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In Vitro Studies of Antibacterial Activities of *Nauclea latifolia* Root Extracts Using Micro Dilution Indicator Technique

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Abstract: *The antibacterial activities of methanolic, aqueous and chloroform root extracts of Nauclea latifolia were microbiologically investigated against some clinical bacterial isolates and reference organisms using microdilution indicator technique and conventional Kirby Bauer Disc diffusion technique. Nauclea latifolia roots extracts were phytochemically screened. Nauclea latifolia, root extracts revealed the presence of bioactive compounds such as alkaloids, tannins, saponins, flavonoids, cardiac glycosides, steroids and terpenoids. The Nauclea latifolia root extracts exhibited antibacterial activities against both the clinical isolates and reference organisms at varying degrees. The chloroform extract of the root of Nauclea latifolia showed highest inhibitory action. The minimum inhibitory concentrations (MIC) of chloroform root extracts ranging between 2.5mg/ml and 10.0mg/ml. The aqueous root extracts had the weakest inhibitory effect with minimum inhibitory concentrations (MIC) ranging between 7.5mg/ml and 12.5mg/ml. The findings suggest that chloroform extracts of the root of Nauclea latifolia that exhibited the highest antibacterial activities could be useful in the treatment of human pathogens.*

Keywords: *Nauclea latifolia root extracts, antibacterial activities, zone of inhibition, minimum inhibitory concentration, and phytochemical constituents.*

I. Introduction

The quest for plants with medicinal properties continues to receive attention as scientists' survey plants, particularly of ethno botanical significance, for a complete range of biological activities. Thus far, plants have provided western medicine with an abundance of drugs and treatments for a variety of health problems (Lewis and Elvin-Lewis, 1995). While species used in traditional medicines continue to be the most reliable sources for the discovery of useful compounds, the screening of plants growing under various conditions (Pernas *et al*., 2000) has provided yet another source for compounds with useful activities against microbes and other health conditions. Traditional healers have long used plants to prevent or cure infectious conditions; and Western medicine is trying to duplicate their successes. Plants rich in a wide variety of secondary metabolites, such as tannins, terpenoids, alkaloids, and flavonoids have been found in vitro to have antimicrobial properties.

N. latifolia is used for several medicinal purposes such as a tonic and fever medicine, chewing stick, toothaches, dental caries, septic mouth and malaria, diarrhea and dysentery, thus suggesting some antimicrobial activity (Lamidi *et al*., 1995; Falodun *et al*., 2007). There are studies showing the root has antibacterial activity against Gram positive and negative bacteria and antifungal activity (Iwu, 1993). It is most effective against *Corynebacterium diphtheriae*, *Streptobacillis sp.*, *Streptococcus sp.*, *Neisseria sp.*, *Pseudomonas aeruginosa*, and *Salmonella sp*. (Deeni, 1991).

It has been reported that *N. latifolia* is one of the six most prescribed medicinal plants among the Igede people of Benue State (Igoli, *et al*., 2005). The plant has also been reported to have antihypertensive and laxative activities (Akpanabiatu *et al*., 2005). The antihelmintic activity of the aqueous extract of the stem bark has been demonstrated (Onyeyili *et al*., 2001). Okoli and Iroegbu (2004) also reported the antimicrobial activity of the ethanolic and cold water root extracts of the plant. Also antibacterial activity of *N. latifolia* stem bark has been reported by Falodun *et al*. (2007) and revealed a broad spectrum antibacterial activity on both clinical isolates and reference strains of Gram positive and Gram negative organisms such as *Bacillus substillis*, *Staphlococcus aureus*, *Escherichia coli*, *Streptococcus varidas*, *Klebsiella pneumonia* and *Pseudomonas aeruginosa*. The aim of this study is to evaluate the antibacterial activities of *N. latifolia* root extracts against some clinical isolates and reference organisms. And also to screen the root extracts phytochemically.

II. Materials and Methods

The root of *Nauclea latifolia* was collected in June 2010 from the farmland behind Ex-Service Officers Estate, Kurudu Abuja, Nigeria. The plant was identified by Dr P.O Egwumah of the College of Forestry, Federal University of Agriculture Makurdi, Benue State, Nigeria.

