



Research Article

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Phytochemical assessment and antimicrobial activity of leaves extract of *Vernonia colorata* (Willd.) Drake on Resistant Germs of *Staphylococcus aureus* and *Pseudomonas aeruginosa*

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ABSTRACT

The emergence of multi-resistant bacteria especially *Staphylococcus aureus* (*S. aureus*) and *Pseudomonas aeruginosa* (*P. aeruginosa*) to antibiotics is one of the health concern issues of the recent decades. Among the possible resolutions of this concern, the secondary metabolites of medicinal plants seem to be privileged. Carried out in this context this work is based on the phytochemical study and the antibacterial activity of leaves extracts of *Vernonia colorata* (Willd.) Drake on resistant *S. aureus* to methicillin and *P. aeruginosa* resistant to ceftazidime and imipenem. The phytochemical assessment proved that the leaves of *Vernonia colorata*, harvested in Yamoussoukro (Côte d’Ivoire), contain flavonoids, saponins, tannins, steroids, terpenoids and cardiac glycosides. The values of minimum inhibitory concentration (MIC) and those of the minimal bactericidal concentration (MBC) of ethyl acetate extract are identical. They are 3.12 mg / mL on *S. aureus* Meti R and 25 mg / mL on *P. aeruginosa* Cefta R & Imp.R. After column purification, the activity was improved leading to MIC and MBC values of 1.56 mg / mL on *S. aureus* Meti R and 12.5 mg / mL on *P. aeruginosa* Cefta R & Imp.R. Antimicrobial tests showed that leaves extract of *vernonia colorata* can develop bactericidal activities on resistive gram-positive and gram-negative germs such of *S. aureus* resistant to methicillin and *P. aeruginosa* resistive to ceftazidime and imipenem.

Keywords: *Vernonia colorata*, *Staphylococcus aureus*, *Pseudomonas aeruginosa*, Phytochemistry, resistance.

INTRODUCTION

In the animal reign, especially in humans, bacterial infections due to bacterial resistance to antibiotics are one of the main causes of diseases with high morbidity and mortality [1, 2]. This resistance described for many species including *Staphylococcus aureus* (*S. aureus*) and *Pseudomonas aeruginosa* (*P. aeruginosa*) [3, 4] because of their epidemic spread is of major concern in hospitals [5]. Moreover, the combination of the deficiency of new antibiotics and the spread of multidrug resistance may lead, in the coming years, to an increased number of bacterial infections with therapeutic impasses [6]. It is therefore appropriate to conduct a research to find simple and inexpensive treatments to fight against multi-resistant pathogens. According to World Health Organization more than 21 000 plants are being in use for medicinal purpose all around the world [7]. Numbers of plants were screened for primary and secondary metabolites for their medicinal values [8, 9, 10]. In recent years, in connection with ancient practices existing in developing and industrialized countries, the plants become one of the main sources of new molecules with antibacterial activity that is clearly marked in the studies [11].

Côte d'Ivoire, with its 761 species of medicinal plants and more than 1421 drug proceeds [12], has high potentialities to explore new antibacterial compounds. Thus, among the plants of Cote d'Ivoire pharmacopoeia, in Africa, *Vernonia colorata* is well known in treatment of diabetes, skin rashes, and acute hepatitis. It is commonly used in the treatment of schistosomiasis, the epileptiform seizures, fevers, diarrhea and hypertension [13, 14]. This plant of the *Asteraceae* family is also sporadically used in Côte d'Ivoire for traditional treatment of Buruli ulcer.

The present work aims to determine the phytochemical profile of *Vernonia colorata* in Yamoussoukro region (central Cote d'Ivoire) and to evaluate the antimicrobial activity of leaves extracts on resistant strains such as *S. aureus* and *P. aeruginosa*, germs of secondary infections of Buruli ulcer.

EXPERIMENTAL SECTION

Plant material: Fresh leaves of *Vernonia colorata* were collected in December 2010 around the Institut National Polytechnique Felix Houphouët-Boigny of Yamoussoukro (Côte d'Ivoire). The leaves were identified by the botanist of the Institute and a sample was deposited in the Herbarium.

