

# Antibacterial activity of the major compound of an endophytic fungus isolated from *Garcinia preussii*

Jean-Bosco Jouda<sup>a</sup>, Judith-Laure Demgne Fopossi<sup>b</sup>, Céline Djama Mbazona<sup>a</sup>, Jean Wandji<sup>a\*</sup>

<sup>a</sup>Department of Organic Chemistry, Faculty of Science, University of Yaoundé I, P.O Box 812, Yaoundé, Cameroon. <sup>b</sup>Department of Biochemistry, Faculty of Science, University of Yaoundé I, P.O Box 812, Yaoundé, Cameroon.

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## ABSTRACT

Our research aimed at the chemical and biological investigations of selected endophytes associated with *Garcinia preussii*. The chemical investigation of the crude extract from the solid state fermentation of *Aspergillus japonicus*, an endophytic fungus harboring leaf of *Garcinia preussii*, by means of Liquid chromatography-Mass spectrometry (LC-MS) led to the isolation of two major compounds. Their structures were elucidated on the basis of spectroscopic analysis and by comparison of their spectral data with those reported in the literature. These isolated compounds were found to be variecolin (1) and neovasifuranone B (2) from the cyclohexane fraction. The antimicrobial studies showed that the isolated compounds exhibits antibacterial activity with inhibition zone diameters varying from 09.0±0.00 to 11.0±0.00 mm. These preliminary results showed promising activity of these compounds thus supporting the immensity of the potential of antibacterial drug discovery from microorganisms.

## INTRODUCTION

Antibiotic resistance is a problem that continues to challenge the healthcare sector in a large part of the world in both developing and developed countries. The spread of multidrug resistant (MDR) bacteria in hospital and community settings remains a widely unresolved problem and a heavy burden to health services (Cosgrove *et al.*, 2003). Natural products produced by plants, animals or microbes are sources of bioactive compounds potential to be developed as medicine. Endophytes are microbes which reside in living plant tissues without causing injury or diseases to the hosts (Petrini *et al.*, 1992; Strobel *et al.*, 2003). Most of endophytes are capable of producing active metabolites and some of these compounds are proven to have medical values (Santiago *et al.*, 2012; Zhao *et al.*, 2009; Weber *et al.*, 2007; Tan *et al.*, 2001). Plants of the genus *Garcinia* (Guttiferae) have been extensively investigated from both

phytochemical and biological points of view, and they are well known as a rich source of natural xanthenes, bioflavonoids and benzophenones (Bilola *et al.*, 2012; Bilola *et al.*, 2013). In this research, an endophytic fungus isolated from *Garcinia preussii* was studied. The fungal isolate *Aspergillus japonicus* CAM231, was found to produce diverse compounds in solid state medium (rice). In this communication, we report the isolation and antibacterial activity of the major compound produced by CAM231.

## MATERIAL AND METHODS

### General experimental procedures

The high resolution mass spectra were obtained with an LTQ-Orbitrap Spectrometer (Thermo Fisher, USA) equipped with a HESI-II source. The spectrometer was operated in positive mode (1 spectrum s<sup>-1</sup>; mass range: 100-1000) with nominal mass resolving power of 60 000 at *m/z* 400 with a scan rate of 1 Hz) with automatic gain control to provide high-accuracy mass measurements within 2 ppm deviation using an internal standard; Bis(2-ethylhexyl)phthalate : *m/z* 391.28428.

\* Corresponding Author

Jean Wandji, Department of Organic Chemistry, Faculty of Science,  
University of Yaoundé I, P.O Box 812, Yaoundé, Cameroon.

