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Antibacterial Activity of Secalonic Acid B Produced by *Clonotachys sp.*, an Endophytic Fungus Isolated from *Picralima nitida* Leaves

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Abstract: Natural products and their derivatives have always been the major source of bioactive compounds used for the treatment of new and existing diseases. The present study was carried out to investigate the antibacterial activity of the crude extract of an endophytic fungus isolated from the leaves of *Picralima nitida*, a medicinal plant of the Apocynaceae family and to isolate and identify the molecule responsible for this activity. Thus, secalonic acid B isolated from the crude extract of an endophytic fungus was tested on six (06) pathogenic bacterial strains by agar diffusion method for strain susceptibility test. And the liquid diffusion test was used to determine the antibacterial parameters (BMC and MIC). The isolated endophytic fungus was identified by molecular biology methods as a strain of *Clonotachys sp*. The antibacterial test performed with the *Clonotachys sp.* extract showed inhibitory activity on *Staphylococcus aureus* (ATCC 25923, 931/18 and 934/18) and *Klebsiella pneumoniae* (815/18) strains with inhibition diameters ranging from 18.43±1.05 to 7.56±0.73 mm. This extract has a bactericidal power on these strains. As for the secalonic acid B isolated from this fungal extract, it also showed efficacy against *Staphylococcus aureus* and *Klebsiella pneumoniae* with bactericidal power. The endophytic fungus *Clonotachys sp*. is therefore a potential source of antibacterial compounds.

Keywords: *Picralima nitida*, Endophyte, *Clonotachys sp.*, Antibacterial Activity

1. Introduction

The emergence of new diseases and the development of resistance of pathogenic microorganisms to antibiotics are the problems encountered continuously in our society. In the face of these problems, natural remedies are the mainstay of traditional medicine [1]. Despite the development of chemistry, active ingredients of natural origin are still sought after for their diverse and proven biological properties [2] and are used as basic compounds for the synthesis of various other molecules. These active molecules and their precursors are usually derived from plants, fungi, bacteria, endophytes (fungi and bacteria), etc. [3] However, the non-reasoned exploitation of natural resources, the search for new cultivable land, advanced urbanization..., are among other harmful actions that contribute significantly to the alteration of natural potentialities [4]. It is therefore necessary to explore different natural sources. In this context, the endophytic fungi of medicinal plants have been the subject of several scientific researches [5]. Interest in these microorganisms has been revived with the discovery of the endophytic fungus *Taxomyces andreanae*, isolated from *Taxus brevifolia*, capable of producing the anticancer agent taxol [6].

In this study, an endophytic fungus was isolated from the leaves of *Picralima nitida*. Picralima nitida is a plant that occupies an

important place in the African pharmacopoeia. It is used against malaria, typhoid fever, anemia, convulsions, jaundice, hypertension, gastrointestinal disorders, hernia, etc. [7-9]. Several bioactive molecules have been isolated from this plant, including akuammicin which has the anti-diabetic and opioid analgesic activities [10]. The objective of this study is to evaluate the antibacterial activity of a compound isolated from the extract of an endophytic fungus from the leaves of *Picralima nitida*.

2. Material and Methods

2.1. Isolation of Endophytic Fungi

Healthy leaves of *Picralima nitida*, collected at the National Floristic Center (CNF) of the Felix Houphouet-Boigny University of Ivory Coast. The leaf surfaces were sterilized according to the protocol described by [11]. Briefly, after rinsing them thoroughly in water, the leaf surfaces were sterilized in 70% ethanol for one minute, then in 3% sodium hypochlorite for four minutes and finally in 70% ethanol for one minute. They were successively rinsed three times in sterile distilled water before being cut into small rectangular pieces with a sterile slide and placed on PDA agar medium. The plates were incubated at 27°C. The sterility test was performed with the rinsing water of the leaves. The isolates obtained were purified by subculturing onto a new agar plate [12].

