

Antibacterial Activity of Verticillin D Produced by Endophyte *Clonostachys Rosea* EC 28

Sagou Dominique

Biology and Health Laboratory, UFR des Biosciences
Félix Houphouët-Boigny University, Côte d'Ivoire

Pakora Gilles Alex

Biology and Health Laboratory, UFR des Biosciences
Félix Houphouët-Boigny University, Côte d'Ivoire

YEO Dodehe

Biology and Health Laboratory, UFR des Biosciences
Félix Houphouët-Boigny University, Côte d'Ivoire

N'Guessan Jean David

Biology and Health Laboratory, UFR des Biosciences
Félix Houphouët-Boigny University, Côte d'Ivoire

ABSTRACT

The objective of this work was to determine the antibacterial effect of endophytic fungi of *Ceiba pentandra* and to identify the compound(s) responsible for this activity. The antibacterial screening of the mycelium of endophytic fungi isolated from the bark of *C. pentandra* was evaluated against multiresistant strains *E. coli* and *S. aureus*. This screening led to the selection of isolate EC 28 for its ability to effectively inhibit the growth of the bacterial strains tested. Analysis of morphological characters and ITS rDNA sequences identified EC 28 as *Clonostachys rosea*. EC 28 was cultivated and extract. From the crude extract, two compounds, ergosterol and verticillin D, were isolated and identified. The in vitro activity of each compound against the growth of five strains bacterial multiresistant were assessed. Ergosterol did not have any activity. Verticillin D was active on all strains of bacterial multiresistant *E. coli* 942, *E. coli* 4814, *S. aureus* 931, *S. aureus* 934, and *K. pneumonia* 815 with respective MICs of 18.75; 18.75; 18.75; 3 and 37.5 µg/ ml.

Keywords: *Clonostachys rosea*; Endophytic fungus; Multi-resistant bacteria; Verticillin D.

INTRODUCTION

Bacterial resistance to antibiotics is a threat to humanity. Currently, the number of deaths related to this scourge is very high, with nearly 30,000 death in Europe and 700,000 worldwide each year [1]. This phenomenon should have consequences dramatic by 2050 if nothing is done. The number of deaths due to bacterial infections multiresistant could exceed the number of those caused by cancer [2]. Countries with low revenue are the most affected [3] Emergence of producing β -lactamase spectra expanded (ESBL) become a real public health problem [4]. These Enterobacteriaceae can in effect resist other family's antibiotics, such as aminoglycosides

and fluoroquinolones [5] hence the notion of bacteria multiresistant (BMR). However, the discovery of new compounds antibiotics does not keep pace with the emergence of strains bacterial resistant [6]. Therefore, the molecules latest of antibiotics are less in less efficient [7]. Therefore, it exists an emergency to orient towards other sources of production of active molecules, in particular the endophytic fungi of plants medicinal. According to the literature, medicinal plants are known to harbor various endophytic fungi that produce antimicrobial compounds [8]. This is the case of 7-amino-4-methylcoumarin, an antibacterial produced by *Xylaria* sp, isolated from the medicinal plant *Ginkgo biloba*. This molecule showed strong antibacterial activity in vitro against *Staphylococcus aureus*, *Escherichia coli*, *Salmonella typhi* and *Salmonella typhimurium* [9]. *Ceiba pentandra* (Bombacaceae) is a tree that has many applications in medicine traditional throughout the world to treat several pathologies such as parasitic infections, diarrhea, dysentery, skin diseases, arthritis, bronchitis, fever and bites insects [10]. From a point of view chemical, the bark of this tree contains isoflavones and terpenoids having properties antibacterial [11], antiplasmodial [12], antiinflammatories [13], an analgesic [14], antidiabetics [15], antispasmodics and diuretics [16]. The present work aims to determine the effect antibacterial of endophytic fungi of *C. pentandra* and identify the compound (s) responsible for this activity.