Email: [jeanwandji@yahoo.fr](mailto:jeanwandji@yahoo.fr)

The spectrometer was attached with an Agilent (Santa Clara, USA) 1200 HPLC system consisting of LC-pump, photodiode array (PDA) detector ( $\lambda = 260$  nm), auto sampler (injection volume 5  $\mu$ l) and column oven (30°C). The following parameters were used for experiments: spray voltage 5 kV, capillary temperature 260 °C, tube lens 70 V. Nitrogen was used as sheath gas (50 arbitrary units) and auxiliary gas (5 arbitrary units). Helium served as the collision gas. The separations were performed by using a Nucleodur C18 Gravity column (50 x 2 mm, 1.8  $\mu$ m particle size) with a H<sub>2</sub>O (+ 0.1% HCOOH) (A)/acetonitrile (+ 0.1% HCOOH) (B) gradient (flow rate 300  $\mu$ l min<sup>-1</sup>). Samples were analyzed by using a gradient program as follows: 80% A isocratic for 1 min, linear gradient to 100% B over 18 min, after 100% B isocratic for 5 min, the system returned to its initial condition (80% A) within 0.5 min, and was equilibrated for 4.5 min. The separation was carried out by preparative HPLC run for 20 min on a Gilson apparatus with UV detection at 220 nm using a Nucleodur C18 Isis column (Macherey-Nagel, Düren, Germany), 5  $\mu$ m (250 × 16 mm) with a H<sub>2</sub>O (A) / CH<sub>3</sub>OH (B) gradient (flow rate 4 ml.min<sup>-1</sup>). Samples were separated by using a gradient program as follows: 60% A isocratic for 2 min, linear gradient to 100% B over 18 min, after 100% B isocratic for 5 min, the system returned to its initial condition (60% A) within 0.5 min, and was equilibrated for 4.5 min. The NMR spectra were recorded on a Bruker DRX-500 MHz spectrometer. Chemical shifts ( $\delta$ ) were quoted in parts per million (ppm) from internal standard tetramethylsilane and coupling constants ( $J$ ) are in Hz. Silica gel [Merck, Kieselgel 60 (0.063–0.200 mm)] was used for column chromatography.

### Isolation and identification of endophytic fungi

The fungus was isolated from the leaf of *Garcinia preussii* collected in February 2013 in Foumban, in West region Cameroon. The plant material was identified by Mr. Victor Nana, botanist at the Cameroon National Herbarium (Yaoundé) under a voucher specimen (55520/HNC).

A healthy leaf of *G. preussii* was washed with tap water, and then air-dried. Before surface sterilization, the leaf was cut into small fragments using sterile surgical blades, 12 segments from each leaf. Sample fragments were successively surface sterilized by immersion in 95% ethanol for 30 s, 5% sodium hypochlorite solution for 2 min, 95% ethanol for 30 s and sterile distilled water for 3–5 s. The surface-sterilized fragments were placed on water agar (supplemented with 50 mg.L<sup>-1</sup> tetracycline and ampicillin to suppress bacterial growth) and incubated at 25 °C until the outgrowth of endophytic fungi was discerned. Hyphal tips originating from plant segments were transferred to potato dextrose agar (PDA) without antibiotics. Each fungal isolate was checked for purity and transferred to the new medium by the hyphal tip method. One of the isolates, CAM231 was selected for further studies based on its morphotype and the LC-MS profile of its crude extract from small scale fermentation.

CAM231 Fungal molecular identification based on ITS sequence analysis revealed that the isolate CAM231 belong to

*Aspergillus* genera. The alignment of this sequence with the 18S rRNA genes sequences retrieved from the GenBank database evidenced a high degree of similarity (99%) with the *Aspergillus japonicus*.

### Bioassay

The *in vitro* antibacterial activity of variecolin was evaluated using a panel of clinical isolates collected from the Pasteur Centre (Yaoundé-Cameroon). The Gram positive bacteria *Streptococcus pneumoniae* and Gram-negative bacteria *Klebsiella pneumoniae*, *Pseudomonas aeruginosa*, *Aeromonas* sp., *Esherichia coli*, one fungal species, *Candida albicans* and two reference bacteria (*Staphylococcus aureus* ATCC 25922 and *Esherichia. coli* ATCC 13565) all from the American Type Culture Collection.