2.2. Molecular Identification

Molecular identification of the isolate was performed by analyzing the 18S ribosome DNA sequence of the endophytic fungus using polymerase chain reaction (PCR) cloning technology. For this purpose, the fungal strain was grown on PDA, the dishes were then incubated at 28°C for 7 to 15 days for mycelium growth. Genomic DNA was extracted as described by [13] with slight modifications. Briefly, 0.5 g of mycelium was weighed and ground in a mortar with a pestle, the mycelium powder was suspended in extraction buffer (Tris HCl 100 µM, EDTA 100 µM, NaCl 100 µM, SDS 2%, pH 8) (1:1, w/v). After centrifugation at 16100g for 5 min, the supernatant was collected and the proteins were precipitated with 5 M sodium acetate, pH 5.5 (1:10, v/v) for 1 h at 4°C. After centrifugation at 16100g, 5 min, DNA was precipitated with isopropanol (v/v) for 18h at -20°C. After centrifugation at 16100g for 30 min, DNA was washed with cold 70% ethanol and suspended in sterile ultrapure water. The DNA was stored frozen at -20°C.

Fungal isolate was identified by sequencing the ITS (Internal Transcribed Spacer) regions of the rDNA (Eurofins Genomics ITS5 and ITS4). 4 µL of 5x Master Mix buffer (Slis biodyne FIREPOL), 0.75 µL of each fungal primer at 10µM, 5 µL of fungal DNA were combined in a final volume of 25 µL. PCR amplifications were performed with the ITS5 and ITS4 primers according to the following program: 4 min at 94°C, followed by 35 cycles of 1 min at 94°C, 1 min at 55°C and 2 min at 72°C, with a final elongation of 15 min at 72°C. Analysis and comparison of the sequence obtained after PCR was performed with nucleotides contained in the GenBank database (BLASTn) (http://www.ncbi.nlm.nih.gov) [14]. The identification of the isolate was done by local alignment search [15].

2.3. Extraction of Secondary Metabolites

The fungus has been grown on rice media and then incubated at room temperature for metabolite production for three to weeks depending on the isolates. After the growth of the mycelium, the extraction of the metabolites was done by maceration of the mycelium in ethyl acetate [16]. After three successive filtrations on absorbent cotton, the filtrate was concentrated using a rotary evaporator.

2.4. Antibacterial Activity

In this study, two reference strains and four multi-resistant strains were used. They were provided by the Laboratory of Bacteriology and Virology Laboratory of the Pasteur Institute of Côte d'Ivoire.

Table 1. *Profiles and origins of bacterial strains.*

Souches	Profil	origines
S. aureus ATCC 25923	-	Reference strain
S. aureus 931/18	KTG resistant	Patient blood
S. aureus 934/18	KTG resistant	Patient blood
K. pneumoniae 815/18	ESBL	Patient urine
E. coli ATCC 25922	-	Reference strain
E. coli 942/18	ESBL, fluoroquinolone resistant	Patient pus

2.4.1. Preparation of the Inoculum

Bacterial inoculum was prepared from colonies less than 24 h old in Mueller Hinton broth (MHB). An isolated colony from the bacterial culture was picked with a platinum loop and homogenized in 10 ml of the broth and incubated for 3 to 5 h at 37°C to have a pre-culture. A volume of 0.1 ml or 1 ml was taken respectively for *Enterobacteriaceae* and *Staphylococcus* and was added to 10ml of sterile BMH. This bacterial suspension made is estimated to be about 106 cells/ml and constitutes the 100 dilution or pure inoculum [17].

2.4.2. Sensitivity Test

The sensitivity of the strains to plant extracts was performed by the agar diffusion technique [18]. Mueller Hinton media were inoculated by flooding. Using sterile forceps, sterile disks were placed on the agar. Each disk received 20 µl of the test substance at concentrations of 5, 2.5 and 1.25 mg/ml. After 15 min of diffusion at laboratory temperature, the Petri dishes were incubated at 37°C for 18-24 h. The presence or absence of a zone of inhibition was observed. Interpretation was done according to Duraffourd et al, Ponce et al, [19, 20].