MATERIALS AND METHODS

Strains bacterial multiresistant

The biological material consisted of 5 strains clinics multiresistant provided by the Laboratory of Bacteriology and Virology of the UFR of medical sciences of the University Félix Houphouët-Boigny from Abidjan (Table 1).

Table 1. Origin of products organic and strain phenotypes bacterial.

Bacteria	Origin	Profile
<i>S. aureus</i> 934/18	Blood	KTG resistant
<i>Escherichia coli</i> 942/18	Pus	ESBL, resistant to fluoroquinilones
<i>S. aureus</i> 931/18	Blood	KTG resistant
<i>K. pneumoniae</i> 815/18	Urine	ESBL
<i>Escherichia coli</i> 4814/20	Urine	Carbapenem resistant

Isolation of endophytic fungi and screening of their activity antibacterial

The isolation of endophytic fungi has realized according to the method described by Toghueo *et al.*, [17] with some modifications. The stem bark of *C. pentandra* harvested has summer rinsed under running tap water for ten minutes, then they have cut in small 5x5 mm pieces and immersed in 70% ethanol for 5 minutes. They have placed in 1% sodium hypochlorite (NaOCl) for 15 minutes, then placed in 70% ethanol for 2 minutes, and finally rinsed three times with water distilled sterile for 1 minute each time. The pieces have dried on paper filtered sterile, placed aseptically in petri dishes containing Potato Dextrose Agar (PDA) medium previously autoclaved at 121°C for 15 minutes, and supplemented with 150 mg/l of chloramphenicol and are incubated at 27°C for 4 days. After purification of the isolates fungal, an activity screening antibacterial has been realized to select active endophytic fungi. *E. coli* 942 and *S. aureus* 934

have been of the target bacteria. Thereby a mycelial explant 6 mm of diameter from each isolate 10 days old cultured on PDA was collected then placed on a mat bacterium on MHA medium. Cultures have incubated at 37°C for up to 24 hours and the diameters inhibition have observed and measured.

Identification of the fungal endophyte EC 28

Identifying the isolate coded EC 28 was made first from of a 10-day old pure culture on PDA medium in observing the color and the growth rate of the mycelium. Then microscopic observation of the conidiophores and conidia was made on the morphology of fruiting bodies [18; 19] and finally the ITS regions of rDNA have amplified by PCR and sequenced DNA extraction genomics of the mycelium of EC 28 has been realized according to the method described by White *et al.*, [20] with some changes. EC 28 grown for 5 days in 20 ml of PDB medium. The culture has been centrifuged at 10,000g for three (3) min. After removal of the supernatant, the pellet was homogenized in 400 µl of TBS buffer (0.4 M NaCl, 10 mM Tris-HCl at pH8 and 2 mM EDTA at pH8) and vortexed for one minute. Then 40 µl of 20% SDS and 8 µl of proteinase K (20 mg/ml) were added, and the mixture was incubated at 60°C for 3 hours. After incubation, 300 µl of 6 M NaCl has introduced in the various tubes, which also vortexed for 30 seconds then centrifuged at 10000 g for 30 min at 4°C. The supernatant was transferred in a 2 ml tube to which was added equal of 100% isopropanol. The tubes have incubated at -20°C for one hour and centrifuged at 10000g for 20 min at 4°C. The pellet was washed with 70% ethanol then centrifuged again for 10 min at speed maximum the nerve resulting from centrifugation then collected and dried at 37°C for 5 min. DNA as well got was handed over suspended in 300 µl of sterile TE (Tris-EDTA) buffer and stored at -20°C. PCR amplification and sequencing of ITS rDNA were performed using the following set of primers: ITS4 (5-TCCTCCGCTTATTGATATGC-3) and ITS5 (5-GGAAGTAAAAGT CGTAACAAGG-3') [21] The PCR was realized in a reaction volume of 23.5 µl containing 5 µl of DNA template, 4 µl of Premix (5x Firepol Master mix SOLIS BIODYNE), 13 µl of biomol water and 1.5 µl of ITS4 couples and ITS5. PCR amplification was done according to the program next: a denaturation initial 94°C for 4 min, followed by primer annealing time 94°C for 1 min, 55°C for 1 min, and 72°C for 2 min for 35 cycles. A final elongation of 72°C for 15 min. PCR products were analyzed by agarose gel electrophoresis. The resulting ITS sequence of EC 28 rDNA was analyzed with the NCBI Gen Bank Sequence Database (<http://www.ncbi.nlm.nih.gov>) to identify related species closer.

