

Research Article

Exploration of the antistaphylococcal activity of *Vitex doniana* (Verbenaceae) stem bark extracts

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Abstract: The goal of that study was to test the antibacterial activity of the different extracts of *Vitexdoniana* on three strains of the *Staphylococcus aureus*: *S. aureus* Meti-S, *S. aureus* Meti-R and *S. aureus* ATCC 25923. Two methods have been used: they are agar well diffusion and the macrodilution method in liquid medium (macrobroth dilution method). Among all the tested strains, ethanolic 70 %, methanolic and ethyl acetate extracts have given the inhibition diameters included between 11 and 26 mm whereas the aqueous extract has only been active on *S. aureus* Meti-S with an inhibition diameter of 17 mm. The values obtained with the Minimum Inhibitory Concentration of (MIC) are between 0.39 and 53.57 mg/mL and those of Minimum Bactericidal Concentration (MBC) are between 0.39 and 107.14 mg/mL. Moreover, the ethyl acetate extract has shown the best activity against all the tested strains with 3.12, 0.39 and 3.12 mg/mL as MBC respectively on *S. aureus* Meti-R, *S. aureus* Meti-S and *S. aureus* ATCC 25923.

Keywords: *Vitex doniana*, medicinal plant, bactericidal, *Staphylococcus aureus*, Côte d'Ivoire.

INTRODUCTION

The growing resistance of the *Staphylococcus* against the antibiotics formerly recognized for their efficiency is today a real problem of public health [1]. In human pathology, the *Staphylococcus* are responsible for many infections: respiratory tract diseases (pneumonia, bronchitis), skin, wound and mucous infections, sinusitis, endocarditis, osteomyelitis, food poisoning and carbuncles. They are also the germs frequently met during surgical wound infections which are often provoked by the use of intravascular catheters or by the spread of bacteria from another source of infection [2].

Presently, the sequencing has allowed counting many species of strains belonging to the *Staphylococcus* family [3]. Most of these species is part of commensal human flora so they live in harmony with the host organism. For immunodepressed and generally when the conditions become unfavourable, these species become quickly pathogenic. So for populations at risk especially the drug addicts, the HIV positive and even the former prisoners, the rate of *S. aureus* resistant to methicillin is very high [4, 5, 6, 7]. The *Staphylococcus aureus* is the most pathogenic among all the species of *Staphylococcus* and it is responsible for almost 25 % of septicemias met in hospitals [8]. Generally, the treatment of infections caused by *Staphylococcus* is long and expensive [9]. Many scientific researches have allowed to bring out the therapeutic properties of some natural substances from plants in the prevention and the treatment of pathologies caused by the microorganisms

resistant to antibiotics commonly used [10, 11, 12]. That makes the medicinal plants some potential sources of new molecules to explore. It's in this context, that our team of research was interested in *Vitex doniana* (Verbenaceae), a plant species of the savanna that can be also found in the tropical forests of Africa [13]. Many reports have reported to use of the fruits and the leaves of *Vitex doniana* in African traditional medicine [14, 15, 16, 17] in the treatment of many diseases caused by infections.

This present work aims at evaluating the effects of the stem bark of *Vitex doniana* on the growth in vitro of three strains of *Staphylococcus aureus* which are *S. aureus* Meti-R, *S. aureus* Meti-S and *S. aureus* ATCC 25923.

EXPERIMENTAL SECTION

Plant material

Some freshly stem barks of *Vitex doniana* was collected in January 2012 in northern Côte d'Ivoire precisely in Lataha a village located 8 km far from Korhogo. The plant was authenticated by Professor Ake-Assi of the National Floristic Center (NFC) of the Felix Houphouet-Boigny University of Cocody, Abidjan where a voucher specimen was deposited.

Bacterial stains

The bacteria used for the biological tests are *Staphylococcus aureus* sensitive to Methicillin (*S. aureus* Meti-S), *Staphylococcus aureus* resistant to

Methicillin (*S. aureus* Meti-R) and *Staphylococcus aureus* ATCC 25923 (reference strain).

