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Original Research Article

Antimicrobial activities of two secondary metabolites isolated from *Aspergillus niger*, endophytic fungus harbouring stems of *Acanthus montanus*

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Emergence of multidrug-resistant pathogenic microorganisms has prompted a worldwide search for new antibiotics from various sources. Endophytic fungi from unique habitats that are much easier to grow are considered as potential sources of novel bioactive compounds including antimicrobials. The objective of this work was to isolate, identify and characterize compounds produced by endophytic fungi associated with *Acanthus montanus stems. Aspergillus niger* was isolated from the stems of *Acanthus montanus* and subjected to solid-state fermentation on rice medium. Its secondary metabolites were extracted using ethyl acetate. The obtained crude extract was sequentially fractionated by column chromatography. The structures of isolated compounds were identified based on their spectral studies using nuclear magnetic resonance and comparison with published data. The antimicrobial and cytotoxicity of these compounds were determined using broth microdilution method. Two compounds were isolated from *Aspergillus niger* and identified as Trypacidin A and Methylsulochrin. They showed different degrees of antibacterial activities against the tested pathogenic bacteria. Methylsulochrin was most active on *Enterobacter cloacae* and *Enterobacter aerogenes* with MIC between 7.8–31.2 µg/mL. Trypacidin A was inactive on all the tested microorganisms Methylsulochrin isolated from *Aspergillus niger* crude extract is a potential candidate for the development of anti- infectious diseases molecules.

Key words: *Acanthus montanus*, endophytic fungi, *Aspergillus niger*, Trypacidin A, Methylsulochrin, antibacterial activity.

INTRODUCTION

Development of multiple drug-resistant microbes raised the need to search for novel and potent antimicrobials for treatment of human and animal infections (Wise, 2008). An intensive search for newer and more effective agents to deal with these infections is a continuous process. Endophytic fungi are a diverse group of microorganisms which spend all or part of their life cycle inter- and/or intra-cellularly, colonizing healthy tissues of their host

plants, typically causing no apparent disease symptoms (Azevaldeo et al., 2013; Sunitha et al., 2013). An increasing number of compounds with interesting biological activities and a high level of biodiversity (Tiwari, 2015) are being isolated from these microorganisms.

Since the bioactive compound paclitaxel was successfully discovered from the endophytic fungus *Taxomyces andreanae* in 1993, many scientists have increased their interest in studying fungal endophytes as potential producers of novel and bioactive compounds. Over the past two decades, many valuable bioactive compounds with antimicrobial, insecticidal, cytotoxic and anticancer activities have been discovered from endophytic fungi (Golstime et al., 2015; Gunatilaka, 2006; Shukla et al., 2006; Silva et al., 2005). These bioactive compounds could be mainly classified as alkaloids, terpenoids, steroids, quinolones, isocoumarins, lignans, phenylpropanoids, phenols and lactones (Yu et al., 2010; Zhang et al., 2006).

Acanthaceae is a family of dicotyledonous flowering plants containing almost 250 genera and about 4,000 species. Most are tropical herbs, shrubs, or twining vines; some are epiphytes (Prasad, 2014). *Acanthus montanus* (Nees) T. Anders. (Family: Acanthaceae) is a small shrub with sparse branches and soft stems, widespread in Africa, the Balkans, Romania, Greece and Eastern Mediterranean. In the Center, West and South Region of Cameroon, its leaves are frequently used to treat various ailments such as cough, constipation, diarrhea, indigestion and nausea (Adeyemi et al., 2004; Focho et al., 2009). In other regions of Africa it is used to alleviate urethral discharge, chest pain, emesis, constipation, rheumatic pains, and syphilis. Despite all these potential beneficial effects, scientific reports on biologically active ingredients from endophytic fungi harbouring *Acanthus montanus*, are still lacking. In view of the afore mentioned facts, this work was designed to isolate endophytic fungi associated to *Acanthus montanus* stems, as well as to extract phytochemicals attributing antibacterial properties to the fungi.

MATERIALS AND METHODS

Collection of plant materials

Samples of *Acanthus montanus* (leaves and barks) were collected from Kala Mountain neighborhood of Yaoundé (Longitude 11°21’00’’E and Latitude 03°50'121’’N), and was identified at the Cameroon National Herbarium, where a voucher specimen has been deposited under the reference N0.2127/SRFK.