The leaves were dried for two weeks out of direct sun light and then crushed in a traditional mortar. The resulting powder was stored in polyethylene bags at 4°C until extraction.

Extract preparation: In a 2L Erlenmeyer flask, protected from light, 200 g of powdered leaves were macerated in 1L hexane for 12 hours at ambient temperature (28°C). This operation, aimed at cleaning up powders from wax and paraffin, was repeated once. After drying, the residual powder was exhaustively extracted with ethyl acetate under the same conditions. At each extraction cycle, the ratio of plant material - solvent was maintained at 1: 5 (w / v).

The organic layers were filtered through cotton wool and Whatman® paper N°3. The filtrates were evaporated to dryness at 40°C under reduced pressure using a rotavapor Büchi 161 Water Bath type leading to 16.71 g of greenish powder (8.35%).

Ten grams (10 g) of this powder were fractionated on a column of silica gel eluting with hexane, ethyl acetate, and methanol in the following proportions 100/0/0, 50/50/0 ; 0/100/0, 0/50/50 (v / v / v). Four fractions (F1, F2, F3 and F4) were collected according to the chromatographic profiles. The fractions residues were stored at 4°C under nitrogen until the phytochemical screening and antimicrobial tests.

Chemicals used: All chemicals and drugs used were obtained commercially and of analytical grade.

Bacterial strains: The bacterial strains used for biological tests were provided by the antibiotics unit of natural substances and Survey of Resistance of Micro-organisms for anti-infective (ASSURMI) Department of Bacteriology at Pasteur Institute of Côte d'Ivoire (IPCI). The strains used were:

Staphylococcus aureus sensitive to methicillin (*S. aureus* Meti S);

Staphylococcus aureus resistant to methicillin (*S. aureus* Meti R);

Pseudomonas aeruginosa sensitive to ceftazidime and imipenem (*P. aeruginosa* Cefta S & Imp S);

Pseudomonas aeruginosa resistant to ceftazidime and imipenem (*P. aeruginosa* Cefta R & Imp R).

Referenced strains of *S. aureus* ATCC 25923 and *P. aeruginosa* ATCC 27853 were also tested.

For getting young colonies for the tests, the different bacterial strains were subcultured by streaking method and incubated in an oven at 37°C for 18 to 24 hours.

Efficiency test substances: The efficiency test was used to detect biological activity of a substance. For this test, the agar and Mueller Hinton broth were the main culture media [15]. The mixture of DMSO / distilled water in proportion 1: 1 (v / v) was used as solvent to prepare the solution of leaves extracts. Non-impregnated discs of 6 mm of diameter, purchased from Biorad® were also used. The tests were performed on bacterial inoculums of 5.10⁶ CFU / mL.

Each disc was impregnated with 40µL of extract or fractions solutions at 200 mg/mL concentration. The choice of 200mg/mL concentration for this test was literature guided. After drying, the discs were placed on the agar previously seeded with micro bacterial strains and incubated at 37 ° C for 18 to 24 hours [16]. The observation of an inhibition zone reflected the existence of antimicrobial activity. Observation of an inhibition zone can be used to judge the efficiency of substances in extract or fractions. Control tests were carried out using discs impregnated with 40µL of appropriate solvent used to prepare extract or fractions.

To confirm the resistance of bacteria, tests on young colonies using oxacillin (OX-5 μ g) and ceftaxitin (FOX-30 μ g) for *S. aureus* and the ceftazidime (CAZ-30 μ g) and imipenem (IMP 10 mg) for *P. aeruginosa* were made under the same conditions.

Antimicrobial screening: A concentration range of plant extract was prepared by the method of double dilution with concentrations ranging from 100.00 to 0.39 mg / mL for ethyl acetate extract and from 50.00 to 0.19 mg/mL for its fractions.

The antimicrobial screening was performed using the method proposed by [17]. The tests were performed by introducing into a series of hemolysis tubes 1 mL of the solution of plant extract and 1 mL of bacterial inoculums as described by Moroh and al. [18]. At the same time, in control tube, 1 mL of the solvent used to solve the extract (DMSO / distilled water to 1: 13 v / v) and 1 mL of bacterial inoculums were introduced. All the tubes were incubated at 37°C for 18 to 24 hours.