2.1 Test Organisms

The microorganisms used were hospital isolates of *Staphylococcus aureus, Escherichia coli, Salmonella typhi, Pseudomonas aeruginosa* and *Streptococcus varidans* and reference strains of *Staphylococcus aureus (ATCC 28923), Escherichia coli (ATCC 25922)* and *Pseudomonas aeruginosa (ATCC 27853).*

2.2 Drugs and Their Sources

The following drug was used in the study:

1) Ciprofloxacin (CIPRO-J$^{(R)}$, Juhel, Nigeria) 500mg white scored caplet marked 'Cipro/500' on one side and 'Juhel' on the reverse.

2.3 Extraction Procedure

The root of *Nauclea latifolia* collected was sun dried and pulverized by pounding. One hundred grams of the powdered root of *Nauclea latifolia* was placed in a corked bottle, and 500 ml of solvent (methanol, chloroform or water) were added in the cold (cold extraction). The resulting suspensions were allowed to stand in a tightly covered bottle for 48 hours at room temperature, after which were filtered using Whatman's No.1 filter paper into a round bottom flask. The flask containing the filtrates were placed in the water bath and allowed to evaporate off the extraction solvent to obtain the crude extract. The crude extract were placed in sterile sample bottles and labeled appropriately. The yield were weighed and recorded in grams.

2.4 Microbiological Test- Determination of Minimum Inhibitory Concentration

2.4.1 Preliminary Test: The micro dilution assay and disc diffusion were carried out using Ciprofloxacin against the test organisms both the reference strains and clinical isolates to confirm the sensitivity of Ciprofloxacin against the test organisms.

2.4.2 Micro Dilution Assay Technique

The antibacterial activities of the various extracts were assayed using modified micro dilution techniques of Drummond and Waigh (2000), as described by Satyajit *et al*. (2007). Micro-titer plates were prepared under aseptic conditions. A sterile 96-well micro-titer plate was used and properly labeled. One hundred microlitres (100 µL) of test material in 10% (v\v) dimethylsulphoxide (a stock concentration of 10mg/ml) was pipetted into the first row of wells in triplicates. Sterile nutrient broth (50 µL) was added to all other wells. The extract was serially diluted two-folds using a multichannel pipette. Tips were discarded after use so that each well had 50 µL of the test material in serially descending concentrations. To each well 10 µL of resazurin indicator dye solution in sterile water was added. Bacterial suspension (10 µL) in nutrient broth ($5x10^6$ cfu/ml) was added to each well to achieve a concentration of $5x10^5$ cfu/ml.

Two columns were used as controls: positive control (comprising broad-spectrum antibiotic Ciprofloxacin); and negative control containing bacterial suspension, resazurin indicator dye and 10%v/v dimethylsulphoxide without the test extract. Finally each plate was sealed with paraffin film to ensure that the bacteria did not become dehydrated. Plates were incubated at 33°C for 18-22hrs. The plates were observed macroscopically for colour change and turbidity under the reading mirror. The minimum inhibitory concentration, which is the lowest concentration of the extract that caused bacterial growth inhibition, was read off and recorded.

2.4.3 Disc Diffusion Assay Technique

Antibacterial screening using the disk diffusion method as described by the NCCLS (2004) was carried out. Twenty milliliters (20ml) of molten sterile Mueller-Hinton agar was poured into sterile petri dishes and allowed to solidify. A loop full of inoculums containing $5x10^5$cfu/ml bacterial suspensions was uniformly streaked on the surface of the agar. Pre-sterilized filter paper discs of 3 mm diameter were impregnated with the different concentrations of extracts, and placed on the seeded agar. Ciprofloxacin discs (10µg) used as a positive control and discs saturated with sterile water (negative control) were placed at the center of the seeded plates and incubated for 18-22hrs at 33 to 37^{o}C. At the end of incubation period, diameter zones of inhibition in all three replicates were measured in millimeters using measuring slide and the mean of the three determined.

2.4.4 Determination of Minimum Bactericidal Concentration

The minimum bactericidal concentration was determined by sub-culturing the minimum inhibitory concentrations onto a sterile surface of Mueller-Hinton agar plates. The plates were incubated at 33^0C for 18-22 hours. The plates were observed for growth. Plates without colonies indicated minimum bactericidal concentration.