Culture media

Potato Dextrose Agar (PDA) and rice were used for fungal growth and isolation. Grated potato (200 g) and water (1L) were boiled for 60 minutes. The mixture was cooled, mashed and the extract was collected by squeezing through a pre-cleaned cloth filter. Twenty grams of Dextrose were added to the filtrate and the volume of the extract was adjusted up to 1L with distilled water. The medium was supplemented with 50 µg/mL of chloramphenicol and agar (15 g/L) was added for solidification to obtain PDA used in the study. To obtain enough quantity of extract, we proceed to a large scale cultivation of the fungi. 100g of rice (Bijou, white rice, Thailand) was mixed with distilled water (1:1 m/v) and autoclaved in 1L glass bottle for 30 minutes.

For the culture of enterobacteria strains, Mueller Hinton Agar (MHA) and Mueller Hinton Broth (MHB) were used and prepared according to the instructions of the manufacturer Sigma-aldrich (38g/L for MHA and 21g/L for MHB). Finally, mycobacterium strain was cultured and tested using 7H10 and 7H9 media, enriched with 10XADS (Albumin-Dextrose-Saline).

Isolation of endophytic fungus

The fungus of interest was isolated from the stems of *Acanthus montanus* according to the procedure described by Schulz (Schulz et al., 1993), with slight modifications. The stem was washed several times under tap water. It was sliced into small pieces and surface sterilized by sequential washing in 70% ethanol for 30 sec, then in 5% sodium hypochloride (NaOCl) solution for 5 mins and finally 3 times in sterile water for 3 mins. The plant material was dried on a sterile filter paper and put on a 50 µg/mL of chloramphenicol-supplemented PDA plates. All the plates were incubated at 28 °C to promote the endophytic fungal growth and were regularly monitored for any microbial growth contamination. Each time a fungal growth was observed, it was sub-cultured. Each endophytic culture was checked for purity and transferred to freshly prepared PDA plate. The effectiveness of surface sterilization was checked by making an imprint of the treated portion on agar media (Schulz et al., 1998).

Identification of the endophytic fungus

Cultures were grown on PDA at 28 °C under 12 h light/ 12 h darkness cycles. The isolate code AMSt4 formed abundant colonies that filled the Petri dishes for 8 days. The isolate was identified after macroscopic and microscopic examinations of its morphological features.

The Mueller Hinton Agar (MHA) was used for the activation of bacteria. The Mueller Hinton Broth (MHB) and nutrient agar (Hi-Media) were used for the Minimal Inhibitory Concentration (MIC) determination. 7H9 and 7H10 media were used respectively for the activation and for the determination of the MIC of compounds on *Mycobacterium smegmatis*.

Fungal culture and extraction of secondary metabolites

Aspergillus niger was cultured in 12 flat culture bottles containing 100 g of rice and 100 mL water, autoclaved at 121 °C for 45 min. Each flask received about 5 small pieces (3 x 3 mm) of *A. niger*-growing medium excised from PDA

plate, at the strain growth front under sterile conditions. After 40 days of growth at 28 °C, ethyl acetate (500 mL) was added to each bottle, homogenized and filtered after 24 h and concentrated to dryness in a Rotavapor to obtain a crude extract. The final weight of the obtained extract was 27.4 g.

Fractionation and isolation of compounds

Crude ethyl acetate extract (24.0 g) was submitted to column chromatography using silica gel as stationary phase and using an increasing polarity gradient of Hex/AE and AE/MeOH as follows: Hex 100%, Hex/AE 95:5, Hex/AE 90:10, Hex/AE 80:20, Hex/AE 65:35, Hex/AE 50:50, Hex/AE 25:75, AE 100%, AE/MeOH 95:5, AE/MeOH 85:15, AE/MeOH 85:15, AE/MeOH 75:25, AE/MeOH65:35, AE/MeOH 70:30, MeOH 100%. This gave a total of 78 fractions of 30 ml which were grouped on the basis of their Thin Layer Chromatography (TLC) profiles into ten sub-fractions. Preparative thin layer chromatography coupled with sephadex gel exclusion chromatography led to the isolation of 2 compounds: C222 from F20 (eluted with Hex/AE 4:1 v/v) and C271 from F27 (eluted with Hex/AE, 3:1 v/v). These compounds were identified based on their H and C13 NMR and MS spectral data in comparison with literature (Natalia et al., 2015; Pinheiro et al., 2013).

