Effect Of Crude Ethanol Extract Of *Nauclea Latifolia* On Some Clinical Isolates Of Food Importance And Its Toxicological Potentials

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Abstract: The leaves of *Nauclea latifolia* used in traditional medicine for the treatment of diarrhea were extracted in hot and cold ethanol with the aim of determining their antibacterial activities and toxicological potentials. Soxhlet extraction method was used for the hot ethanol extraction while for the cold ethanol ground samples were soaked in ethanol for 48h. The Agar diffusion method was used for the antibacterial assay at different concentrations on *Escherichia coli, Staphylococcus aureus, Pseudomonas aeruginosa* and *Salmonella typhi.* Albino rats were used for toxicological studies by injecting varying doses of the extracts through the intraperitoneal route for 14 days. The growth of *S. typhi* was not inhibited by the extract. However the hot ethanol extract had minimum inhibitory concentrations (MICs) of 3.24mg/ml, 3.28mg/ml and 4.82mg/ml on *E. coli*, *S aureus* and P. *aeruginosa* respectively, while the cold ethanol extract produced MICs of 4.74mg/ml, 5.14mg/ml and 5.61 mg/ml respectively on the isolates. Hematological analyses revealed that RBC, PCV and Hb values decreased with increase in doses of extract while ESR and WBC values increased. MCHC values were lower than that obtained from the control. However, statistical analyses revealed that there were significant differences in the values obtained for RBC, PCV and Hb at the lower doses and the controls. The extracts contained alkaloids, tannins and saponins. The antibacterial assay justifies the use of the plant in palm wine preservation and possible use in preservation of other foods, and the treatment of diarrhoea.

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Key words: Antibacterial; toxicological; preservation; ethanol extracts; *Nauclea latifolia;* clinical isolates.

Introduction

Throughout recorded history, spices and herbs have been used for flavouring foods and beverages, and for medicinal purposes. Also a wide range of natural products from plants can be useful in extending shelf life of foods, reducing or eliminating pathogenic bacteria, and increasing overall quality of food products (Droughon, 2004).

There are estimations that as much as 250,000-500,000 plant species are on Earth (Boris, 1996) and thousands of compounds have been isolated from these plants which are claimed to posses antimicrobial or medicinal properties (Schultes, 1978; Cowan, 1999). Although numerous studies have been done in vitro to evaluate the antimicrobial activity of botanicals, only a few have been done with food products (Doughon, 2004; Hancock and Harrison, 2002; Tsigarida et al., 2000; Gill et al., 2002; Diaz et al., 2002; Lemay et al., 2002).In Nigeria also some ethnobotanical texts are available which describe the species that posses antimicrobial and medicinal properties (lwu, 1993; Soforowa, 1982; Igoli et al., 2005). Some have been found to inhibit the growth of some food and clinical isolates (Akujobi et al., 2004; Esimone et al., 1998; Ogbulie et al., 2004; Ogueke et al., 2006; Ogbulie et al., 2007; Ogueke et al., 2007a; Ogueke et al., 2007b). Amongst these plants that posses medicinal properties is *Nauclea latifolia*.

Nauclea latifolia belongs to the family Rubiaceae (Ntiejumokwu and Kolawole ,1991) and is a common plant in the tropical forests of South Eastern Nigeria. It is commononly used by palm wine tappers as a preparative ("*nche*") for palm wine. In traditional medicine the leaves and the stem bark are used in the treatment of diarrhea (Igoli et al., 2005). It is called "African quinine in Northern Nigeria, a cold infusion of the bark is taken as a diuretic and anthelmintic. The Fulanis in Nigeria uses the leaf extract to regularly deworm animals (Adebowale 1993). The ethanol extract have been found to decrease the level of parasitaemia in a dose-dependent manner in mice experimentally infected with a *Trypanosoma brucei* (Morah, 1998). Different indolo-quinolizidine alkaloids and glycol-alkaloids have been isolated from the root bark. The former has been identified and named anguistine, angustoline. The glyco alkaloids have been identified as cadambine 3-a-dihydro cadambine (Hottellier *et al*, 1975).