The results of antimicrobial screening were read looking through at daylight using human eye [19]. The transparency of the tubes indicated the antimicrobial effect of the tested extract, while its turbidity shows its ineffectiveness (a sign of bacterial growth). The Minimum Inhibitory Concentration (MIC) will correspond to the concentration of the extract in the first tube with a clear content.

The minimum bactericidal concentration (MBC) is the lowest concentration of extract that kills at least 99.99% of bacteria in culture. For its determination, the content of control tube was diluted to 10⁻⁴, corresponding to 0.01% of survival bacteria in culture. The experimental tubes sowed antimicrobial effect from the CMI's one are transplanted by streaks of 5cm on Mueller Hinton agar and incubated at 37°C for 24 hours. The first experimental tube in which the number of determined germs is less or equal to the dilution concentration (10⁻⁴) corresponds to the CMB.

Phytochemical study: The phytochemical study of the leaves of *Vernonia colorata*, based on color and / or precipitation tests was carried out on the powder of crushed leaves, ethyl acetate extract and the active fraction [20, 11]. The target molecules families of this screening were saponins, tannins, flavonoids, alkaloids, steroids, terpenoids and cardiac glycosides.

RESULTS AND DISCUSSION

Tests of efficiency: The tests of efficiency conducted prior to the determination of microbiological parameters of extract at 200 mg/mL give the results summarized in Table 1.

Table 1 Results of effectiveness tests of the extract, fractions and antibiotics

Bacterial strains	Tested substances								
	Observed inhibition diameter (mm)								
	AcOEt Extract	F1	F2	F3	F4	Ox	Fox	Caz	Imp
<i>S. aureus Méti S</i>	13	0	08	14	0	43	32	-	-
<i>S. aureus Méti R</i>	12	0	09	15	0	0	0	-	-
<i>S. aureus ATCC 25923</i>	11	0	08	14	0	28	30	-	-
<i>P. aeruginosa Cefta & Imp.</i>	0	0	0	0	0	-	-	30	30
<i>P. aeruginosa Cefta & Imp.</i>	0	0	0	0	0	-	-	0	0
<i>P. aeruginosa ATCC 27853</i>	0	0	0	0	0	-	-	26	25

Not tested (-); Oxacillin (Ox); Ceftaxitin (Fox); Ceftazidime (Caz); Imipenem (Imp).

The tests confirm the effectiveness of antibiotic resistance and sensitivity of received organisms.

During the efficiency tests of *Vernonia colorata* leaves extract and its fractions, zones of inhibition were observed only in strains of *S. aureus*. Inhibition zone diameters for extract and its fractions are lower than those of antibiotics. Basing on the inhibition diameter, the fraction F3 appears more active than the crude extract and fraction F2. At the same time we observed no inhibition area for the fractions F1 and F4. These results indicate the presence of bioactive substances in the extract and active fractions able to inhibit the growth of germs at the concentration of 200 mg/mL.

Antimicrobial parameters: Although efficacy trials showed a biological activity of the extract and its fractions only on *S. aureus* at the concentration of 200 mg/mL, the antibacterial parameters were sought on all of the studied seeds using only the ethyl acetate extract and the fraction F3. The obtained results are summarized in Table 2.

Table 2: Antimicrobial parameters of ethyl acetate extract (EtOAc) and fraction F3

Bacterial strains	Ethyl acetate extract (AcOEt)			Fraction F3		
	CMI (mg/mL)	CMB (mg/mL)	CMB / CMI	CMI (mg/mL)	CMB (mg/mL)	CMB / CMI
<i>S. aureus Meti S</i>	3,12	3,12	1	1,56	1,56	1
<i>S. aureus Meti R</i>	3,12	3,12	1	1,56	1,56	1
<i>S. aureus ATCC 25923</i>	3,12	3,12	1	0,78	1,56	2
<i>P. aeruginosa Cefta & Imp.S</i>	25,00	25,00	1	12,50	12,50	1
<i>P. aeruginosa Cefta & Imp.R</i>	25,00	25,00	1	12,50	12,50	1
<i>P. aeruginosa ATCC 27853</i>	25,00	25,00	1	12,50	12,50	1