2.5 Phytochemical Test

Phytochemical test was carried out on the extracts for the following bioactive constituents: tannins, saponins, cardiac glycosides, alkaloids, steroids and flavonoids. Chemical tests were carried out on the extracts of the root using standard procedures to identify the constituents as described by Sofowara (1993), Trease and Evans (2002), and Harborne (1998).

2.5.1 Test for Tannins: Small portion of extracts of root each was stirred with 1ml absolute methanol and $FeCl_3$ (aq) added in a test tube. A blue black precipitate is indicative of the presence of tannins.

2.5.2 Test for Saponins:

i. Frothing test: Small portion of extracts of root each was shaken with water in a test tube. Frothing which persisted on warming is indicative of the presence of saponins.

ii. Emulsion test: Five drops of olive oil was added to 3ml of extracts of root in a test tube and the mixture was vigorously shaken to form stable emulsion. Formation of stable emulsion indicates the presences of saponins.

2.5.3 Test for Flavonoids: 2ml of solution of extracts was added to Magnesium chip and few drops of H_2SO_4 in a test tube. Light yellow precipitate in brown solution is indicative of flavonoids.

2.5.4 Test for Alkaloids:

i. Small portion of the extracts of root was dissolved in 2ml 0.1 HCl (aq) in a test tube. To this was added 2 drops of Mayer's reagent, a light yellowish white precipitate indicates the presence of alkaloids.

ii. Dragendroff's reagent, 0.85g basic bismuth nitrate in 10ml glacial acetic acid into 40ml water was added to small portion of extracts of root in a test tube and the mixture was heated for 2minutes. Faint yellowish orange precipitate indicates the presence of alkaloids.

2.5.5 Test for Cardiac glycosides: Keller-Killani test: To a small portion of solution of extracts in glacial acetic acid containing 2 drops of $FeCl_3$ (aq) was added 1.5ml conc. H_2SO_4. Lower conc. H_2SO_4 layer that is colourless, with upper acetic acid layer is indicative of the presence of glycosides.

2.5.6 Test for Steroids: The extracts were dissolved in 2ml of acetic anhydride and cooled ice conc. H_2SO_4 was carefully added. A colour change from violet to blue then to green indicates the presence of steroids.

III. Results

The phytochemical investigation are as shown on table 1, revealed that the plant *N. latifolia* root extract contained some of the phytochemicals such as alkaloid, saponin, flavonoids, cardiac glycosides and steroids especially for the chloroform extract. The result of the antibacterial activities of the root extracts of *Nauclea latifolia* using modified indicator based micro dilution technique as shown on table 2, revealed that the chloroform extract had the highest inhibitory effects on the organisms both reference strains and hospital isolates. The MIC values ranging between 2.5mg/ml and 10.0mg/ml. *E.coli ATCC 25922*, hospital isolates of *E.coli* and *S. aureus* had 2.50mg/ml. *S. aureus ATCC 28923*, *Streptococcus varidans*, *Salmonella typhi* and *P. aeruginosa* had 5.0mg/ml while *Pseudomonas aeruginosa ATCC 27853* had 10.0mg/ml. The MIC of the methanol extract ranged between 5.0mg/ml and 12.5mg/ml. *E.coli ATCC 25922* had 5.0mg/ml, the hospital isolate of *Strep. varidans, Sal. typhi* and *E. coli* had 7.5mg/ml respectively. *S. aureus ATCC 28923* and hospital isolate of *S. aureus* had 10.0mg/ml. *P. aeruginosa ATCC 27853* and hospital isolate of *P. aeruginosa* had 12.5mg/ml. The aqueous extract had the weakest inhibitory effect with MIC 7.5mg/ml against *E. coli ATCC 25922* and MIC 10.0mg/ml against *S. aureus ATCC 28923*, hospital isolate of *Strep. varidans, Salmonella typhi*, and *E.coli.* 12.5mg/ml against hospital isolate of *S. aureus* while *P. aeruginosa ATCC 27853* and hospital isolate of *P. aeruginosa* were resistant to the available concentrations.