2.4.3. Determination of the Minimum Inhibitory Concentration (MIC)

In a series of eight hemolysis tubes numbered C1 to C8, 1mL of pure inoculum was introduced. Then, 1mL of plant extract was added to the tubes according to the prepared concentration range. This distribution of plant extract was done so that 1ml of 10 mg/ml plant extract was transferred to tube C1, tube C2

received 1ml of 5 mg/ml and so on to tube C7 which received 1ml of the 0.15 mg/ml solution. Tube C8 received 1 ml of sterile BMH instead of the plant extract, which served as a growth control. Due to the volume/volume dilution thus achieved, the concentration in the tubes was reduced by half. These tubes were incubated at 37°C for 24 h. The MIC is the lowest concentration of the substance for which there is no growth visible to the naked eye after an incubation time of 18 to 24 h. It was determined by observing the growth-induced cloudiness of the test organisms in each tube [21].

2.4.4. Determination of the Minimum Inhibitory Concentration (MIC)

The minimum bactericidal concentration (MBC) is the lowest concentration of substance that leaves no more than 0.01% of germs surviving. Using a sterile loop, the contents of tubes in which no cloudiness was observed were collected and plated on Mueller-Hinton agar starting with the MIC tube. Inoculation was done in 5 cm long parallel streaks on the agar surface. This plate was numbered: Plate A. At the same time, four (04) successive 10-fold dilutions of the pure inoculum (100) were made. We obtained 4 dilutions at 10^{-1}, 10^{-2}, 10^{-3}, 10^{-4}. These different dilutions as well as the pure inoculum were inoculated with a calibrated loop of 2 µl per 5 cm long streak on Mueller Hinton agar and incubated at 37°C for 24 h. This plate was numbered: Plate B.

After 24 h of incubation at 37°C, the number of colonies on the streaks was compared to those on the inoculum counting plate (Box A). Thus, the first experimental tube whose number of germs on its streak is less than or equal to that of the 10^{-4} dilution will correspond to the minimum bactericidal concentration [22].

2.5. bio-guided Purification of the Endophyte Extract

The initial fractionation process of the crude extract of the fungal isolate was performed using an open glass column on silica gel. Successive elutions were performed from the column with gradients of cyclohexane / ethyl acetate (with increasing polarity), then AcOEt / MeOH (8:1; 5:5) and finally a column wash step with methanol. The eluates from this column were collected in six (06) fractions (F1 to F6) according to the similarity of their profiles obtained by thin layer chromatography (TLC). Fractions 3 and 4, dissolved in ethyl acetate, presented precipitates at the bottom of the tubes. These precipitated compounds were put together to constitute the fraction F7. So in total seven (07) fractions numbered from F1 to F8. All these fractions (F1 to F7) were tested on the bacterial strains. The most active fraction (s) were retained for further purification. The active fraction was further purified by chromatography on silica column using dichloromethane/Methanol (of increasing polarity). From this column, four (04) subfraction were obtained. These sub-fractions were tested for their efficiency on the studied germs. The most active sub-fraction was purified to give a compound. The elucidation of the structure of the isolated compounds followed a standard scheme through mass spectrometry (MS) and NMR spectra.

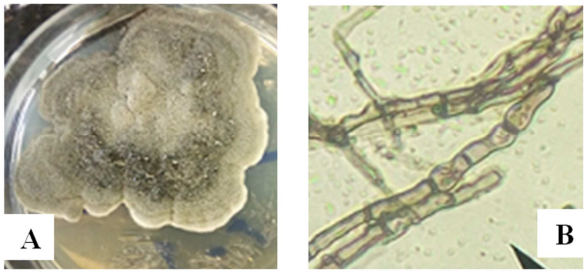
2.6. Statistical Analysis

An analysis of variance (ANOVA-ONE WAY) was performed to compare the means of inhibition diameters, using GraphPad Prism 5.0 software.