Production, purification and characterization of secondary metabolites EC 28

A volume of 2 ml of a suspension of EC 28 spores was distributed over sterilized rice medium (40 g of rice and 40 ml of distilled water supplemented with 5% glucose, 5 g/l of peptone and extract yeast) and incubated at 28°C for 21 days. At the end of the 21 days of incubation, the cultures of EC 28 were extracted with ethyl acetate. The organic phases were combined, dried (MgSO₄) and evaporated under vacuum at 35°C. The crude extract obtained (2.5g) was fractionated by chromatography on open silica gel column to give 4 fractions numbered from F1 to F4. According to the analysis of the thin layer chromatography profiles, the F2 and F3 fractions were further purified. Fraction F2 (627 mg) was chromatographed on silica gel with an elution system (C₆H₁₂/EtOAc, 70:30) to give compound **1** (325 mg). Fraction F3 (254 mg) was also subjected to silica gel column chromatography with an elution system (C₆H₁₂/EtOAc, 60:40/40:60), to give compound **2** (117mg).

Antibacterial activity of secondary metabolites

The strain susceptibility test bacteria to secondary metabolites insulated from EC 28 has carried out by the technique of diffusion in agar medium. In petri dishes containing Mueller Hinton Agar medium, a bacterial suspension of the strains at a concentration of 10^6 CFU/ml inoculated by spreading using a swab. Discs antibiogram have impregnated with 15 μ l of the molecular solution of each compound at a concentration of 0.5 and 0.25 mg/ml. A positive control was carried out with a reference antibiotic ampicillin prepared at 30 μ g/disk. A negative control was carried out with DMSO diluted to 1/10. Petri dishes have incubated at 37°C for 24 hours. After incubation, petri dishes have observed and the diameters of the zones of inhibition have measured. The interpretation was done according to Ponce *et al.*, [22].

Determination of Bacterial MIC and MBC Parameters of verticillin D

The method described by Fawole *et al.*, [23] was used, in 96-well microplate with some modifications. On a 96-well plate, 100 μ l of MHB was distributed in the wells. 70 μ l of bacterial suspension of each strain was added. 30 μ l of each concentration of verticillin D prepared at 500; 250; 125; 60; 30 and 15 μ g/ml by the double dilution method was added to the media. This amounts to a respective final concentration of 75; 37.5; 18.75; 9; 3 and 1.5 μ g/ml. A negative control was carried out with 50 μ l of bacterial suspension added to 150 μ l of MHB broth. The positive control was prepared with 100 μ l of MHB broth supplemented with 50 μ l of bacterial suspension and 50 μ l of Ampicillin at 30 μ g/disk. Plates were incubated at 37°C for 24 hours. The lowest concentrations that gave no growth after sub culturing were considered the minimum inhibitory concentration (MIC) values [24]. The minimum bactericidal concentration (MBC) was estimated by inoculating 2 μ l of each well which does not show cloudiness on Mueller Hinton Agar (MHA), and observation of bacterial colonies after incubation at 37°C for 24 hours. The MBC corresponds to the lowest concentration where no bacterial colonies are observed. The final evaluation of the results is indicated by the report of the MBC and the MIC.

Statistical analysis

Analysis statistic has been carried out by ANOVA-one-way analysis of variance followed by Tukey's test for comparison between verticillin D and ampicillin concentrations on the one hand and between tests. All results obtained have cast in mean $\pm$ standard deviation. Probability values less than ($P < 0.05$) have considered as statistically significant. All results have analyzed using the software analysis statistical *GraphPad Prism 5*.

