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Original article

Antimicrobial Activity of Nauclea Latifolia Smith on Multiresistant Bacterial Strains

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Abstract

Nauclea latifolia Smith (Rubiaceae) is a plant with medicinal properties much better known in sub-Saharan Africa in the traditional pharmacopoeia for its numerous pharmacological activities. It is a very abundant tree or shrub in West and Central Africa. Its wide use in traditional medicine has prompted us to carry out an in vitro evaluation of its antimicrobial potential on multidrug-resistant strains in order to ensure consistency between the prescriptions and the potential activities of the chemical constituents present. N. latifolia Sm. due to the presence of numerous chemical families has interesting biological activities. The aqueous and hydroalcoholic extracts from the bark and roots of the plant have proven antimicrobial activity. These extracts inhibit the proliferation of multidrug resistant strains of S. aureus, E. coli and K. pneumoniae. On the other hand, these extracts showed no efficacy on the strains of C. albicans at the doses used (200 mg/ml; 100 mg/ml and 50 mg/ml).

Keywords: ***Nauclea latifolia Smith; antibacterial activity; multiresistant; antimicrobial; medicinal plant***

Introduction

Since their inception, antibiotics have remained the most preferred means of fighting bacterial infections. Among these antibiotics, beta-lactams are nowadays the most widely used across the world and more particularly in developing countries like the Republic of Congo. The reason for this preference would be the extent of their action spectrum, harmlessness, effectiveness and especially their costs meeting all budgets (Livermore,1995). In contrast, inadequate and abusive self-medication of antibiotics by humans has led to the emergence of multidrug-resistant strains. Numerous cases of multidrug resistance have been reported for the Congo and other African countries (Savard, 2003). Faced with therapeutic failures using benchmark antibiotics, the antibiotics of tomorrow will have to target new targets for action in bacteria. There are many avenues of research, but the exploration of secondary metabolites of aromatic and medicinal plants appears to be the most promising because, by virtue of their biological diversity, these constitute the largest reserve of bioactive substances. According to the World Health Organization (WHO), nearly 80% of populations depend on traditional medicine for primary health care (OMS,2002). Significant economic gains in the development of this medicine and in the use of medicinal plants for the treatment of many diseases have been observed (Muthu *et* al.,2006). However, it was not until the beginning of the 20th century that scientists began to take an interest in it (Yano *et* al., 2006).

In this context, the objective of this study is to evaluate, in vitro, the antimicrobial activity of the total aqueous and hydroalcoholic extracts of the bark and roots of Nauclea latifolia against a number of multiresistant bacterial and fungal strains.

Materials and Methods

Materials

Plant material

The plant material used consisted of the bark and roots of *Nauclea latifolia Smith* collected in the central basin department, north of Congo-Brazzaville from December 2016 to March 2018, the chemical study of which was carried out at the National Institute of Research in Health Sciences (INRSSA).

Bacterial strains

Consists of two (2) reference bacterial strains and six (6) strains provided free of charge by the National Public Health Laboratory (LNSP) of Congo, five (5) of which are multi-resistant.

Table I: Profile of the tested microorganisms

Strains	Group	Profile	Origins
Escherichia coli LNSP	Gram -	BLSE	Urine
Escherichia coli BLSE/AmpC		BLSE	Urine
Klebsiella pneumoniae LNSP		Sensitive to C3G	Urine
Klebsiella pneumoniae C3GR		C3G resistant	Urine
Staphylococcus aureus CIP103429	Gram +	Sensitive to methicillin	Reference
Staphylococcus aureus SARM/LNSP		Methicillin resistant	Pus
Candida albicans LNSP	Yeasts	Fungizone resistant	Vaginal swab
Candida albicans CIP2503		Sensitive to Fungizone	Reference

Methods

Preparation of plant extracts

The barks and roots of *Nauclea latifolia Smith* were cut into small pieces and dried at room temperature for four weeks, then made into a fine powder using an IKA Labortechnik type MFC grinder. This powder was used for the preparation of aqueous and hydro-ethanolic extracts according to the methods (Ahon *et al.*, 2011).