Antibacterial assay

(a)Pathogenic microorganism tested

A total of 11 bacterial strains were tested: 10 Enterobacteriaceae (2 strains of *Escherichia coli* MEcl and ATCC35218, 02 strains of *Staphylococcus aureus*: SAcl and ATCC43300, 01 strain of *Enteroccocus faecalis* ATCC51299, 01 strain of *Klebsiella pneumonia* ATCC13883, 02 isolates of *Enterobacter cloacae* K2 and BM67, 02 isolates of *Enterobacter aerogenes* CM64 and EA289) and a strain of mycobacterium (*Mycobacterium smegmatis* ATCC700084). The bacterial strains were maintained on agar slant at 4 °C and subcultured on a fresh appropriate agar plates 24 h prior to any antibacterial test.

(b) Inoculation

Suspensions of bacteria were prepared in MHB from cells arrested during their logarithmic phase growth (18 h) on MHA at 37 °C. The turbidity of the microbial suspension was read spectrophotometrically at 600 nm and adjusted to an OD of 0.5 with physiological saline, which is equivalent to 1.5×10^8 CFU/mL. From this prepared solution, other dilutions were made with MHB to yield 1.5 x10^6 CFU/mL.

(c) Determination of minimum inhibitory concentration (MIC)

The minimal inhibitory concentration of extract, fractions and compounds were assessed using the broth microdilution method recommended by the National Committee for Clinical Laboratory Standards (NCCLS, 1999; NCCLS, 2000), with slight modifications. Each test compound was dissolved in dimethylsulfoxide (DMSO) to give a stock solution. The 96-well round bottom sterile plates were prepared by dispensing 100 µL aliquot of the stock solution of test substances. Two-fold serial dilution was performed and 100 µL of the inoculated broth (1.5 x10^6 CFU/mL) were added into each well to give a final concentration range from 500 to 3.8 µg/mL for extract, fractions and compounds. The final concentration of DMSO in each well was < 0.5% (a control test with 1% (v/v) DMSO did not inhibit the growth of the test organisms). Dilutions of Ciprofloxacin and Isoniazid served as positive controls, for bacteria and *Mycobacterium smegmatis* respectively, while broth with 1% DMSO was used as negative control. Finally, 100 µL of liquid medium were added. Then, plates were covered and incubated for 18 h at 37 °C. After incubation, MIC values were determined upon addition of rezasurin chromophore.

***In vitro* assay for cytotoxic activity (MTT assay)**

Vero cells were seeded at a concentration of 1 × 10^4cells/well in 100 µL culture medium and pre-incubated for 4 h at 37°C, 5 % CO_2 and humidified atmosphere for cell adherence. Various concentrations of fractions and compounds (1000 µg/mL-125 µg/mL) in 4 µL were thereafter added into 96 well flat bottom microplates treated for cell culture (Corning), as previously described by Mosmann (Mosmann, 1983). The stock solutions of tested substances were prepared in DMSO at concentration of 100mg/mL. The final concentration of DMSO was 0.2%. DMSO at 0.2% was used as a control in the non-treated cells, while10% DMSO was used as positive control. The experiment was performed in triplicates. Vero cells were incubated for 24 h under 5% CO_2 at 37°C. Twenty microliters of MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] solution (5 mg/mL) were added and incubated for 3 h. The medium was removed, 100 µL of DMSO were added to solubilize the formazan crystals. Absorbance was measured using a microplate reader (Tecan infinite M200) at wavelength 570 nm. The effect of the compound on the viability of Vero cells were expressed as the % of viability using the following formula.

$$\%\ cell\ viability = \frac{A570\ of\ treated\ cells}{A570\ of\ controlled\ cells} x100$$

The percentage cell growth inhibition or percentage cytotoxicity was calculated by the following formula:

$$\%\ cytotoxicity = 100 - \%\ cell\ viability$$

The percentage of cell growth inhibition was used to determine the IC_{50} (concentration that inhibits 50% of cell growth) using Graphpad prism 7.0 version. The One Way Analysis of Variance (ANOVA, Version 16.0) was used to analyze the data, which are presented as means ± SEM values of IC50 and differences between means at 95 %