There is therefore need to authenticate the antibacterial effects of the crude plant extract, especially on the diarrheal causing microorganisms, and determine their toxicological potentials, at least on laboratory animals. This is a prelude to determining their preservative potentials in a food system. Thus the objectives of this, work were to authenticate the antibacterial properties on some clinical isolates of food importance and determine the toxicological potentials on albino rats.

Materials and Methods

Plant collection and identification: Fresh leaves of *N. latifolia* were obtained from Obinze, Owerri West Local Government Area of Imo State, Nigeria in August, 2009. The plant was certified by Dr. I. I. Ibeawuchi of the Department of Crop Science Technology, Federal University of Technology, Owerri. Specimen voucher was deposited in the herbarium of the Department of Crop Science Technology, Federal University of Technology, Owerri, Nigeria.

Sample preparation and extraction procedure

The fresh leaves were air dried and ground into fine powder using a mechanical grinder. Two methods, the hot ethanol extraction and cold ethanol extraction techniques were employed for the extraction of the plant's active principles.

For the hot ethanol extraction technique, 20g of the ground plant material were wrapped in Whatman filter paper, each wrap containing 2.0g. They were put in the timble of a Soxhlet apparatus. Then 250 ml of 95 % ethanol was put in a round bottom flask and this was used to mount the Soxhlet apparatus. The round bottom flask was heated and extraction of the plant material was stopped after seven refluxes. The solution was then evaporated to dryness using a rotary evaporator (model type 34/2; Corning Ltd, England). Yield of 17.95% was obtained in relation to the powdered material.

For the cold ethanol extraction technique 20g of the ground plant sample was weighed into 250 ml of ethanol (95%) in a conical flask. It was covered, shaken every 30 mins, for 6h and thereafter allowed to stand for 48h for extraction. The solution at the end of extraction was shaken and filtered using Whatman filter paper. The filtrate was subsequently evaporated to dryness using a rotary evaporator. Yield of 17.60% was obtained in relation to the powdered plant material.

The extracts obtained were thereafter stored at 10^{o}C in amber coloured bottles until required.

Preparation of stock solutions of extracts

The method of Akujobi et al., (2004) was adopted for the preparation of stock solutions. The crude extracts obtained were diluted with 20% dimethyl sulphoxide (DMSO) solution to obtain 50mg/ml, 25mg/ml, 12.5mg/ml, 6.25mg/ml and 3.2mg/ml concentrations. They were stored at 10^{o}C in amber coloured bottles until required.

Test microorganisms and their sources

The bacterial isolates used for the study were clinical isolates of food importance. These were *Escherichia coli (5 isolates), Staphylococcus aureus (5 isolates), Salmonella typhi (7 isolates)* and *Pseudomonas aeruginosa (3 isolates).* They were obtained from the Microbiology Laboratory of Federal Medical Centre (FMC) Owerri, Nigeria. They were re-identified, sub-cultured on Nutrient agar slants for *Escherichia coli and Pseudomonas aeruginosa*, Baird Parker agar slant for *Staphylococcus aureus*, Deoxycholate citrate agar slant for *Salmonella typhi* and stored at 4^{o}C until required.

Evaluation of antibacterial activity

The well in agar diffusion method as described by Esimone et al.,(1998) and Osadebe and Ukwueze (2004) was adopted for the study. Standardized Nutrient broth cultures of the test isolates containing

approximately 10^7 cells/ml organisms were used. 0.1ml of the broth cultures were introduced into sterile Petri dishes and 15mls of molten Nutrient agar poured into the Petri dishes. The contents were thoroughly mixed and allowed to solidify. Three holes each measuring 5.0mm in diameter were made in each of the solid agar plates using a sterile cork borer. 0.04ml of the different concentrations of plant extracts were transferred into the holes using a Pasteur pipette. Two Petri dishes containing a particular bacterium were used for each concentration of the extracts. The plants were thereafter allowed to stand for one hour for pre-diffusion of the extracts (Esimone et al., 1998) and were subsequently incubated at 37° C for 24 h.

After incubation, the plates were collected and the zones of growth inhibition were measured. The minimum inhibitory concentrations (MICs) of the extracts were determined by plotting a graph of the log of concentrations used (x - axis) against the squares of the zones of growth inhibition (y- axis). A regression line was then drawn through the points and the antilogarithm of the value at the intercept on the x- axis gave the MIC values (Osadebe and Ukwueze, 2004; Esimone et al., 1998).