The powder produced from the bark and the roots underwent extraction (Zirihi and Kra, 2003), as described: 100 g of powder were macerated in one liter of distilled water by grinding in a blender. The resulting homogenate was first drained in a square piece of cloth, then filtered successively twice through cotton wool and once through Whatman 3 mm paper. The volume of the filtrate obtained was reduced using a Büchi-type rotary evaporator at a temperature of 60° C. The paste was collected and lyophilized. The extract thus obtained is the total aqueous extract noted Ex.aq. The ethanolic extract was produced by fractionating the aqueous extract (Zirihi and Kra, 2003): 10 g of Ex.aq were dissolved in 200 ml of a hydroalcoholic solution (V/V 30/70). This mixture was separated into two phases using a separatory funnel for five hours. The upper alcoholic phase obtained was collected and dried in an oven at 50° C; the product thus obtained is the ethanolic extract (Ex.OH). This cycle of aqueous and ethanolic extraction was repeated three times. The extracts were placed in pre-sterilized containers. Hermetically sealed, they were stored in the refrigerator at 4° C.

Study of the antibacterial activity of plant extracts

Measurement of the rate of inhibition of bacterial growth: HD medium (environment) was used for the culture of bacteria. The media (environments) were autoclaved at 121° C for 20 minutes. The incorporation of the various plant extracts into the culture medium was carried out using the double geometric bond dilution method ½ (Leroux and Credet, 1978). Seven (7) concentrations were selected for Ex.aq (100 mg ml, 50 mg/ml, 25 mg/ml, 12.5 mg/ml, 6.25 mg/ml, 3.12 mg/ml and 1.56 mg/ml), against six for Ex.ETOH, in particular all those of Ex.aq, except the strongest (100 mg/ml). The witness received no addition of the extracts. The different culture media were poured at 40°C. into Petri dishes 90 mm in diameter. Three Petri dishes were used for each concentration and the test was repeated 3 times under the same experimental conditions. Petri dishes were sealed with adhesive film and incubated in an oven for 24 hours at 37 ± 2° C. The rate of inhibition of bacterial radial growth was measured daily for 5 days compared to the control. This inhibition rate was calculated according to the formula ([Zirihi and Kra, 2003):

T (%) = (D - d) / D) x 100

T: inhibition rate,
D: bacterial growth in the control Petri dishes,
d: bacterial growth in the test boxes.

Determining the rate of inhibition of bacterial growth of each strain made it possible to define, for each extract, the minimum inhibitory concentration (MIC) and the minimum bactericidal concentration (MBC).

Sensitivity test

The sensitivity of the strains to plant extracts was achieved by the technique of diffusion in agar medium. Mueller Hinton's circles were seeded by flooding. Using a sterile Pasteur pipette, wells approximately 5 mm in diameter were made in the nutrient agar. Each well received 80 µl of the test extract at concentrations of 100, 50 and 25 mg/ml. After 40 minutes of diffusion at room temperature, the Petri dishes were incubated at 37° C for 48 h. The presence or absence of a zone of inhibition has been observed (Bssaibis *et al.*, 2009). The interpretation was made (Duraffourd *et al.*, 1990; Ponce *et al.*,2003) method by measuring the diameters of the radial inhibition halos compared with the result obtained with the reference molecule.

Preparation of the inoculum

The bacterial inoculum was prepared from colonies less than 24 hours old in Mueller Hinton liquid medium (MHB). A colony isolated from our bacterial culture was taken using a platinum loop and homogenized in 10 ml of the culture broth, then incubated for 4 or 5 h at 37° C to have a preculture. A volume of 0.01 ml and 1 ml was taken for *Staphylococci and Escherichia* respectively and was added to 10 ml sterile MHB. respectively for Staphylococci and *Escherichia* and was added to 10 ml sterile MHB. This bacterial suspension produced is evaluated at approximately 10^6 cells/ml and constitutes the 100 dilution or the pure inoculum.

Inoculum count

The inoculum count was performed by a 10th dilution from the pure inoculum. 4 dilutions were obtained at 10^{-1}, $10^{-2.}$ 10^{-3}, $10^{-4.}$ These various dilutions as well as the pure inoculum were inoculated using an automatic calibrated 2 µl micropipette on an HD agar, and then incubated at 37° C for 24h. This preparation constitutes box B.