The result of the MIC of the *Nauclea latifolia* root extract using Disc Diffusion Technique as shown on table 3, revealed that the MIC of chloroform extract on the reference strains and hospital isolates was 5.0mg/ml except *P. aeruginosa ATCC 27853* with MIC 10.0mg/ml. The aqueous extract had the MIC of 7.5mg/ml on *Strep. varidans*, 10.0 mg/ml on the *S. aureus ATCC 28923*, *E. coli ATCC 25922* and *S. typhi*, the MIC

12.5mg/ml on *E.coli* and *S. aureus. P. aeruginosa ATCC 27853* and hospital isolate of *P.aeruginosa* were resistant. The MIC's of the methanolic extract ranged between 7.5mg/ml and 12.5mg/ml. *E.coli ATCC 25922* and *Streptococcus varidans* had 7.5mg/ml. The MIC of *S. aureus ATCC 28923*, hospital isolates of *S. aureus, S. typhi* and *E. coli* were 10.0mg/ml. *P. aeruginosa ATCC 27853* and hospital isolate of *P.aeruginosa* had 12.5mg/ml. The chloroform root extracts exhibited the highest bactericidal effect against the organisms with MBC value of 5.0mg/ml except for *P. aeruginosa ATCC 27853* with MBC of 10.0 mg/ml. The methanol and aqueous extracts had bactericidal effect of MBC values ranging between 7.5mg/ml and 12.5mg/ml as shown on table 4. The chloroform extracts had the highest zone of inhibition between 15mm and 20 mm. Aqueous extract had zone of inhibition between 8mm and 12mm meanwhile the zone of inhibition of *P.aeruginosa ATCC 27853* and *P. aeruginosa* were not determined. Methanol extracts had zone of inhibition 10mm to13mm as shown on table 5.

Table 1: Phytochemical constituents of the root extracts

Test	Methanol Extract	Chloroform Extract	Water Extract
Alkaloid	+	+	+
Saponin	+	+	+
Tannins	+	-	+
Flavonoids	+	+	+
Cardiac glycosides	+	+	-
Terpenoids	+	-	+
Steroids	+	+	-

Key: +: Presence of constituent; -: Absence of constituent

Table 2: Minimum inhibitory concentrations (mg mL^{-1}) of root extracts obtained by the Micro Dilution Technique

Organisms	Methanol	Water	Chloroform	Ciprofloxacin	Viability Control
Reference Strains					
S. aureus ATCC 28923	10.0	10.0	5.00	0.003	-
E. coli ATCC 25922	5.0	7.5	2.5	0.003	-
P. aeruginosa ATCC 27853	12.5	-	10.0	0.003	-
Hospital strains					
Streptococcus varidans	7.5	10.0	5.00	0.003	-
Salmonella typhi	7.5	10.0	5.00	0.003	-
P. aeruginosa	12.5	-	5.00	0.003	-
Escherichia coli	7.5	10.0	2.50	0.003	-
Staphylococcus aureus	10.0	12.5	2.50	0.003	-

Key: - : growth uninhibited

Table 3: Minimum inhibitory concentrations (mg mL^{-1}) of root extracts obtained by Disc Diffusion Assay Technique

Organisms	Methanol	Water	Chloroform	Ciprofloxacin	Viability Control
Reference Strains					
S. aureus ATCC 28923	10.0	10.0	5.0	0.003	-
E. coli ATCC 25922	7.5	10.0	5.0	0.003	-
P. aeruginosa ATCC 27853	12.5	-	10.0	0.003	-
Hospital strains					
Streptococcus varidans	7.5	7.5	5.0	0.003	-
Salmonella typhi	10.0	10.0	5.0	0.003	-
P. aeruginosa	12.5	-	5.0	0.003	-
Escherichia coli	10.0	12.5	5.0	0.003	-
Staphylococcus aureus	10.0	12.5	5.0	0.003	-

Key: - : growth uninhibited

Table 4: Minimum bactericidal concentrations (mg mL^{-1}) of root extracts obtained by the Micro Dilution technique

Organisms	Methanol	Water	Chloroform	Ciprofloxacin	Viability Control
Reference Strains					
S. aureus ATCC 28923	10.0	10.0	5.0	0.003	-
E. coli ATCC 25922	7.5	7.5	5.0	0.003	-
P. aeruginosa ATCC 27853	12.5	-	10.0	0.003	-
Hospital strains					
Streptococcus varidans	7.5	10.0	5.0	0.003	-
Salmonella typhi	10.0	10.0	5.0	0.003	-
P. aeruginosa	12.5	-	5.0	0.003	-
Escherichia coli	10.0	12.5	5.0	0.003	-
Staphylococcus aureus	10.0	12.5	5.0	0.003	-