RESULTS

Isolation of endophytic fungi and screening of their activity antibacterial

At the end of the antibacterial screening, a single endophytic fungus was active against the Gram- bacterium (*E. coli* 942) (Figure 1) and the Gram+ bacterium (*S. aureus* 934) with respective inhibition diameters of 16 mm and 21mm. This active endophytic fungus was coded EC 28.

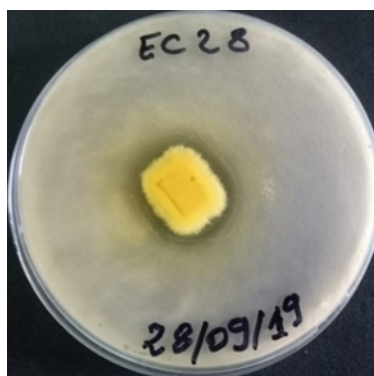


Figure 1: Effect antibacterial of isolate EC 28 on the strain *E. coli* 942

Identification of the fungal endophyte EC 28

Isolate EC 28 identified as belonging to the genus *Clonostachys* based on characteristics culture and the morphology of fruiting bodies and spores. The colonies were white with a light-yellow outline, producing orange pigments on the cultures (figure 2). The conidiophores were like verticillium, bearing phialides as first described by Schroers et al., [25]. The ITS region of EC 28 rDNA amplified by PCR then sequenced. The BLAST of the sequence obtained in the GENEBANK NCBI allowed to identifying the EC28 isolate with 98.55 % identification as being *Clonostachys rosea* under the number of accession NR_145021.1.

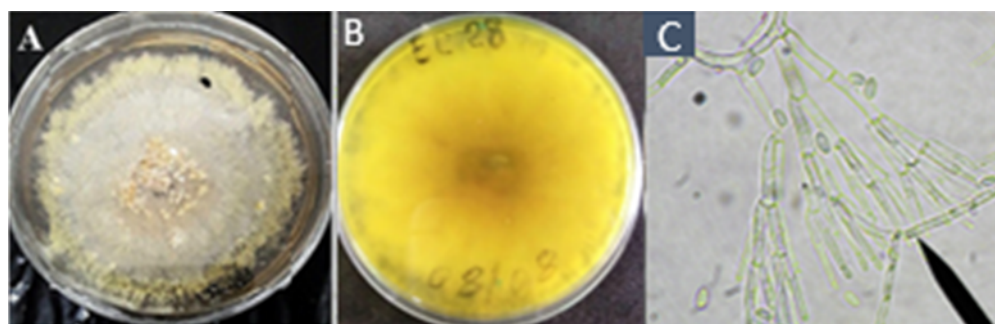


Figure 2. Macroscopic and microscopic description of *C. rosea*
Macroscopic and microscopic description of *C. rosea*. Macroscopic and microscopic identification of *C. rosea* showed a white color with a yellow outline with medium growth (10 days) on PDA medium (A). The back of the box is yellow (B). Under microscopy, the conidiophores are fan-shaped without conidia (C)

Identification of secondary metabolites

Fraction F2 gave compound **1** as white crystals of mass 325 mg. Analyzes of the proton ^1H NMR 1D spectrum and the carbon ^{13}C spectrum (DEPT-Q) as well as the 2D NMR showed that compound **1** at ESI $[\text{M}-\text{H}]^-$ m/z 395.3297 (calc. for $\text{C}_{28}\text{H}_{44}\text{O} - \text{H}^+$: 395.330) corresponds to ergosterol (figure 3). Fraction F3 led to compound **2** as a white powder of mass 117 mg. Analysis of the mass spectrum of compound **2** made it possible to demonstrate a high resolution molecular ion peak ESI $[\text{M}+\text{H}]^+$ m/z 757.123 (calc. for $\text{C}_{32}\text{H}_{32}\text{N}_6\text{O}_8\text{S}_4 + \text{H}^+$: 757.124). This analysis, combined with the 1D (proton ^1H and DEPT Q) and 2D NMR data, made it possible to determine the chemical structure of the compound in accordance by the data in the literature. It was verticillin D [26] (figure 4).