Preparation of the concentration range of plant extracts

The concentration range of hydro-ethanolic plant extracts was carried out in seven test tubes numbered from 1 to 7 by the double dilution method according to a geometric progression of ratio ½.

Inoculation

In a series of eight hemolysis tubes numbered T1 to T8, 1 ml of the pure inoculum was added. Then, 1 ml of plant extract was added to the tubes according to the prepared concentration range. This distribution of extract was made so that 1 ml of plant extract of 200 mg/ml was transferred into tube T1, tube T2 received 1 ml of 100 mg/ml and so on up to tube T7 which received 1 ml of the 3.1 mg/ml solution. Tube T8 received, instead of the plant extract, 1 ml of sterile MHB which served as a growth control. Due to the

volume/volume dilution thus achieved, the concentration in the tubes was reduced by half. All of these tubes were incubated at 37° C for 24h.

Determination of the minimum inhibitory concentration (MIC)

The MIC is the lowest concentration of the test substance for which there is no growth visible to the naked eye after an incubation time of 24h. Its determination was made by observing the disorder induced by the growth of the microorganisms studied in each tube. The MIC was the smallest concentration for which there was no disturbance observed.

Determination of the minimum bactericidal concentration (MBC)

The minimum bactericidal concentration (MBC) is the lowest concentration of substance which leaves at most 0.01% of surviving microorganisms. Using an automatic calibrated 2 µl pipette with sterile tips, the contents of the tubes in which no cloudiness was observed were removed and inoculated on HD agar, starting with the MIC tube. The inoculation was done by parallel streaks 5 cm long on the surface of the agar (Box B1). After 24h incubation at 37° C, the number of colonies on the streaks was compared to that of the inoculum count box (Box B2). Thus, the first experimental tube in which the number of microorganisms present on its streak is less than or equal to that of the dilution 104 will correspond to the MBC.

Results

Sensitivity tests

The results below have shown that the hydro-ethanolic extract used exhibits good antibacterial activity both on strains *of Escherichia coli* and on those of *Staphylococcus aureus*, with intervals of the diameters of inhibition respectively from 12 to 22 mm and 15 to 26 mm. These diameters are obtained with concentrations of between 50 and 200 mg/ml. However, weak antibacterial activity was observed with *Klebsiella pneumoniae C3GR strains*, with barely detectable inhibition diameters at the 50 mg/ml concentration; 11 mm for 100 mg/ml and 16 mm for 200 mg/ml.

Table II: result of the in vitro effect of extracts of roots and bark (RAEC: 1/1)

Strains	Origines	Hydro-ethanolic extracts (mg/ml)			Aqueous extracts (mg/ml)		
		200	100	50	200	100	50
Escherichia coli LNSP	Urines	22	18	13	16	14	10
Escherichia coli BLSE/AmpC	Urines	19	15	12	16	13	09
Klebsiella pneumoniae LNSP	Urines	18	18	13	19	18	12
Klebsiella pneumoniae C3GR	Urines	16	11	07	15	10	00
Staphylococcus aureus CIP103429	Reference	26	21	16	17	15	13
Staphylococcus aureus SARM/LNSP	Pus	25	21	15	18	14	11
Candida albicans LNSP	Vaginal swab	Executed (E)	E	E	E	E	E
Candida albicans CIP2503	Reference	E	E	E	E	E	E

Table III: result of the in vitro effect of Root extracts

Strains	Origines	Hydro-ethanolic extracts (mg/ml)			Aqueous extracts (mg/ml)		
		200	100	50	200	100	50
Escherichia coli LNSP	Urines	15	12	8	8	6	Unfinished
Escherichia coli BLSE/AmpC	Urines	13	11	9	7	5	Unfinished
Klebsiella pneumoniae LNSP	Urines	13	12	7	10	6	Unfinished
Klebsiella pneumoniae C3GR	Urines	12	08	12	10	5	Unfinished
Staphylococcus aureus CIP103429	Reference	17	14	10	9	8	Unfinished
Staphylococcus aureus SARM/LNSP	Pus	14	13	10	10	7	Unfinished
Candida albicans LNSP	Vaginal swab	E	E	E	E	E	Unfinished
Candida albicans CIP2503	Reference	E	E	E	E	E	Unfinished