The compounds were dissolved in methanol 50% at a concentration ranging from 50-1000  $\mu$ g/mL. We used the standard antibiotic difloxacin as reference in parallel to reveal the comparative antimicrobial efficacy of compounds against the tested organisms. The assay was performed according to Jorgensen and Turnidge (2007) with some modification. Nutrient agar (NA) media was used for test bacteria, and PDA media was used for test yeasts. The test organisms were inoculated over the surface of the sterilized media, with 1.5 x 10<sup>8</sup> CFU/mL (colony-forming units) for bacteria and 4 x 10<sup>7</sup> CFU/mL for yeasts. Sterile disks of Wattman No. 3 (6.0 mm in diameter) were loaded with 40  $\mu$ g/mL of each compound, dried, and then placed on agar surface of freshly inoculated medium with the test microorganisms. A control test for the solvent was also performed. The Petri dishes were kept in a refrigerator for one hour to permit homogenous diffusion of the antimicrobial agent before growth of the test microorganisms, and the plates were then incubated at 37°C for 24 hours. The appearance of a clear inhibition zone around the disk in the inoculated Petri dishes is an indication of antimicrobial activity, and the diameters of the clear zones surrounding the discs were measured.

## RESULTS AND DISCUSSION

### Fermentation, extraction and isolation

*Aspergillus japonicus* CAM231 was cultured in 20 flasks (1L each) containing 100 g rice and 100 ml water enriched with 0.3% peptone each, autoclaved at 121°C for 45 min. Each flask received about 5 small pieces of mycelium from PDA plate under sterile conditions. After 30 days growing at 25°C, ethyl acetate (20 x 400 ml) was added to each bottle, homogenized and filtered after 24 h and taken to dryness. The obtained EtOAc extract (16.4 mg) was firstly subjected to HPLC-MS (Fig. 1) for screening purpose and then partitioned with cyclohexane and the resulting fraction (9.6 g) was subjected to column chromatography (CC) on silica gel employing a step gradient of cyclohexane-ethyl acetate and ethyl acetate-methanol to give twenty four fractions Fr1-Fr24. Fraction 5 (930 mg) was subjected to column chromatography on silica gel using a step gradient of cyclohexane-ethyl acetate to

afford variecolin (**1**) (16.8 mg,  $t_R = 15.22$  min), the major compound both of this fraction and crude extract. Fraction Fr22 (254 mg) was purified by reversed-phase preparative HPLC eluting with a gradient of MeOH-H<sub>2</sub>O+ 0.1%TFA to yield neovasifuranone B (**2**) (7.7 mg,  $t_R = 6.41$  min). The structures of these compounds were determined by the combination of their HRLC-MS, spectral data together with the dereplication and partial identification using Antibase (Laatsch, 2013) that suggested that they belonged to the ophiobolin (Hessens *et al.*, 1991) and furanone (Furumoto *et al.* 1995) class of compounds respectively.

**Variecolin (1)**: white powder; HRESIMS  $m/z$  369.27820 [M+H]<sup>+</sup> (calcd. 369.27936); Molecular formula C<sub>25</sub>H<sub>36</sub>O<sub>2</sub>; UV (MeOH)  $\lambda_{max}$ : 244 and 260 nm.