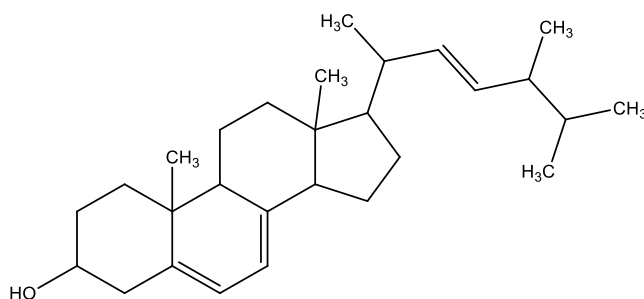


Figure 3: Structure of ergosterol

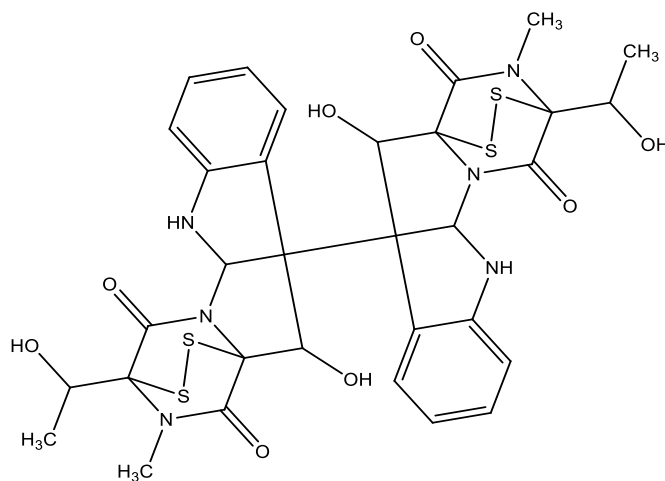


Figure 4: Structure of verticillin D

Antibacterial activity of isolated compounds

Verticillin D showed antibacterial activity on all multidrug resistant strains tested. The test results showed inhibition diameters for both concentrations tested. The *S. aureus* 934 strain was the most sensitive with inhibition diameters of 23 ± 0.5 mm to 30.33 ± 0.3 mm respectively for the concentrations of 0.25 mg/ml and 0.5 mg/ml. As for the *E. coli* 4814 stain, it was the least sensitive to concentrations of 0.25 mg/ml and 0.5 mg/ml for respective inhibition diameters of 20.33 ± 0.3 mm and 21.67 ± 0.3 mm (Table 2). Statistical analyzes showed no significant difference ($P > 0.05$) for the concentrations of 0.5 mg/mL and 0.25 mg/ml of verticillin D to that of ampicillin on the strain *K. pneumoniae* 815 (figure 5). The ratio of antibacterial parameters (MBC/MIC) showed that verticillin D has a bactericidal effect on all the germs tested in particular (table 3). Ergosterol showed no activity on all strains tested.

Table 2. Verticillin D inhibition diameter (mm)

Bacterial Strains	verticilline D		Ampicillin 30µg/disk
	0.5 mg/ml	0.25 mg/ml	
<i>S. aureus</i> 931	26 ± 0.5	23.33 ± 0.8	20 ± 0.5
<i>K. pneumoniae</i> 815	23 ± 0.5	20 ± 0.5	21 ± 0.5
<i>S. aureus</i> 934	30.33 ± 0.3	23 ± 0.5	20 ± 0.5
<i>E. coli</i> 4814	21.67 ± 0.3	20.33 ± 0.3	10 ± 0.5
<i>E coli</i> 942	24.67 ± 0.8	22 ± 0.5	20.33 ± 0.3