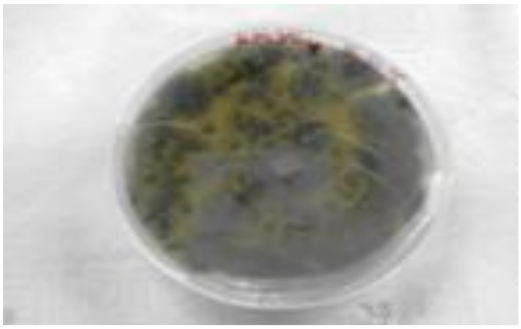


Figure 1a: Macroscopic Face view AMSt4

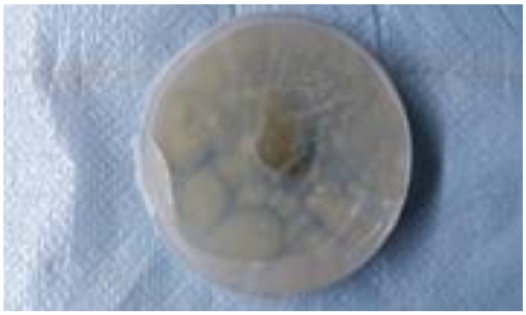


Figure 1b: Macroscopic bottom view AMSt4

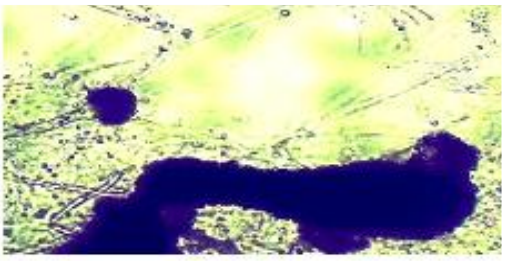


Figure 2: Microscopic aspect of *Aspergillus niger*

confidence interval were compared using the Waller Duncan test.

RESULTS

Identification of the fungus AMst4

Macroscopic and microscopic examinations of the isolate revealed that colonies were whitish then become black after 4 to 5 days (Figure 1a). The opposite side (bottom) of cultures Petri dish was brown (Figure 1b). The aspergillum-like spore-bearing structure was clearly observed (Figure 2). The strain was then identified using the standard taxonomic keys. Moreover, cultural and morphological features of the strain AMSt4 enabled its reliable placement in the genus Aspergillus. There were noticeable microscopic morphological similarities with *Aspergillus niger*, a specie generally known as ubiquitous, but that can be isolated as endophyte from other different host plants. With reference to recent revision of species concept in Aspergillus, specific determination requires molecular techniques, not available yet in our laboratory and is thus one limitation to confirm the identification of the species.

Fractions and compounds isolated

Fraction 1 was oily, and its fractionation was not continued. Fraction 8, 15, 17 and 30 did not show any activity, and were not further purified. Further fractionation of F6 and 11, initially active, yielded sub-fractions which showed no antibacterial activity and thus further isolation was discontinued.

Fractions 20 and 27 retained some biological activities. Further fractionation of F20 yielded C201 and C222, that all lost their activity. From F27, compound C271 retains its antibacterial activities.

C222 from F20 (eluted with Hex/AE 4:1 v/v) and C271 from F27 (eluted with Hex/AE, 3:1.

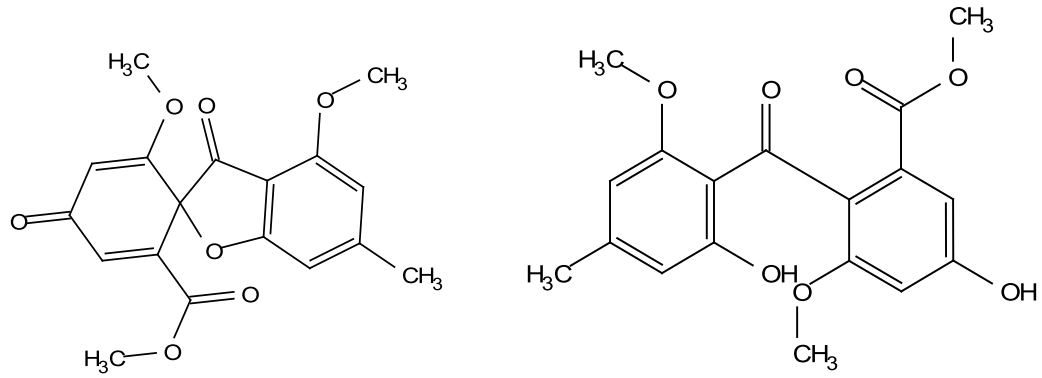
By comparing physical and spectroscopic data with literature values, the two compounds were identified as:

C222 : Spiro(benzofuran-2(3H),1'-(2,5) cyclohexadiene)-2'-carboxylic acid, 4,6'-dimethoxy-6-methyl-3,4'-dioxo-, methyl ester, with trivial name Trypacidin A (C271) (Natalia et al., 2015; Pinheiro et al., 2013; Turner, 1965; Frivad et al., 2009), Figure 3a.