Table IV: result of the in vitro effect of extracts from the bark

Strains	Origines	Hydro-ethanolic extracts (mg/ml)			Aqueous extracts (mg/ml)		
		200	100	50	200	100	50
Escherichia coli LNSP	Urines	20	17	11	14	11	07
Escherichia coli BLSE/AmpC	Urines	17	13	11	14	11	08
Klebsiella pneumoniae LNSP	Urines	16	15	13	15	14	10
Klebsiella pneumoniae C3GR	Urines	16	11	08	16	10	06
Staphylococcus aureus CIP103429	Reference	25	22	15	14	12	10
Staphylococcus aureus SARM/LNSP	Pus	23	20	16	15	10	07
Candida albicans LNSP	Vaginal swab	E	E	E	E	E	E
Candida albicans CIP2503	Reference	E	E	E	E	E	E

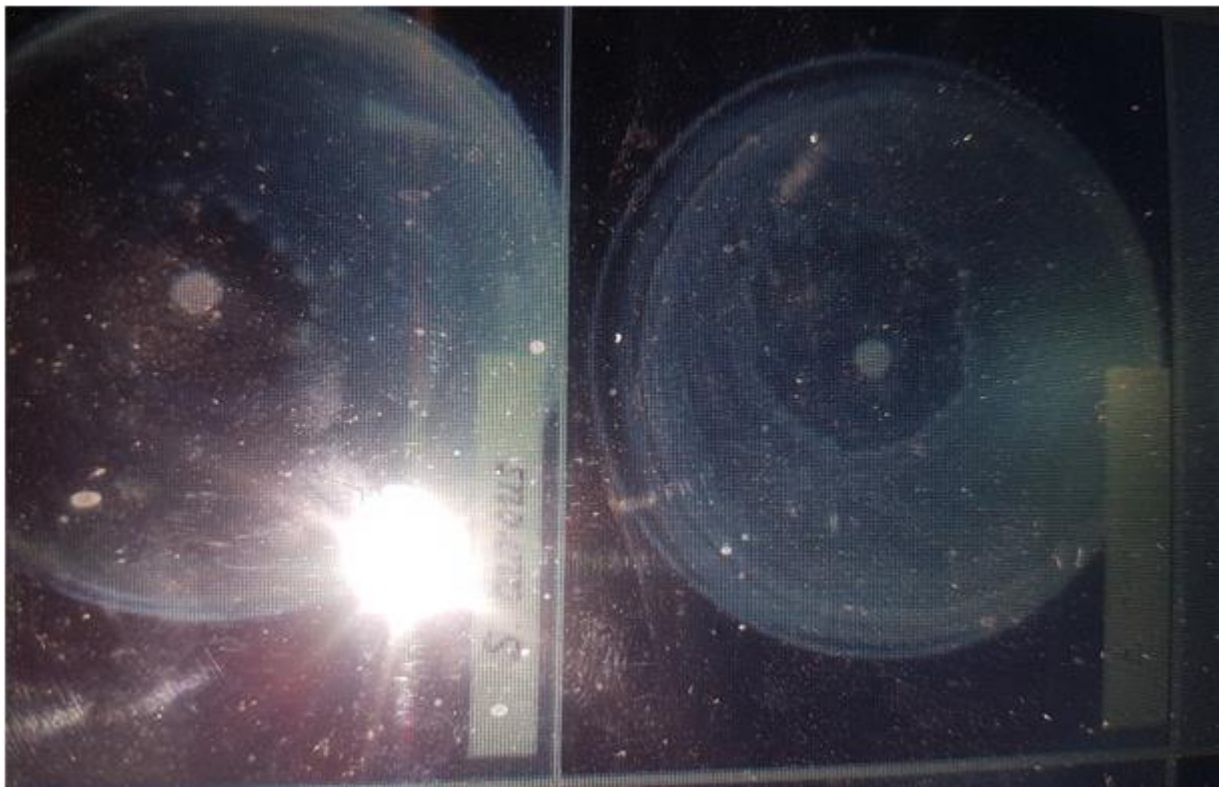

Figure 1: Sensitivity of the S. aureus and E. coli strains to the RAEC hydroalcoholic extract (100 mg / ml) of N. latifolia.