3. Results

3.1. Identification of the Isolate

Macroscopic observations showed on the PDA medium, flat and grey white colonies with a black back side. The contours are coated and the growth is slow (10 days for a diameter of 10 mm). In microscopy, the mycelium is transparent, branched, and irregularly septate. Conidiophores are observed in some places on the mycelium in the form of vesicles. The production of spores is slow on PDA.



A: macroscopic image on PDA medium; B: microscopic observation

Figure 1. *Images of the endophytic fungus isolated from P. nitida leaves.*

Molecular biology methods were used to obtain the query sequence, a 470 bp DNA fragment corresponding to the sample. This sequence was used to run a BLASTn with the Megablast program. The database used was RNA_typestrains/ITS_RefSeq_Fungi. The information collected is contained in the following table.

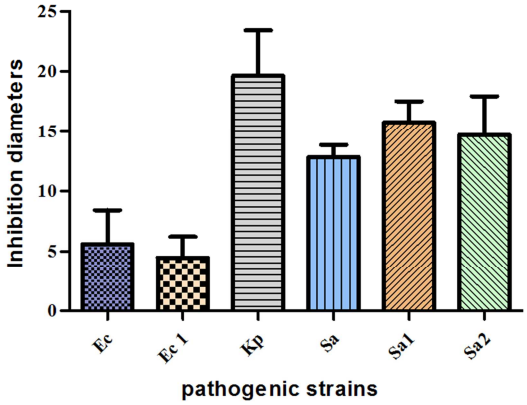


Figure 2. *Diameters (mm) of inhibition zones induced by extracts.*

Ec: *E. coli* ATCC 25922; Ec 1: *E. coli* 942/18; Kp: *K. pneumoniae* 815/18; Sa: *S. aureus* ATCC 25923; Sa 1: *S. aureus* 931/18; Sa 2: *S. aureus* 934/18

Table 2. *Fungal isolate identifier.*

source	Fungal DNA Sequence (FASTA format)	Fungus Name
Picralima nitida	5'-TGTCTCTACTTCGCAGAGGAGGCCACGACGGGTCCGCCACTAGATTTAGGGGCCGGCCGTCCCTC GCGGGCTTTGGCCGATCCCCAACACCACGCCCTAGGGGCATGAGGGTTGAAATGACGCTCAGACAG GCATGCCCGCCAGAATACTGGCGGGCGCAATGTGCGTTCAAAGATTCGATGATTCACTGAATTCTGCA ATTCACATTACTTATCGCATTTCGCTGCGTTCTTCATCGATGCCAGAACCAAGAGATCCGTTGTTGAAA GTTTTTATTTATTTGTAAAAACTACTCAGAAGATTCCAAAATAAAACAAGAGTTAAGGTTCCTAGGCGG GCGCCGGATCCGGGGCACACGAGGCGCCCGGGGCGATCCCGCCGAAGCAACGATAGGTATGTTCACA TGGGTTTGGGAGTTGTAAACTCGGTAATGATCCCTCCGCTGGTTCACCAACGGAGACCTTGTTCAT -3'	Clonotachys sp.

3.2. Antibacterial Activity

3.2.1. Sensitivity Test

The sensitivity test performed with the leaf extract of *P. nitida* showed no zone of inhibition on E. coli ATCC and *K. pneumoniae* 815/18. But weak zones of inhibition ranging from 4.6±0.30 to 8.57±1.20 mm in diameter on S. aureus (ATCC 25923, 931/18 and 934/18) and E. coli (942/18) strains. As for the crude extract of the endophyte, it showed low activity on E. coli ATCC 25922 and E. coli 942/18 strains with diameters of 5.56 and 4.63 mm respectively. On the other hand, on the S. aureus and *K. pneumoniae* strains tested, it showed a strong inhibitory activity with inhibition diameters ranging from 12.54±1.70 to 19.23±1.04 mm. Ampicillin used as a reference molecule showed inhibitory activity on all strains tested with inhibition diameters greater than 23 mm.