C271: benzoic acid, 5-hydroxy-2-(2'-hydroxy-6'-methoxy-4'-methylbenzoyl)-3-methoxy-, methyl ester, trivially known as Methylsulochrin (C222) (Natalia et al., 2015; Pinheiro et al., 2013; Turner, 1965; Frivad et al., 2009), Figure 3b.

Compound C222 was isolated as a white powder. The ^{1}H NMR spectrum showed four olefinic proton signals at δ_H 7.11, 6.55, 6.38 and 5.77 ppm. In the highfield, three methoxyl groups at δ_H 3.95 and 3.69 and 3.66, one methyl group at δ_H 2.44 ppm was observed. These data suggested the compound 1 to be Trypacidin A isolated for the first time by Turner, 1965. This was evident on the ^{13}C-NMR spectrum (Figure 4) with the resonance of carbonyl at δ_c 190.5, 185.6, 174.3 ppm and characteristic peak of unsaturared quaternary carbons at δ_c 169.4, 163.4, 158.3 and 152.1 ppm.

Compound 271 was isolated as a white powder. Its ^{1}H NMR spectrum (Figure 5) showed four olefinic proton signals at δ_H 7.02, 6.66, 6.342 and 6.14 ppm. Similarly as compound 1, in the highfield, three methoxyl groups at δ_H 3.95 and 3.69 and 3.66, one methyl group at δ_H 2.44 ppm indicating that compound 271 is a derivative of compound C222. The main differences were the appearance of a carbonyl signal at δ_C 200.3 and the disappearance of the peaks at δ_C 190.5, 185.6, 174.3ppm in C222. The combination of these spectroscopic data and the comparison with those reported in the literature allowed us to assign to compound C271 the structure of Methylsulochrin (Natalia et al., 2015; Pinheiro et al.,



Trypacidin A Methylsulochrin

Figure 3 Chemical structures of compounds isolated from *Aspergillus niger* haboring *Acanthus montanus* stems. 3a: Trypacidin; 3b: Methylsulochrin

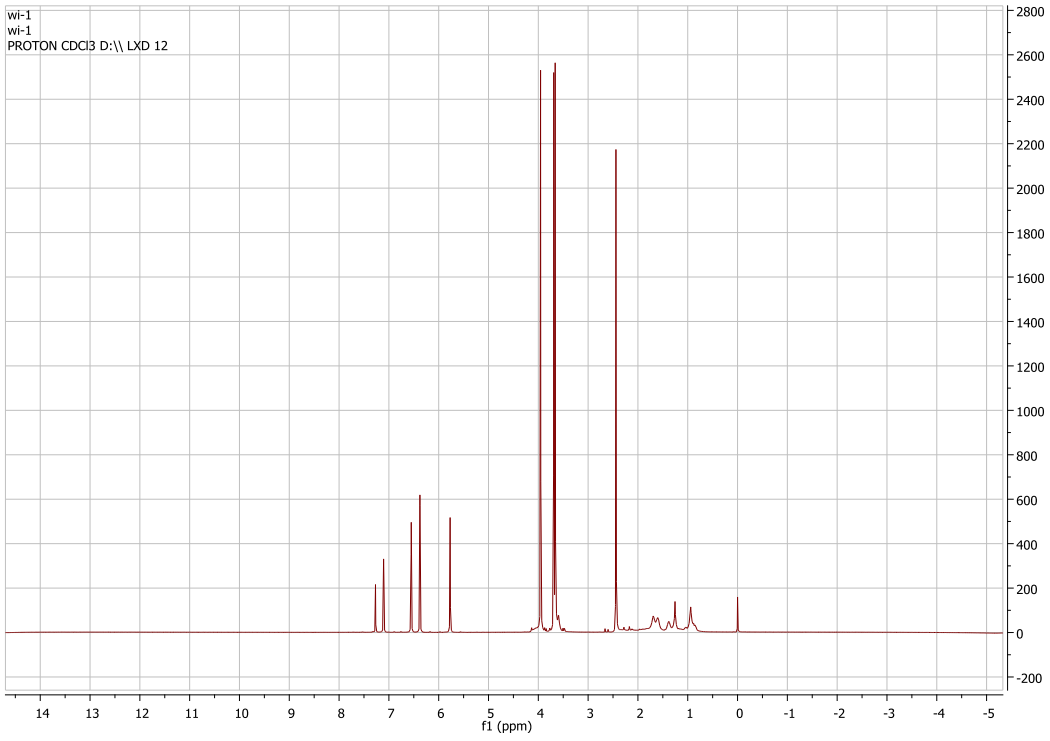


Figure 4(a)