Determination of antibacterial parameters (MIC and MBC)

It was found that the degree of turbidity induced by bacterial growth decreased with increasing concentration of the plant extract in the incubation tubes. *S. aureus and E. coli* were more sensitive with MIC (6.2 and 12.5 mg/ml) and MBC (6.2 and 25 mg/ml). The highest MBC value was obtained with *the K. pneumoniae C3GR strains* (100 mg/ml), while the same strain gave the same MIC value as that obtained with the *S. aureus* strains (12.5 mg/ml). Only the MBC/MIC ratio of the K. pneumoniae strains was greater than 8.

Table V : Antimicrobial endpoint results

Strains	Origines	MIC (mg/ml)	MBC (mg/ml)	MBC/MIC	Interpretation
Escherichia coli LNSP	Urines	12,5	25	2	Bactericidal
Escherichia coli BLSE/AmpC	Urines	6,2	50	4	Bactericidal
Klebsiella pneumoniae LNSP	Urines	12,5	25	2	Bactericidal
Klebsiella pneumoniae C3GR	Urines	12,5	100	> 8	Bacteriostatic
Staphylococcus aureus CIP103429	Reference	6,2	12,5	2	Bactericidal
Staphylococcus aureus SARM/LNSP	Pus	6,2	6,2	1	Bactericidal
Candida albicans LNSP	Vaginal swab	E	E	/	/
Candida albicans CIP2503	Reference	E	E	/	/

Discussion

The in vitro tests of the antibacterial and antifungal activity of the bark and root extracts of *Nauclea latifolia Smith* against the various multidrug-resistant strains were qualitatively and quantitatively evaluated by the presence or absence of zones of inhibition and determination of the MIC and MBC.

Reading the results given in chart I showed that the hydroalcoholic extract of the bark exhibits a strong antibacterial activity against *Staphylococcus aureus* CIP103429, and strains of clinical origin such as: *Staphylococcus aureus MRSA/LNSP*, *Escherichia ESBL/AmpC coli*, and *Escherichia coli LNSP*. This extract is more active than the aqueous extract, except for their effect on strains of *Klebsiella pneumoniae* C3G R where the aqueous extract has shown significant activity like that of the hydroalcoholic extract. *Saccharomyces aureus, Escherichia coli and Klebsiella pneumoniae* having shown a very significant sensitivity (14 ± 1.7mm) with respect to the ethanolic extract, were therefore retained for the microdilution test in culture broth in order to determine it. The MIC and MBC, *Candida albicans* on the other hand showed no sensitivity to the same extract (9.3 ± 1.5 mm).

Contrary to the results obtained (Toty *et al*.,2013) with the aqueous and ethanolic extract of the trunk bark of *Harungana madagascariensis* on the in vitro growth of bacterial strains of *E. coli* and *S. aureus* multi-resistant at the 50 and 100 mg/mL concentrations which were less sensitive.

The difference in sensitivity observed between the bacterial strains of Escherichia coli and Klebsiella pneumoniae (gram negative) on the one hand, and Staphylococcus aureus (gram positive) on the other hand, with respect to the same extract, can be explained by the fact that the wall of Gram-bacteria contains a lipid layer making them less permeable and therefore more resistant than Gram + bacteria which lack it.

Furthermore, we have found that the inhibitory activity of the extract used is dose-dependent on all the strains studied, this testifies to the close relationship that could exist between the active principle of our extract and the microbial strains. In addition, the

bacterial resistance factors, which make most of the reference antibiotics used ineffective, would have no effect on our extract.

The activity of a plant substance depends both on the method of extraction and on the concentration of active ingredients (Wagner,1993; Badiaga,1984). Also, it should be remembered that *Nauclea latifolia Smith* contains flavonoids, anthracenes, sterols, coumarins, triterpenes, alkaloids and tannins. These compounds have known antibacterial properties, in this case, polyphenols (flavonoids), their presence could therefore explain the antimicrobial properties (Scalbert ,1991).