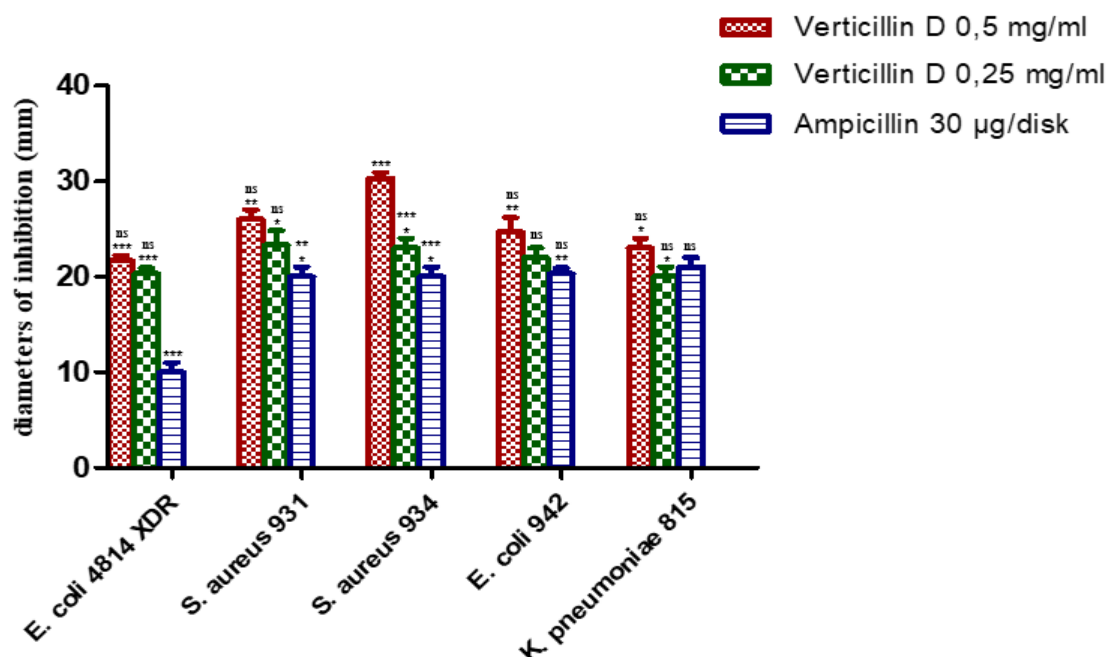


Figure 5: Sensitivity histograms of ESBL multi-resistant strains to verticillin D
ns: ($P > 0.05$), difference not significant

*: significant difference between the different concentrations of verticillin D on the one hand and between the concentrations of verticillin D and Ampicillin on the other hand ($P < 0.05$).

**: highly significant difference between the different concentrations of verticillin D on the one hand and between the concentrations of verticillin D and Ampicillin on the other hand ($P < 0.001$).

***: very highly significant difference between the different concentrations of verticillin D on the one hand and between the concentrations of verticillin D and Ampicilline on the other hand ($P < 0.0001$).

Table 3. Antibacterial Parameters of verticillin D

Bacterial Strains	MIC (µg/ml)	MBC (µg/ml)	MBC/MIC	Effect
<i>S. aureus</i> 931	18.75	37.5	2	Bactericida
<i>K. pneumoniae</i> 815	37.5	75	2	l
<i>S. aureus</i> 934	3	3	1	Bactericida
<i>E. coli</i> 4814	18.75	37.5	2	l
<i>E. coli</i> 942	18.75	37.5	2	Bactericida
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DISCUSSION