These bacterial strains were provided by the department of Bacteriology and Virology at Pasteur Institute of Cote d'Ivoire (IPCI).

Preparation of extracts

The stem barks of *Vitex doniana* collected were washed, cut up and have been dried shelter from the sun light for two weeks and reduced to powder by a type IKAMAG-RCT grinder. According to the methods described by Guede-Guina *et al.* and Bagre *et al.* [18, 19], 100 g of plant powder have been macerated in 1 L of distilled water then homogenized under magnetic agitation for 24 hours at 25 °C with a IKAMAG-RC Ttype agitator. The homogenate obtained has been filtered successively two times through hydrophilic cotton (cotton wool) then once through whatman paper n°2. The volume of filtrate obtained is first reduced with a rotavapor Büchi at 60°C. Then, the rest of the filtrate is evaporated with a Med Center Venticell drying oven at 50°C to provide a grayish powder which is the aqueous extract (Etaq).

The same process was carried out by using ethanol 70%, methanol or ethyl acetate instead of distilled water to obtain respectively ethanolic extract 70% (Eeth_{70%}), methanolic extract (Emet) or ethyl acetate extract (Eace) [20,21]. All plant extracts obtained are kept in refrigerator till they are used for antibacterial tests.

Antibacterial activity of different extracts

In order to obtain a microbial suspension with a turbidity similar to that of Mc Farland 0.5 (10⁶CFU/mL) for each bacterial strain tested, an inoculum has been prepared by homogenizing 0.3 mL of three hours opalescence suspension in 10 mL of Mueller-Hinton broth (Biorad, France). By the double dilution method, one range of concentration varying from 100 to 0.39 mg/mL was also prepared for every extract studied [22].

The susceptibility tests have been carried out on Mueller-Hinton agar (Biorad, France) by using well methods [23, 24, 25]. So, like in the case of classic antibiogram realization, every well or hole (6 mm of diameter) has been filled with 80 µL of a 200 mg/mL concentration extract by taken care to separate two holes from at least 20 mm. A reference well has been carried out for each bacterial strain with a 80 µL mixing DMSO solution/sterilized distilled water in proportion 0.5: 0.5 (V/V) [26]. After 45 minutes prediffusion at ambient temperature under hood, the plates have been incubated in an oven at 37°C for 18 to 24 hours. After that period, the extracts action is appreciated by measuring a zone of inhibition (absence of colonies) around the wells. At the same time, the Oxacillin (5 µg) and the Cefoxitin (30 µg) were served as positive control and comparison.

The antibacterial parameters (MIC and MBC) have been obtained by introducing into a series of hemolysis tubes numbered from T₁ to T₁₀ 1 mL of bacterial inoculums. Then, 1 mL of plant extract with a known concentration according to the range of prepared concentrations has been added in the same tubes. That sharing out of plant extracts has been done so that 1 mL of plant extract of 100 mg/mL may be transferred to the tube T₁ that of 50 mg/mL to tube T₂ and so on, and so forth till to tube T₉ that will receive 1 mL of plant extract of 0.39 mg/mL. The tube T₁₀ received instead of plant extract, 1 mL of DMSO/distilled water (1/13: V/V) which been use as reference. That plant extract preparation with a known concentration in each of the tubes containing previously 1 mL of inoculums has brought back the concentration of plant extract medium to its half. So, the tube T₁ concentration moved from 100.00 mg/mL to 50 mg/mL. That of the tube T₂ from 50 mg/mL to 25 mg/mL till to tube T₉ with a real concentration of 0.19 mg/mL. That experience has been carried out in the same way for each tested extract. The nine (9) first tubes (from T₁ to T₉) are called « experimental tubes » and last tube (T₁₀) is called « reference tube or tube of growth ». These full tubes have been incubated in an oven for 24 hours at 37°C. The experience has been reported three times.

The Minimum Inhibitory Concentration (MIC) corresponds to the first tube concentration where we can't observe any trouble visible to naked eye. From the MIC, the smallest concentration that allows at most 0.01 % of bacteria in the first suspension to survive within 24 hours corresponds to Minimum Bactericidal Concentration (MBC). It's determined by streaking on solid medium of 0.1 mL of the content of each tube that has a concentration superior or equal to the MIC. Therefore, the calculation of the ratio MBC/MIC of the extracts has permitted to determine their antibacterial power.