Evaluation of haematological toxicity on albino rats

Initial LD_{50} studies were carried out to determine the maximum dose of extracts that will not produce any death on the rats. Based on the LD_{50} studies, four groups of albino rats (male) each comprising three rats, randomly selected and weighing 132.5g was used. Doses of 30.2 mg/kg body weight, 60.4mg/kg body weight, 120.8 mg/kg body weight and 241.6 mg/kg body weight were injected into each group through the intraperitoneal route (Iyaniwura et al., 1991, EFPIA/ECVAM, 2001) on daily basis for 14 days. The control group was injected with the diluent (20% DMSO solution). Food and water were provided adlibitum.

On the 15^{th} day, the animals were collected and blood samples drawn from the sublingual vein according to the method described by Zeller et al., (1998). This method has been found suitable for laboratory animal's well being as stated in EFPIA/ ECVAM (2001). 3.0ml of blood sample was immediately transferred to ethylene di-amine tetra acetic acid (EDTA) treated bottles for hematological assay. They were analyzed within 3h of collection for total erythrocyte (RBC), leukocyte (WBC) counts, packed cell volume (PCV), haemoglobin (Hb) contents, serum glutamate pyruvic transaminase (SGPT) and serum glutamic oxaloacetic transaminase (SGOT) according to the methods described by Okeudo et al., (2003). ESR was determined according to the method described by Okeudo et al.(2003)and Iheukwumere et al.(2002). Varied haematological indices were calculated from the results obtained. These included mean corpuscular volume (MCV), mean corpuscular haemoglobin (MCH), and mean corpuscular haemoglobin concentration (MCHC).

Preliminary phytochemical analysis of extracts

These were carried out according to the methods described by Trease and Evans (1989) for determination of alkaloids, tannins, saponins, flavonoids, cardiac glycosides and cyanogenic glycosides.

Analysis of data

Statistical analyses were conducted on the data obtained from the study using Analysis of Variance (ANOVA). The means were separated using Fisher's Least Significant Difference (Sanders, 1990).

Results

The results obtained from the study showed that in general the hot ethanol extracts produced greater inhibitory effect on the isolates than the cold ethanol extracts.

Table 1 shows the antibacterial activity of the crude hot ethanol extract of the plant on the isolates. *E. coli* and *S. aureus* were inhibited by all the concentrations applied while *S. typhi* was not affected by any of the concentrations. The 3.2 mg/ml concentration of the extract did not inhibit the growth of *P. aeruginosa* while the other concentrations had inhibitory effects on the bacterium.

Table 2 shows the antibacterial effect of the cold ethanol extracts on the isolates. All the isolates were not inhibited by the 3.2mg/ml concentration of the extract. *S. typhi* was not affected by all the concentrations of the extract.

Table 3 shows the MICs of the extracts on the bacterial isolates. The lowest MIC was produced by the hot ethanol extract on *E. coli* with MIC value 3.24 mg/ml. The highest MIC value was obtained from the cold ethanol extract with a value of 5.61 mg/ml on *P. aeruginosa*. In general the hot ethanol extract produced lower MIC values than the cold ethanol extract.

Table 4 shows the results of the haematological analyses of the blood samples obtained from rats injected with different doses of the extracts. It was observed that increase in doses of extract resulted in decrease in the RBC, PCV and Hb values of the blood samples. However there was no significant difference in these values with those obtained from the control up to 60.4 mg/kg body weight. ESR and WBC values increased with increase in doses applied.

Statistical analysis revealed that there was no significant difference in values and the control at 30.2 mg/kg body weight dose for ESR and WBC respectively. However, for MCV, MCH and MCHC, there was no ordered pattern in the values as doses administered increased. Values obtained for MCV and MCH were higher than that obtained for the control while the values of MCHC were lower than the control.

Table 5 shows the result of the preliminary phytochemical analyses of the extracts. Tannins, alkaloids and saponins were identified in the extracts.

