganisms.

These results obtained in the phytochemical screening correlate with the work done by Hotellier *et al.,* (1979) and Morah (1995) who reported that Nauclea latifolia contains terpenes, alkaloids, glycoalkaloids and tannins.

The methanolic extract showed remarkable activity against all the microorganisms used for this work. When compared with the standard augmentin, it is about 78% as effective on Pseudomonas aeruginosa, 65% as effective on E.coli, 79% on Staphilococcus aureus, 65% as effective on Bacillus subtilis and 98.4% on S.dysentariae when compared with augmentin. On fungi, it is 68% as effective as ketoconazole – the standard drug used – on Candida albican and 63% on Aspergillus niger when also compared with ketoconazole. Using Analysis of Variance (ANOVA), there was no significant difference ($p>0.05$) between the inhibition zones of methanol extract of leaves at 100mg/ml when compared with Augmentin on *P. Aeruginosa and S. Dysentariae,*the result of the extract also showed a very significant difference ($p<0.05$) on its activity against *E.coli, S. aereus, and B. subtilis.* On fungi, there is a significant difference ($p<0.01$) between the effect of the extract and the standard antifungal agent-Ketoconazole.

CONCLUSION

The plant extract is very effective against the infective microorganisms studied. As a result it should be considered for further Pharmacological work and for inclusion into official compendium

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