3.2.2. Determination of Antibacterial Parameters (MIC & BMC)

As the leaf extract did not have any activity on all the bacterial strains used, we determined the antibacterial parameters of the fungal extract. It was observed that there was a progressive decrease in the intensity of the bacterial growth-induced cloudiness with increasing concentration of the fungal extract in the tubes. The results of the determination of antibacterial parameters are presented in Table 3.

Table 3. *Antibacterial parameters of the fungal extract.*

fungus	Parameters	Gram-negative bacteria			Gram positive bacteria		
		Ec	*K.p*	Ec1	*S.a*	*S. a 1*	*S. a 2*
Clonotachys sp.	MIC (mg/ml)	ND	0,625	ND	5	2,5	2,5
	BMC (mg/ml)	ND	0,625	ND	5	2,5	2,5
	BMC/MIC	ND	1	ND	1	1	1

ND: not determined

3.3. Metabolite Isolated from the Extract of Clonotachys sp.

Bio-guided purification of the crude extract of *Clonotachys sp.* yielded a majority compound. This compound was obtained as a yellow solid. HRESI-TOF mass spectrometry performed in positive mode showed a molecular peak at 607.1816 m/z. This result suggests a molecular formula: $C_{32}H_{31}O_{14}$. The analysis of the different NMR spectra compared to literature data allowed to deduce that it is secalonic acid B.

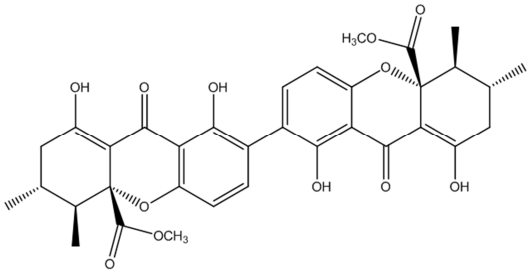


Figure 3. *Structure of secalonic acid B.*

3.4. Antibacterial Activity of Secalonic Acid

Secalonic acid from the purification of the noise extract of *Clonotachys sp.,* was tested on the studied strains. The efficacy of this molecule on these germs was demonstrated by the sensitivity test through the different zones of inhibition observable on Muller-Hinton agar medium (Table 4). The results showed that secalonic acid induced a larger zone of inhibition (25.42±1.05 mm) on *K. pneumoniae*. While that obtained with the reference antibiotic (chloramphenicol) was 31.08±2.46 mm. This result allows us to conclude that secalonic acid could be a potential source of a new antibiotic.

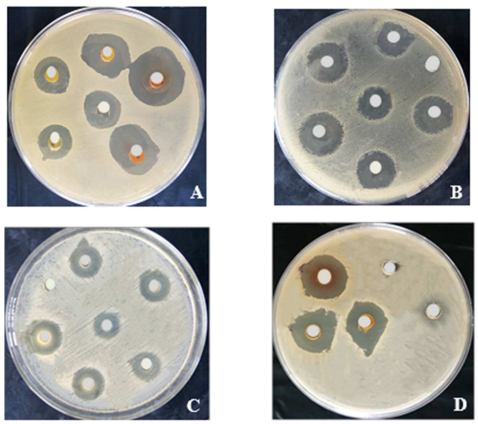
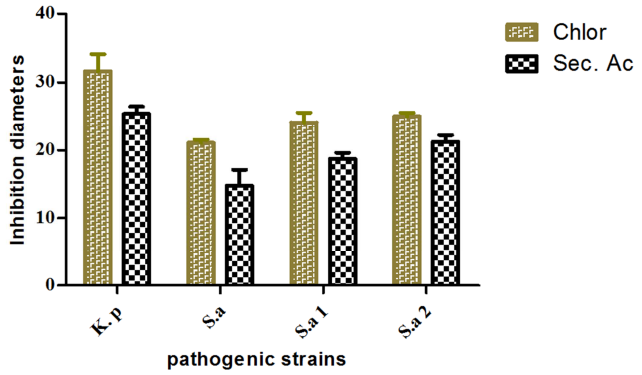


Figure 4. *Images of the zones of inhibition of secalonic acid B on the different strains.*

Action of secalonic acid on: A: *S. aureus* ATCC 25923; B: *S. aureus* 931/18; C: *K. pneumoniae* 815/18, D: *S. aureus* 934/18.