Results

Table 1: *Antibacterial activity of crude hot ethanol extract of *N. latifolia* on isolates

Concentration of Extract (mg/ml)	Mean diameter zone of inhibition (mm) *E. Coli*	*S. aureus*	*P. aeruginosa*	*S. typhi*	
3.2	8.7	6.4	-	-	
6.25	13.8	11.6	7.5	-	
12.5	19.7	16.3	14.8	-	
25.0	26.6	22.3	21.9	-	
50.0	32.2	29.9	25.5	-	

*Results are average of the triplicate determinations of the isolates

Table 2:* Antibacterial activity of crude cold ethanol extract of *N. latifolia* on isolates.

Concentration of Extract (mg/ml)	Mean diameter zone of inhibition (mm) *E. Coli*	*S. aureus*	*P. aeruginosa*	*S. typhi*	
3.2	-	-	-	-	
6.25	7.2	6.3	5.9	-	
12.5	10.5	9.7	9.6	-	
25.0	14.6	13.5	13.8	-	
50.0	17.9	17.1	17.9	-	

*Results are average of the triplicate determinations of the isolates

Table 3: The minimum inhibitory concentration (MIC) of *N. Latifolia* extracts on isolates.

Minimum inhibitory Concentration (mg/ ml)				
Type of extract	*E. coli*	*S. aureus*	*P. aeruginosa*	S.aureus
Hot ethanol	3.24[a]	3.28 [a]	4.82[b]	NIL
Cold ethanol	4.74[a]	5.14[b]	5.61[b]	NIL

a,b....values on the same now with the same superscript are not significantly different [P=0.05]

Table 4: Results of the haematological toxicity tests on rats injected with varying doses of *N. latifolia* ethanol extract.

Doses of extracts administered [mg/kg body weight]					
Parameters	Control	30.2	60.4	120.8	241.6
RBC (x10^6 cells/mm^3)	5.32[a]	4.78[a,b]	4.49 [a,b]	4.01b	3.93[b]
PCV (%)	36.3[a]	35.4[a]	35.0[a]	33.6[b]	32.6[b]
ESR (mm/hr)	3.50[a]	3.60[a]	4.14[b]	5.03 [c]	5.51[c]
MCV (cubic microns)	68.23[a]	74.06[b]	77.95[c]	83.79[d]	82.95[d]
Hb (g /100ml)	9.8[a]	9.5[a]	9.2 [a,b]	9.0[b]	8.4[a]
MCHC(%)	27.00[a]	26.84[a]	26.29 [b]	26.78 [a]	26.69[a]
WBC(X10^3 cells / mm^3)	4.77[a]	4.96[a,b]	5.02 [a,b]	5.39, [a,b]	5.73[b]

a, b..... values on the same row with the same superscript are not significantly different (P = 0.05)

Table 5: Phytochemical analysis of crude hot and cold ethanol extracts of *N. latifolia*.

Type of extract	Alkaloids	Tanins	Saponins	Flavonoids	Cardiac glycosides	Cyanogenic glycosides
Hot Ethanol	+	+	+	-	-	-
Cold ethanol	+	+	+	-	-	-
+ =	Present	- =	Absent			

Discussion

The results obtained from this study showed that the extracts inhibited the growth of the isolates except *S. typhi*. However, the hot ethanol extract had greater inhibitory effect on the isolates than the cold ethanol extract. That the extract inhibited the growth of the isolates is an indication that they contain substance(s) that are active against bacteria. Other workers have also shown that extracts of plants inhibit the growth of various bacteria (Akujobi et al., 2004, Esimone et al., 1998, Nweze et al., 2004 Osadebe and Ukwueze, 2004, Ntiejumokwu and Kolawole, 1991). That the extract did not inhibit the growth of *S. typhi* may be due to the fact that the bacterium posses mechanisms for detoxifing or removing the active principles. Some bacteria such as *S. aureus* detoxify penicillin by converting it to penicillinic acid (Braude, 1982). The observed antibacterial activities of the extracts may be due to tannins, alkaloids and saponins, either singly or in combination, identified in the extracts. Some workers (Hottellier *et al*, 1975) have identified indolo-quinolizidine alkaloids and glycol-alkaloids (cadambine 3-a-dihydro cadambine) in the root barks. These groups of compounds have been identified to posses antibacterial properties (Draughon, 2004).