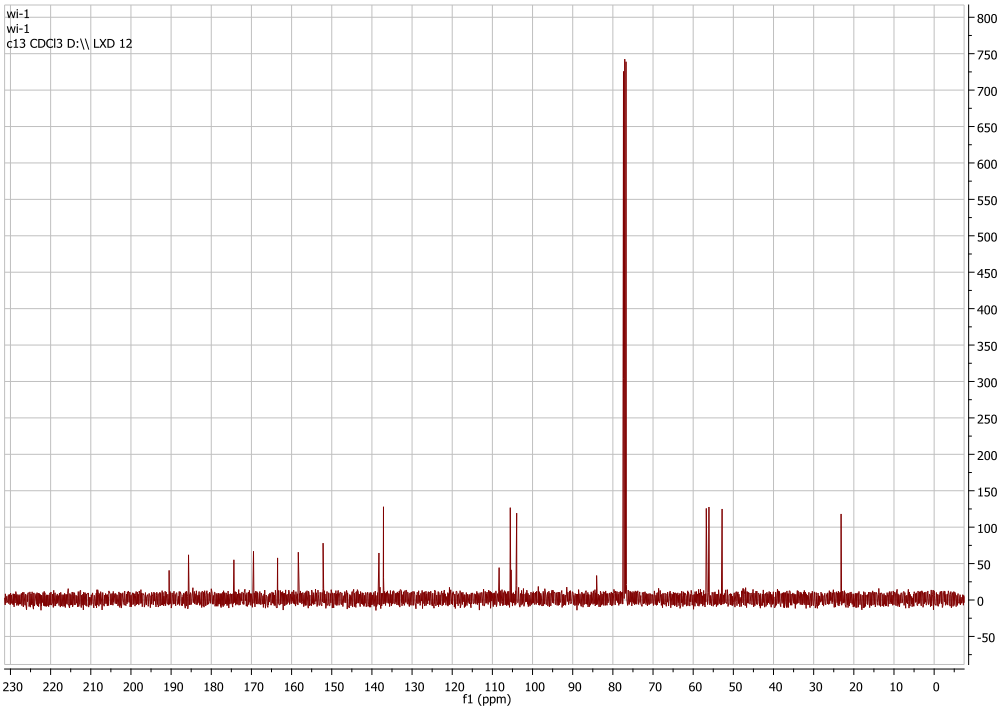
2013; Turner, 1965; Frivad et al., 2009).

Antibacterial activities

The antimicrobial properties of fractions and compounds from crude ethyl acetate extract are given in Table 1. The crude extract showed the best activity on *Mycobacterium smegmatis*, with a MIC value of 125µg/mL. It exhibited a large spectrum activity on enterobacteriaceae, ranging from 250 to 500µg/mL. While C271, methylsulochrin exhibited interesting activity on six of the tested microorganisms, MIC ranging from 500 to 7.8 µg/mL. None of the two compounds showed any activity on *Mycobacterium smegmatis.*