Ceiba pentandra is a tree whose different parts (leaves and bark) are known for their use in traditional medicine. This study led to the isolation of 21 endophytic fungi from the stem bark of *C. pentandra*. The screening of these fungi having an antibacterial activity led to the selection

of isolate EC 28. Unlike the other isolates, the mycelial explant of EC 28 successfully inhibited the growth of a bacterium with Gram- and Gram+ represented respectively by multiresistant strains *E. coli* 942 and *S. aureus* 934. Depending on the method used, this antibacterial activity of EC 28 revealed that this fungus produced antibacterial secondary metabolites, which were diffused through the agar. Indeed, medicinal plants are known to harbor a diversity of endophytic fungi that produce antimicrobial compounds [8]. The literature clearly indicates that the antibacterial secondary metabolites produced by endophytic fungi belong to various classes of compounds such as flavonoids, xanthenes, terpenoids, alkaloids, phenols, steroids, and quinines [27; 28]. The molecular methods used enabled isolate EC 28 to be identified with certainty as being *Clonostachys rosea*. This fungus is known to be a common soil saprophyte [29]. Many studies have revealed that *C. rosea* is a potential biological control agent against many phytopathogenic fungi [30] eg *Botrytis cinerea* cocoa pathogen [30], *Fusarium sp.* ear of wheat pathogen [31]. This study reports the first description of *C. rosea* as an endophytic fungus of a medicinal plant. The chemical study of the crude extract of *C. rosea* led to the identification of ergosterol and vertricillin D. Ergosterol belongs to sterols like cholesterol. Being the main sterol of fungal membranes, it is essential for the growth of fungi. The antibacterial activity achieved with ergosterol thus showed no effect on the multiresistant bacteria tested. Because this compound is devoid of activity. Indeed, ergosterol has a low solubility in oil and is easily crystallized, which represents a problem for its direct use [32]. Moreover, this molecule becomes active when it is combined with other compounds. Additionally, Park and Kim, [32] synthesized ergosterol derivatives with ergosterol unsaturated fatty acids, more specifically, ergosterol oleate and ergosterol linolenate. These authors found that these compounds had strong growth inhibitory activity against certain dairy and cosmetic spoilage bacteria. Joshi et al., [26], first described verticillin D. This compound belongs to epidithiodioxopiperazines. A dimeric molecule contains in its chemical structure two disulfide bridges. Members of this class of molecules exhibit various biological activities including cytotoxic, antifungal, and antibiotic. Epidithiodioxopiperazines are produced by certain fungal species belonging to the genera *Aspergillus*, *Penicillium*, *Bionectra* and *Clonostachys*. The antibacterial activity achieved with verticillin D showed a bactericidal effect on all the multiresistant bacteria tested both on Gram- and on Gram+. The results obtained on the strains *S. aureus* 931 and *S. aureus* 934 are in agreement with those of Zheng et al., [33] who highlighted the antibacterial potential of verticillin D isolated of *Bionectra byssicola* on *S. aureus* CCARM 3167 (MRSA) with a MIC of 3 µg/ml. Our work has shown the antibacterial potential of verticillin D on Gram- bacteria such as *K. pneumoniae* and *E. coli*. This activity could be explained by the presence of two di-disulfide ridges contained in the chemical structure of this molecule. De Clercq et al., [34]; Rightsel et al., [35] reported that the antiviral activity of gliotoxin, an epepidithiodioxopiperazine as due to the presence of the disulfide bridge contained in its chemical structure. Moreover, the removal of sulfur atoms or the addition of the reducing agent dithiothreitol completely suppresses this activity [36; 37].

CONCLUSION

The objective of this work was to determine the antibacterial effect of endophytic fungi of *C. pentandra* and to identify the compound(s) responsible for this activity. Screening for antibacterial activity made it possible to select the active isolate EC 28 among these 21 endophytic fungi. The macroscopic, microscopic and molecular identification methods used made it possible to identify isolate EC 28 as *Clonostachys rosea*. The chemical study of *C. rosea*

subjected to fractionation on a column chromatography led to the identification of ergosterol and verticillin D. Antibacterial tests carried out with these isolated compounds showed that only verticillin D has an antibacterial effect against multi-resistant clinical bacterial strains (*S. aureus* 931, *K. pneumoniae* 815, *S. aureus* 934, *E. coli* 4814, *E. coli* 942).

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