Phytochemical analysis

The phytochemical analysis of the different extracts of *Vitex doniana* have been based on the coloration and precipitation tests [10, 27].

Test for alkaloids

0.5 g of extract was diluted into 10 mL with acid alcohol, boiled and filtered. To 5 mL of the filtrate was added 2 mL of dilute ammonia. 5 mL of chloroform was added and shaken gently to extract the alkaloidal base. The chloroform layer was extracted with 10 mL of acetic acid. This was divided into two portions. Mayer's reagent was added to one portion and Draggen dorff's reagent to the other. The formation of a cream (with Mayer's reagent) or reddish brown precipitate (with Draggen dorff's reagent) was regarded as positive for the presence of alkaloids.

Test for polyphenols and tannins

About 0.5 g of the extract was boiled into 10 mL of water in a test tube and then filtered. A few drops of 0.1% of ferric chloride was added and observed for brownish green or a blue-black coloration.

Test for terpenoids (Salkowski test)

To 0.5 g each of the extract was added 2 mL of chloroform. Concentrated H₂SO₄ (3 mL) was carefully added to form a layer. A reddish brown coloration of the interface indicates the presence of terpenoids.

Test for Glycosides:

Extracts was treated with 2 mL of Glacial acetic acid, add 1 drop of FeCl₃ and 1 mL of concentrated H₂SO₄ appearance of brown coloration indicates the glycosides.

Test for flavonoids

Three methods were used to test for flavonoids. First, dilute ammonia (5 mL) was added to a portion of an aqueous filtrate of the extract. Concentrated sulphuric acid (1 mL) was then added. A yellow coloration that disappears on standing indicates the presence of flavonoids. Secondly, a few drops of 1% aluminium solution were added to a portion of the filtrate. A yellow coloration indicates the presence of flavonoids. Next, a portion of the extract was heated with 10 mL of ethyl acetate over a steam bath for 3 min. The mixture was filtered and 4 ml of the filtrate was shaken with 1 mL of dilute ammonia solution. A yellow coloration indicates the presence of flavonoids.

Test for saponins

To 0.5 g of extract was added 5 mL of distilled water in a test tube. The solution was shaken and observed for a stable persistent froth. The frothing was

mixed with 3 drops of olive oil and shaken after which it was observed for the formation of an emulsion.

Fehling's Test:

Filtrates were hydrolysed with dil. HCl neutralized with alkali and heated with Fehling's A and B solution. Formation of red precipitate indicates the presence of reducing sugars.

Test for steroids and terpenoids

A quantity (9 mL) of ethanol was added to 1 g each of the extracts and refluxed for a few minute and filtered. Each of the filtrates was concentrated to 2.5 mL in a boiling water bath. Distilled water, 5 mL was added to each of the concentrated solution, each of the mixtures was allowed to stand for 1 h and the waxy matter was filtered off. Each of the filtrates was extracted with 2.5 mL of chloroform using a separating funnel. To each 0.5 mL of the chloroform extracts in a test tube was carefully added 1 mL of concentrated sulphuric acid to form a lower layer. A reddish-brown interface showed the presence of steroids. To another 0.5 mL each of the chloroform extract was evaporated to dryness on a water bath and heated with 3 mL of concentrated sulphuric acid for 10 min on a water bath. A grey colour indicates the presence of terpenoids.

Statistical analysis

Data were analyzed by one-way ANOVA followed by Dennett's t-test using Instat® (Graph Pad software, U.S.A). At 95% confidence interval $p < 0.05$ was considered statistically significant

RESULTS AND DISCUSSION

The results of the tests of susceptibility are presented in table 1.

Table 1: inhibition diameters of *Vitex doniana* stem bark extracts against bacterial strains.