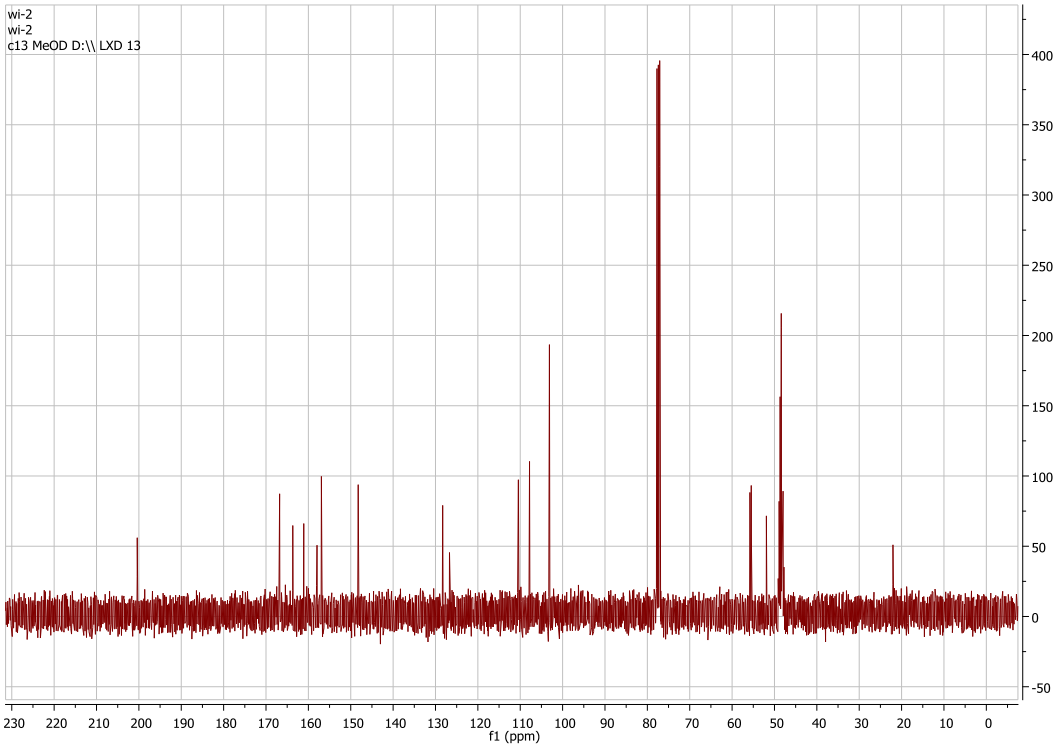
Cytotoxic activities

Cytotoxicity tests were done on Vero cells and the results



4 b

Figure 4a and 4b: NMR spectrum of Compound 222 : Trypacidin. (a)-^{1}H;(b)-^{13}C



5(a)

are presented in Table 2 below :

Fractions F6, 15, 20 and 27 showed weak cytotoxic activity on Vero cells. While F8 and F11 showed no toxicity up to 1000µg/mL. Fraction F27 showed an IC50 value of 350µg/mL. C222 and C271 were not toxic on Vero cells at the tested concentration (1000µg/mL).

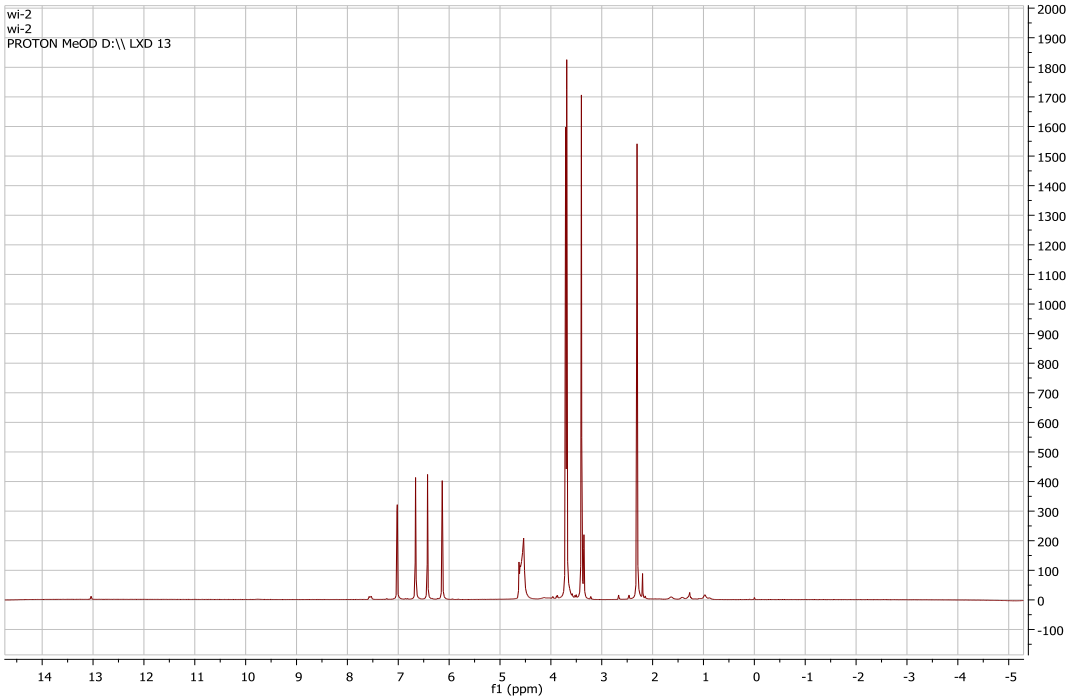


Figure 5a and 5b: NMR spectrum of Compound 271 : Methylsulochrin. (a)-^{1}H;(b)-^{13}C

Table 1. MIC values of fractions and compounds from *Aspergilus niger* on Bacterial strains