Key: - : growth uninhibited

Table 5: Diameter of inhibition zone (mm) of root extracts

Organisms	Methanol	Water	Chloroform	Ciprofloxacin	Viability Control
Reference Strains					
Staphylococcus aureus ATCC 28923	12	10	18	32	-
Escherichia coli ATCC 25922	13	12	20	32	-
Pseudomonas aeruginosa ATCC 27853	11	-	16	28	-
Hospital strains					
Streptococcus varidans	12	10	20	34	-
Salmonella typhi	12	10	18	34	-
Pseudomonas aeruginosa	10	-	15	30	-
Escherichia coli	12	10	18	32	-
Staphylococcus aureus	10	8	16	32	-

Key: Each value represents the mean of three different observations; -: growth uninhibited

IV. Discussion

This study evaluated the phytochemical constituents of *N.latifolia* root extracts. The phytochemical tests are confirmatory of the published report of the key constituents of *Nauclea latifolia* (Iwu *et al.*, 1999). Plants are known to contain secondary metabolites (active principles) these include alkaloids, saponins, terpenoids, tannins, flavonoids and glycosides; these elicit physiological response. The chloroform extracts contained some of the bioactive compounds such as the alkaloid, saponins, flavonoids, and cardiac glycosides. This may be responsible for its antibacterial activities. This result is in conformity with the report of Abiodun *et al*. (2007) as well as the work done by Morah (1995) who reported that *Nauclea latifolia* contains terpenes, alkaloids, glycoalkaloids and tannins, and could be the reason for it significant antibacterial activity against the test organisms. Previous studies reported that the root contains specific type of alkaloid called indole-quinolizidine alkaloids as well as glyco-alkaloid (Iwu, 1999; Shigemori *et al.*, 2003).

Some studies have reported that flavonoids had antitrypanosomal activity (Tarus *et al*., 2002) while Nok (2002) reported anzaanthraquinone to have activity against *T. congolense*.

This study also revealed that the plant *Nauclea latifolia* root extracts possesses significant antibacterial activity against some hospital isolates of *E. coli, P. aeruginosa, Staphylococcus aureus* and their reference types. This finding agrees with the previous antibacterial studies related to this plant and reported by Abiodun *et al*. (2007). This result equally confirmed the reported antibacterial properties of the root of the same plant (Deeni and Hussain, 1991). The significant antibacterial activity of the plant could be attributed to the presence of bioactive compounds in the plant. This result suggests that the plant could be used as a potential broad spectrum antibacterial agent for treatment of bacterial infections.

Antibacterial activity and susceptibility assay of the plant *Nauclea latifolia* varied according to extraction solvent. This indicates that the minimum inhibitory concentration (MIC) and zone of inhibition of the root extracts were affected by the solvent used for extraction. For example the chloroform root extract, which showed the highest inhibitory effect compared to the methanol and water. The high activity may probably be due to the type of bioactive compounds precipitated and present in the extracts as suggested by Abiodun *et al*. (2007) and the plant part which is the root agrees to the findings of Banso and Olatimayin (2001) which stated that extracts from the roots are more effective than those from the bark and leaves. And also may be due to the phytochemical extraction capability of the extraction solvent (Jonaid *et al.*, 2006). Similarly the studies of Umeh *et al*. (2005) showed that chloroform and petroleum ether extracts of the medicinal plants studied have more inhibitory effect than methanolic and aqueous extracts.

Although the inhibitory effects of aqueous and methanolic extracts are often reported (Omer *et al*., 1998). From the results obtained, seven bacteria out of eight were more susceptible to the chloroform root extracts. The reference type; *Pseudomonas aeruginosa ATCC 27853* was the only organism that was less susceptible to the extract. Chloroform extracts exhibited the best inhibitory effects compared to the methanolic and aqueous extracts.

V. Conclusion

Nauclea latifolia chloroform root extracts has exhibited varying degrees of inhibitory effects against several organisms based on the antibacterial activities. Therefore could be considered a potent antimicrobial agent in the treatment of bacterial infections and dental related infections.

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