Table 2 shows different results from Table 1. In liquid medium crude ethyl acetate extract and fraction F3 have antibacterial activity on all studied seeds. The tendency of improved activity of fraction F3 compared to ethyl acetate extract is confirmed. Therefore, depending on the studied seeds, ethyl acetate extract has MIC ranging from 3.12 to 25.0 mg / mL while the MIC of fraction F3 varies from 0.78 to 12.50 mg / mL. Activity of Fraction F3 is twice higher than that of the ethyl acetate extract. Concerning resistive seeds, the MIC for *P. aeruginosa* Cefta & Imp. R is eight times lower than that obtained for *S. aureus* meti R. The same tendencies are observed for the CMB. According to them, the ineffective effect of their extracts could be due to their little diffusion properties in the agar.

The improvement of CMI in fraction F3 and the appearance of antimicrobial activity in liquid medium confirmed that the antimicrobial activity of secondary metabolites of specific plant depends on several factors including the method of extraction, the concentration of active compounds and the mode test applied [21]. In our study, the results are probably linked to increase of concentration of active components and / or their rapid diffusion in liquid medium.

Determination of the type of antimicrobial activity of active substances depends on the MBC / MIC ratio. According to Marmonier [22], when this ratio is less or equal to four (≤ 4) the tested substance is classified as bactericidal otherwise it is a bacteriostatic. For all the studied strains the MBC / MIC ratio was practically equal to one. These results permit to conclude to bactericidal properties on all studied seeds for extracted substances from leaves of *Vernonia colorata*.

Phytochemical screening: To identify the family of molecules responsible for the antimicrobial activity on studied strains, we carried out a phytochemical screening on powdered leaves, ethyl acetate extract and fraction F3. The results of this screening are summarized in Table 3.

Table 3: Secondary metabolite content of leaves, fraction F3 and crud extract of *Vernonia colorata*

Secondary metabolite	powdered Leaves	Crud extract (AcOEt)	Fraction F3
Alkaloids	-	-	-
Flavonoids	++	++	-
Tannins	++	+	-
Saponins	++++	-	-
Steroids -Terpenoids	++	++	+++
Cardiac glycosides	++	++	-

Presence (+); Absence (-); Medium (++); Abundant (+++).

Based on the results of Table 3, the leaves of *Vernonia colorata* of Yamoussoukro contain saponins, tannins, flavonoids, steroids - terpenoids and cardiac glycosides. The presence of these compounds in the leaves of *Vernonia colorata* justifies its traditional use for several disease treatments [13, 23].

The antibacterial activity observed in liquid media suggests a poor diffusion of extracts components when tested in solid medium. These results conform to those reported by [24].

These results are in concordance with those of Paris [25] who conducted special studies on *Vernonia colorata* harvested in Côte d'Ivoire. He found that the plant did not contain alkaloids. But, studies of Fané [26] on *vernonia colorata* harvested in Mali revealed the presence of alkaloids in the sample. So, the absence of alkaloids in the leaves of *Vernonia colorata* of Yamoussoukro could be related to the chemotype.

The phytochemical screening of the fraction F3 gave the prominence of steroids – terpenoids only. Accordingly we can say that the antimicrobial activities observed on studied seeds are connected to these compounds. Inhibition of *Staphylococcus aureus* and *Pseudomonas aeruginosa* growth can justify the traditional use of this plant for Buruli ulcer treatment.

Improvement of antimicrobial activity for fraction F3 could be related to the content of actives components in this fraction.

CONCLUSION

Phytochemical screening confirmed the absence of alkaloids in the leaves of *Vernonia colorata* of Côte d'Ivoire. Antimicrobial tests prove that *vernonia colorata* leaves extract can develop bactericidal activities on resistive gram-positive and gram-negative germs such as *S. aureus* resistant to methicillin and *P. aeruginosa* resistive to ceftazidime and imipenem.

This activity could be due to steroids and terpenoids. These results provided a basis for isolation of antibacterial compounds of interest from *vernonia colorata*, especially using polar solvents like ethyl acetate. Further study is being carried out to isolate and identify the antibacterial compounds present in ethyl acetate fraction of *vernonia colorata* leaves extract.

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