Sec Ac: Secalonic acid B; Chlor.: Choramphenicol

Figure 5. *Diameters (mm) of the inhibition zones induced by the isolated compound.*

Table 4. *Minimum inhibitory concentration (MIC) and bactericidal concentration (BMC) values of total crude extracts in µg/mL.*

Strains	MIC	BMC	BMC/MIC	Power
S. aureus ATCC 29213	650	700	1,07	Bactericidal
S. aureus 931/18	1000	1000	1	Bactericidal
S. aureus 934/18	730	850	1	Bactericidal
K. pneumoniae 815/18	320	400	1,25	Bactericidal

4. Discussion

In the present work, the antibacterial activity was evaluated using a solid and liquid dilution method. The results obtained from the antibacterial tests showed that the leaf extract had no activity on all the tested strains. While *Clonotachys sp.* extract showed inhibitory activity against *K. pneumoniae* and the three strains of *Staphylococcus aureus* at a concentration of 1.5 mg/ml. The BMC/MIC ratio equal to 1, allowed to deduce the bactericidal action of the fungal extract on these strains. This bactericidal action could be due to the production of antibiotic molecule by this fungus able to diffuse in the medium. Antimicrobial activities of secondary metabolites produced by endophytic fungi by numerous works [23, 24]

Previous work on *Clonotachys sp*. revealed that its main metabolites belong to nitrogen-containing compounds, polyketides and terpenoids. Some piperazines, polyketides and terpenoids have been isolated only from *Clonostachys* fungi, presenting obvious biological activities such as cytotoxic, antinematodal and antimicrobial activities [25].

In the present study, bio-guided purification of the crude extract of *Clonotachys sp*. allowed the isolation of a bioactive molecule. Mass spectrometry data, and NMR spectra of this compound identified it as secalonic acid B. This compound is well known as a dimeric tetrahydroxanthone belonging to a class of mycotoxins [26]. Its monomers are linked together by a 2,2'-biphenol bond [27]. This molecule was isolated for the first time from marine fungi by Harada et al then by Kurobane et al [28, 29]. These authors highlighted its anti-tumor and phlogistic activity. Our results show that secalonic acid B is the main compound responsible for the antibacterial activity of the crude extract of *Clonotachys sp.* This compound could be useful as a new therapeutic approach for the prevention of some infectious diseases.

5. Conclusion

Plants have always represented a huge reservoir of potential compounds that have the advantage of having several biological activities. In this study, an endophytic fungus was isolated from the leaves of *Picralima nitida*. molecular biology identified it as *Clonotachys sp*. Antibacterial tests revealed that the crude extract of this fungus was effective against the clinical strains of *Staphylococcus aureus* and *Klebsiella pneumonia* tested with inhibition diameters ranging from 12.54±1.70 to 19.23±1.04 mm. The bio-guided purification of this extract allowed to obtain a majority compound at the origin of this activity. Mass spectrometry and NMR (Nuclear Magnetic Resonance) data confirmed that it was secalonic acid B. This compound showed a bactericidal effect on *Staphylococcus aureus* (ATCC 25923, 931/18 and 934/18) and *Klebsiella pneumonia* (815/18) strains. *Clonotachys sp.* is a potential source of antibacterial compounds. However, further studies to evaluate the toxicity of this compound would be necessary.

Conflict of Interest

All the authors do not have any possible conflicts of interests.

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