Bacterial strains	Inhibition zones in diameter (mm)						
	Etaq	Eeth _{70%}	Emet	Eace	Ox	FOX	T
<i>S. aureus</i> ATCC 25923	0	11	16	20	25	25	0
<i>S. aureus</i> Méti - S	17	23	24	26	31	27	0
<i>S. aureus</i> Méti - R	0	11	14	18	0	0	0

Etaq : aqueous extract ; Eeth_{70%} : ethanolic 70% extract ; Emet : methanolic extract ; Eace : ethyl acetate extract, T : DMSO/distilled water in proportion 0.5 : 0.5 ; V/V ; Oxacillin (OX-5µg) and Cefoxitin (FOX-30µg)

The diameters of zone inhibition have varied from 0.0 to 26 mm. If, ethanolic 70%, methanolic and ethyl acetate extracts of *Vitex doniana* have proved an activity against all the tested microorganisms it's not the case of the aqueous extract for which an inhibition zone has been observed only on *S.aureus* Meti-S (17 mm). On the basis of these inhibition diameters, ethyl acetate extract has been proved as the most active of all the extracts studied with some diameters of 18; 20 and 26 mm respectively on *S. aureus* Meti-R, *S. aureus*

ATCC 25923 and *S. aureus* meti-S. That extract is followed in the same order by the methanolic extract with 14; 16 and 24 mm and then by the ethanolic extract with 11; 11 and 23 mm. Besides any inhibition zone provoked by the Oxacillin (5 µg) and the Cefoxitin (30 µg) can be observed against *S. aureus* Meti-R strain.

From the analysis of these results we noticed that ethyl acetate, methanolic and ethanolic 70 %

extracts are active on the whole tested bacterial strains because they have induced some inhibition diameters superior to 10 mm [28]. In comparison, the aqueous extract is only active on *S. aureus* Meti-S (17 mm). Similar results have been obtained with some methanolic, ethanolic and acetone extracts from the leaves of that plant against a strain of *S. aureus* [29]. We can notice that in spite of the inefficacy of the

commercialized antibiotic against *S. aureus* Meti-R (0 mm), they have been more active against the two other strains of *S. aureus* than the plant extracts.

The values of the MIC and MBC of the extracts of *Vitex doniana* studied against the different *S. aureus* strains tested are presented in table 2.

Table 2: Antibacterial parameters compared of *Vitex doniana* stem bark extracts on bacterial strains.

Bacterial strains	Extracts	Antibacterial parameters (mg/mL)			
		MIC	MBC	MBC/MIC	Antibacterial Effet
<i>S. aureus</i> Méti - S	Etaq	50	100	2	Bactericidal
	Eeth _{70%}	0,78	1,56	2	Bactericidal
	Emet	0,78	1,56	2	Bactericidal
<i>S. aureus</i> ATCC 25923	Eace	0,39	0,39	1	Bactericidal
	Etaq	>200	>200	-	-
	Eeth _{70%}	53,57	107,14	2	Bactericidal
<i>S. aureus</i> Meti-R	Emet	35,71	71,42	2	Bactericidal
	Eace	3,12	3,12	1	Bactericidal
	Eeth _{70%}	53,57	107,14	2	Bactericidal

Etaq : aqueousextract ; Eeth_{70%} : ethanolic70%extract ; Emet : methanolicextract ; Eace : ethylacetateextract

We notice that the values of the MIC agree with that of the diameters of the inhibition zone growth because the extracts that have induced the largest diameter of inhibition have presented the smallest values of MIC on the corresponding strains. In fact, the MIC values of the ethyl acetate extract have varied from 0.39 to 3.12 mg/mL for some diameters of inhibition varying from 18 to 26 mm. The MIC values of methanolic extract have also varied from 0.78 to 35.71 mg/mL for 14 to 24 mm where as those of ethanolic extract has varied from 0.78 to 53.57 mg/mL for 11 to 23 mm. These results agreed with that obtained by [12] on the *Streptococcus pneumonia* and *Candida albicans* strains with some extracts of *Annona senegalensis*.

Taking into account of the MBC and MIC, the values obtained with the ethyle acetate extract are the same no matter the bacterial strain tested. This result in the ratio MBC/MIC is equal to 1. Besides, except for the aqueous extract on *S. aureus* Meti-R and *S. aureus* ATCC 25923, the two other extracts (methanolic and ethenolic70 % extracts) have presented some values of MBC twice superior to that of the found MIC, hence their ratio MBC/MIC equal to 2.