A dose-dependent inhibitory activity of the methanolic extract of *Sebastiania chamaelea* on *E. coli* and *S. aureus*, with an inhibition diameter of 17, 3 ± 0.94 mm and 16.6 ± 0.94 mm respectively at 5 mg extract/disc(Shanthee Sree *et al*, 2010).

Likewise, strong anti-staphylococcal activity of a combination of the essential oils of *Pelargonium graveolens* and *Myrtus communis* has been reported (Chraibi *et al.*,2019), with a MIC of 0.25 µl / ml). Moreover, similar results were obtained on two strains of *Staphylococcus aureus* resistant to methicillin (MRSA) with the resin of balsam fir (*Abies balsamea*), as well as the essential oil of *Monanda didyma* on *Escherichia coli* and *Clostridium perfringens* (Héloïse *et al.*,2019), furthermore, *Staphylococcus aureus* ATCC 25923 demonstrated similar results with *Teucrium polium* L (Fertout-Mouri *et al.,* 2016). Another combination of essential oils of *Pelargonium x asperum* and nisin also gave interesting results on Staphylococcus aureus (ATCC 43300), *Staphylococcus aureus* and *Escherichia coli* with MICs included between 1.98 and 3.96 µl / ml (Ouelhadj *et al.*,2017).

The hydroalcoholic extracts of *Nauclea latifolia* have also shown antimicrobial activity. The bioguided fractionation of these extracts showed that polyphenolic compounds were responsible for them, compounds which would act by a common or synergistic action.

The result obtained by microdilution in culture broth of the ethanolic extract has a MIC and MBC equal to 6.2 mg/ml on S. aureus and 12.5 mg/ml on Escherichia coli, confirms the antibiotic power (MBC/MIC ≤ 4) of *Nauclea latifolia*. Furthermore, this extract is bacteriostatic at a concentration of 12.5 mg/ml on *Klebsiella pneumoniae*. However, the extract has no major effect on *Candida albicans* at any concentration.

Furthermore, the extracts from the roots/bark recipe (RAEC extract) were found to be more active on the strains studied than the root extracts exclusively. One might think that the active ingredient would be more abundant in the bark than in the roots, then that the tests carried out with extracts from the bark exclusively showed an antibacterial activity very close to that obtained with the RAEC extract.

Conclusion

The present work has demonstrated the antimicrobial activity of the ethanolic extract of Nauclea latifolia Smith on multidrug-resistant strains of *Esherichia coli, Klebsiella pneumoniae* and *Staphylococcus aureus*. This activity would be dependent on the presence and the richness in polyphenolic compounds, in particular the flavonoids. These compounds are very probably responsible for the better antibacterial activity of the ethanolic extract against *Staphylococcus aureus* due to their low lipid wall, thus conferring a certain membrane permeability. In addition, it made it possible to realize that the active principle would be more accumulated in the bark. This result could be useful for optimizing the development of an Improved Traditional Medicine (ITM).

This preliminary work may serve as a basis for determining sufficient and effective concentrations for in vivo studies, with a view to alleviating treatment failures due to antibiotic resistance by phytomolecules active against infections caused by *Escherichia coli, Klebsiella pneumoniae and Staphylococcus aureus*. These results may justify the traditional use of the plant in the treatment of certain diseases of bacterial origin.

Ethics approval and consent to participate

"Not applicable".

Data Availability

The data are those recorded in this text. For more information contact the corresponding author

Conflicts of Interest

"The author(s) declare(s) that there is no conflict of interest regarding the publication of this paper."

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Authors' contributions

Raoul Ngassaki is the main author of this article. He is involved in all stages of this work.
Raoul Ampa is involved in the analysis of the results of the antibacterial activities of plant extracts
Tsiba Gouollaly extracted the different fractions of plant extracts.
Rachel Moyen is involved in the analysis of the antibacterial activities of plant extracts.
Gabriel Ahombo coordinated all the activities of this work.

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