That the hot ethanol extract produced greater inhibitory effect than the cold ethanol extract shows that the extraction method employed affects the yield of the active principles even when the same solvent is used. Probably application of vigorous heat in an enclosed system such as is found when using the Soxhlet apparatus increases the rate and yield of extraction of the active principles. It could therefore be advised that such method be employed for extraction of plant active principles. Some workers (Ogbulie et al., 2004) have also shown that Soxhlet extraction method is more efficient in the extraction of plant active principles than other methods of extraction.

The results obtained from the determination of the MICs indicate that the extracts, especially the hot ethanol extracts, could inhibit these isolates at low concentrations. This is most evident in the MIC of the hot ethanol extract on *E. coli.* Such extracts that could produce such low MIC on microorganisms could be of great importance in food preservation especially as they are natural, where they could be used in place of the common synthetic preservative substances. This will reduce the usual health risks associated with the use of these substances. Several works are in literature showing that plant extracts can be used for preservation of certain foods (Hancock and Harrison, 2002; Tsigarida et al., 2000; Cate et al., 2000; Elgayyar et al., 2001 Diaz et al., 2002; Lemay et al., 2002; Pszozola, 2002; Leuschner and Zamparini, 2002; Gill et al., 2002). Thus upon purification to remove other compounds in the extract, better results could be achieved. Some workers have shown that further purification of extracts could enhance their antibacterial properties (Okoli and Iroegbu, 2004).

The results are also of significance in the health care delivery system; since they could be used as alternatives to orthodox antibiotics in the treatment of infections caused by these organisms, especially as these organisms frequently develop resistance to the orthodox antibiotics (Singleton, 1999). The level of growth inhibition exhibited by the extract especially on *E. coli* justifies the use of the plant by traditional medical practitioners in the treatment of diarrhea (Igoli et al., 2004) and its use in the preservation of palm wine by palm wine tappers. *E. coli* is known to be a major cause of various diarrhoegenic infections (Adams and Moss 1999) in the developing countries.

Results from the haematological analyses of the blood samples revealed that RBC, PCV and Hb values decreased with increase in dose of extract administered. The consistent decrease in the RBC count with increasing level of extract administered is an indication that in its present crude form the extract may be destroying circulating erythrocytes or may have impaired the blood forming (erythropioetic) centres of the rats. However, statistical analysis revealed that up to 60.4 mg/kg body weight, the values were not significantly different from the values obtained from the control, indicating that at those lower doses the extract may not adversely affect the erythrocytes. Some other workers (Aniagu et al., 2005) however, have shown that some other plant extracts could remarkably increase the level of red blood cells.

Since PCV is an indication of the ratio of the red cell component to the total blood volume (Smith et al., 1974; Fischbach, 1980), it would seem that increase in dose of the extract decreased the red cell component, thus indicating that the effect of the extract was on the circulating red cells. However, statistical analysis also revealed that up to 60.4 mg/kg body weight, the values were not significantly different from the value obtained from the control.

The consistent decrease in Hb with increase in dose of extract is an indication that the extract destroyed the haemoglobin in the red blood cells or they impaired the uptake and utilization of iron by the

rats. Sokunbi and Egbunike (2000) and Iheukwumere et al., (2000) have suggested that some plant extracts affect iron uptake and utilization by animals. It could also be that the extract affected the uptake and utilization of copper, an element necessary for the utilization of iron in the production of haemoglobin (Smith et al., 1974). Statistical analysis, however, showed that up to 60.4 mg/kg body weight the values were not significantly different from that obtained from the control.

Results obtained for MCV and MCHC show that the extract induced hypochromic macrocytic anaemia in these rats. Smith et al. (1974) stated that the outpouring of less mature erythrocytes (reticulocytes) in response to the destruction of circulating red blood cells usually results in increased MCV and also usually contain lower percentage of Hb per erythrocyte.

For the white blood cells (WBC) the values increased with increase in doses of extracts. It could be that the presence of the extract stimulated the production of more WBC, probably to fight an intruder (the extract). This however, is normal. Statistical analysis revealed that there was no significant difference between the values obtained at different doses and the control.

The results obtained from the study are of significance as the plant is used in traditional medicine for the treatment of diarrhea. More studies should be conducted to determine the effectiveness of the extract upon refinement on preservation of food, and the administration of the extract on the vital organs of the body.

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