The determination of the antibacterial effect of some active substances depends on the ratio MBC/MIC [30]. According to that author, when this ratio is

inferior or equal to 4, the substance is considered bactericidal and bacteriostatic in the ratio is superior to 4. That means these extracts have bactericidal powers against *S. aureus* tested strains. These results confirm that already obtained by [29] with some extracts of *Vitex doniana* leaves against *S. aureus*. *Vitex agnus-castus* has also revealed some bactericidal activities against *S. aureus* Meti-R [31]. Otherwise, a bactericidal action of the methanolic extract of that bark has also been demonstrated on some enterobacteriaceae especially *S. typhy*, *S. dysenteriae* and *E. coli* (Kiliani, 2006). Furthermore, some interesting biological properties of the aqueous extract of *Vitex doniana* bark have also been pointed out through different works of which that realized by James *et al.* [13], Olusola *et al.* [33] and Abdulrahman *et al.* [34].

Nevertheless, in that present work, the bactericidal or bacteriostatic effect of the aqueous extract of *Vitex doniana* has not been determined through 200 mg/mL. According to Ejikeme and Henrietta, (2010), the aqueous extract of the leaves of the plant obtained through decoction and maceration have had some bactericidal powers against *S. aureus* with a MBC of 1600 mg/mL. These results confirm that the antimicrobial activities of some secondary metabolites of some plants depend on many factors such as the extract origin, the extraction method, the solvent nature, the concentration in active compounds, the nature of the tests applied as well as the strain tested

[26, 35]. If we only refer to these authors, we can say that contrary to the aqueous extract, the other extracts

have a best concentration of the plant active compounds.

Table 3: Phytochemical analysis of *Vitex doniana* stem bark extracts.

Extracts	Phen	Cat tan	Gal tan	Flav	Steroid- terp	Red comp	Card glyco	sapo	alkaloides	
									D	M
Etaq	+	-	+	++	-	+	+	+	-	-
Eeth _{70%}	++	-	++	++	-	++	+	-	-	++
Emet	++	++	++	++	+	+	++	-	-	-
Eace	+++	+	++	+++	+	-	+	+	-	-

- : absence + : Present in small amount (concentration)
++ : moderately present +++ : present in large amount

Phen : phenolic compounds ; Cat : Catechin tannins ; Gal : Gallic tannins ; Flav : flavonoids ; Steroid-terp: steroids and terpenoids ; Red comp : reducing compounds ; Card glyco: cardiac glycosides ; sapo : saponins ; D : Dragendorff's ; M :Mayer; Etaq : aqueous extract ; Eeth_{70%} : ethanolic 70% extract ; Emet : methanolic extract ; Eace : ethyl acetate extract

The phytochemical analysis results are recorded in table 3. These results allow distinguishing diverse group of compounds responsible for biological activities especially tannins, flavonoids, steroids and terpenoids, cardiac glycosides, saponins, phenolic compounds, alkaloid and reducing compounds. Many similar chemical groups have been found in the plant leaves [29]. Among these compounds, the tannins, flavonoids, steroids and terpenoids are already known for their antimicrobial activities [36, 37, 38, 39]. The high concentration of these compounds in the ethyl acetate extract might justify the high antibacterial activities compared with the others.

CONCLUSION

This study has allowed to demonstrate that the *Vitex doniana* stem barks have some bactericidal activities against the *S. aureus* strains even against those that are multiresistant . Meeting with the different extracts, the *S. aureus* Meti-S strain was revealed more sensitive compared to the other strains.

Ethyl acetate extract has shown the best activity on the whole tested strains. The concentration to which this extract remains active allows us to say that, that that plant could be used against *Staphylococcus* infections. This work is a contribution for a better knowledge of some medicinal plants in Côte d'Ivoire which can become a new source of antibacterial agents. Some works are in progress on these promising extracts in order to make some improved traditional medicine (MTA) after purifying and evaluation of their toxicity.

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