Bacterial strains	***E coli***		***Staphyloccocus Aureus***	***Enterobacter***					***Enterococcus faecalis***	***Klebsiella pneumoniae***	***Mycobacterium smegmatis***
Test Substances	**MEcl**	**ATCC35218**	**SAcl**	**ATCC43300**	***Cloacae* BM67**	***Cloacae* K2**	***Aerogenes* CM64**	***Aerogenes* EA289**	**ATCC51299**	**ATCC13883**	**ATCC700084**
CE	500	-	250	500	500	500	500	250	-	-	125
20	-	-	-	-	-	-	-	-	-	-	125
27	-	-	-	-	500	-	-	-	500	-	250
C271	500	-	-	**15.6**	**7.8**	**31.2**	**7.8**	**15.6±**	-	-	-
RA	0.03	0.03	0.31	0.15	0.07	0.15	0.07	0.15	0.31	0.62	078

RA: reference antibiotic (ciprofloxacin for enterobacteria and isoniazide for Mycobacterium)
- : Not active at 500µg/mL
C271: methylsulochrin

Table 2. IC_{50} values of fractions and compounds on Vero cells

Test substances	F6	F8	F11	F15	F17	F20	F27	C222	C271
IC_{50} (µg/mL)	254.6±2.39[a]	/	/	320.7±1.44[c]	NT	277.7±1.41[b]	350.6±1.38[d]	/	/

Data are reported as mean of IC50 values ± SEM; /: IC50 values > 1000 µg/mL, Values with the same superscripted letter are not significantly different at P<0.05, Waller Duncan; NT : not tested.

DISCUSSION

Acanthus montanus is a plant largely used by the indigenous population in Cameroon and some other countries of Africa for the treatment of many ailments. This plant also harbors endophytic fungi that possibly produce metabolites of interest. This plant has previously shown anti-mycobacterial activity on the virulent strain of *Mycobacterium tuberculosis* (Nkenfou et al., 2015). From *Acanthus montanus,* eight endophytic fungi have been isolated. From all of them, one was identified as *Aspergillus niger*.

The bioguided fractionation of the acetyl acetate crude extract of *Aspergillus niger* lead to several fractions and pure compounds. Along the fractionation, some of them were not active. Fractions 20 and 27 kept their activity from which two compounds were isolated. Trypacidin A and Methylsulochrin respectively. Their structures were clearly elucidated. The activity of the compound namely Trypacidin A and Methylsulochrin were evaluated on *E. coli, Enterobacter aerogenes, Enterococcus faecalis, Staphylococcus aureus and Mycobacterium smegmatis.*

Contrary to other studies that showed Trypacidin A activity on *E. coli* (Pinheiro et al., 2013), our results showed no activity on tested microorganisms. This may be due to the fact that the strains that were used in this study were resistant strains. Nevertheless, this compound could have activity on virus, fungi or even trypanosomes. Trypacidin (Balan et al., 1963) has been found as a result of biological screening on *Trypanosoma* species and was found active mainly on *Trypanosoma cruzi* (5 – 10 µg/mL) and *Toxoplasma gondii* (10 – 20 µg/mL).

On the other hand, Methylsulochrin was active on 06 of the tested strains. These results corroborate those of (Pinheiro et al., 2013), who found that methylsulochrin was active on *E. coli, Pseudomonas aeruginosa*, *Bacillus subtilis* and *Staphylococcus aureus* at the concentration of 31.25µg/mL. These activities may be due to the presence of several functional groups on his structure such as ketones, hydroxyl and hydroxymethyl functions.

According to (Garbi et al., 2015), the criteria used to categorize the cytotoxicity extracts and fractions is as follows: $IC_{50} \leq 100$ µg/mL = highly cytotoxic, IC_{50} ranged between 21 and 200 µg/mL = moderately cytotoxic, IC_{50} ranged between 101 and 500 µg/mL = moderate cytotoxic and $IC_{50} > 200$ µg/mL = weak cytotoxicity. Referring to this scale, Methylsulochrin was not cytotoxic. This could comfort our conclusion for further investigations of this compound.

CONCLUSION

The overall results of this study indicate that two compounds Trypacidin and Methylsulochrin, isolated from the crude extract of *Aspergillus niger* endophyte harboring *A. montanus* stems. Trypacidin could be further evaluated for its activity on other parasites. Methylsulochrin can be further exploited for the development of a phytomedicinal preparation, acting against Enterobacteria.

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Consent for publication: Non applicable

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Competing interests: The authors declare they have no competing interests.

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Authors' contributions

IKM, AN, J-BJ and LPK carried out the study and wrote the manuscript, PE and PTF realized the microscopic identification; CNN and J-RK conceived the study, interpret the results and read the manuscript. All authors approved the final manuscript.

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