<sup>1</sup>H-NMR (500 MHz, CDCl<sub>3</sub>,  $\delta$  (ppm),  $J$  (Hz)):  $\delta$  1.57 (1H, d, 2.0, H-1 $\alpha$ ), 1.23 (1H, m, H-1 $\beta$ ), 2.67 (1H, m, H-2), 2.44 (1H, m, H-3), 2.40 (1H, m, H-4 $\alpha$ ), 2.53 (1H, m, H-4 $\beta$ ), 3.59 (1H, d, 10.4, H-6), 6.92 (1H, d, 5.2, H-8), 2.28 (1H, obs, H-9 $\alpha$ ), 2.87 (1H, d, 17.2, H-9 $\beta$ ); 2.22 (1H, m, H-10), 1.04 (1H, obs, H-12 $\alpha$ ), 1.91 (1H, dt, 13.7, 4.5, H-12 $\beta$ ), 1.48 (1H, m, H-13), 2.41 (1H, m, H-15), 1.45 (1H, m, H-16), 1.38 (1H, m, H-17 $\alpha$ ), 2.00 (1H, m, H-17 $\beta$ ), 1.24 (1H, m, H-18 $\alpha$ ), 1.45 (1H, m, H-18 $\beta$ ), 0.81 (3H, d, 7.3, H-19), 9.20 (1H, s, H-20), 0.95 (3H, s, H-21), 0.85 (3H, s, H-22), 4.64 (1H, s, H-24 $cis$ ), 4.47 (1H, s, H-24 $trans$ ), 1.72 (3H, s, H-25) and <sup>13</sup>C-NMR (125 MHz, CDCl<sub>3</sub>,  $\delta$  (ppm)):  $\delta$  42.2 (C-1), 39.2 (C-2), 34.7 (C-3), 46.2 (C-4), 220.6 (C-5), 50.3 (C-6), 139.8 (C-7), 160.7 (C-8), 31.5 (C-9), 40.6 (C-10), 39.1 (C-11), 35.4 (C-12), 35.2 (C-13), 43.2 (C-14), 48.4 (C-15), 48.6 (C-16), 29.9 (C-17), 39.8 (C-18), 15.8 (C-19), 193.0 (C-20), 21.8 (C-21), 18.0 (C-22), 150.3 (C-23), 110.6 (C-24), 19.0 (C-25).

**Neovasifuranone B (2)**: Yellowish oil; HRESIMS  $m/z$  283.19000 [M+H]<sup>+</sup> (calcd. 283.19093); Molecular formula C<sub>16</sub>H<sub>26</sub>O<sub>4</sub>; UV (MeOH)  $\lambda_{max}$ : 260 and 276 nm.

<sup>1</sup>H-NMR (500 MHz, CDCl<sub>3</sub>,  $\delta$  (ppm),  $J$  (Hz)):  $\delta$  5.47 (1H, s, H-5), 3.75 (1H, d, 6.7, H-7), 1.52 (1H, m, H-8), 1.10 (1H, m, H-9a), 1.34 (1H, m, H-9b), 0.91 (3H, t, 7.4, H-10), 2.66 (2H, m, H-11), 1.27 (3H, t, 7.6, H-12), 4.30 (2H, s, H-13), 0.87 (3H, d, 6.7, H-14), 1.70 (3H, d, 1.1, H-15), 1.50 (3H, s, H-16), 3.48 (1H, s, 7-OH) and <sup>13</sup>C-NMR (125 MHz, CDCl<sub>3</sub>,  $\delta$  (ppm)):  $\delta$  190.3 (C-1), 112.0 (C-2), 206.1 (C-3), 88.7 (C-4), 122.8 (C-5), 143.6 (C-6), 80.9 (C-7), 37.2 (C-8), 26.1 (C-9), 11.5 (C-10), 22.5 (C-11), 10.5 (C-12), 53.0 (C-13), 13.8 (C-14), 13.3 (C-15), 24.1 (C-16).

#### Antimicrobial activity

The isolated compounds exhibited significant inhibitory activity against a wide range of pathogenic test microorganisms on disc diffusion assay. Compound **1** showed an antibacterial activity with diameters of inhibition zones ranging from 9 to 11 mm at 50 $\mu$ g/mL against all bacteria (Table 1), while **2** was active only against *Candida albicans* with an inhibition zone diameter of 10 mm at the same concentration.

The activity of variecolin (**1**) against all the tested bacteria would be attributed to the presence of aldehyde function C-20 in its structure. Although ophiobolin compounds have been reported to possess interesting activity such as immunosuppressive activities (Fujimoto *et al.*, 2000), inhibition of the human chemokine receptor CCR5 (Yoganathan *et al.*, 2004) and angiotensin II receptor binding (Hessens *et al.*, 1990), to the best of our knowledge, no study has been reported on the activity of the variecolin against these types of pathogenic strains.

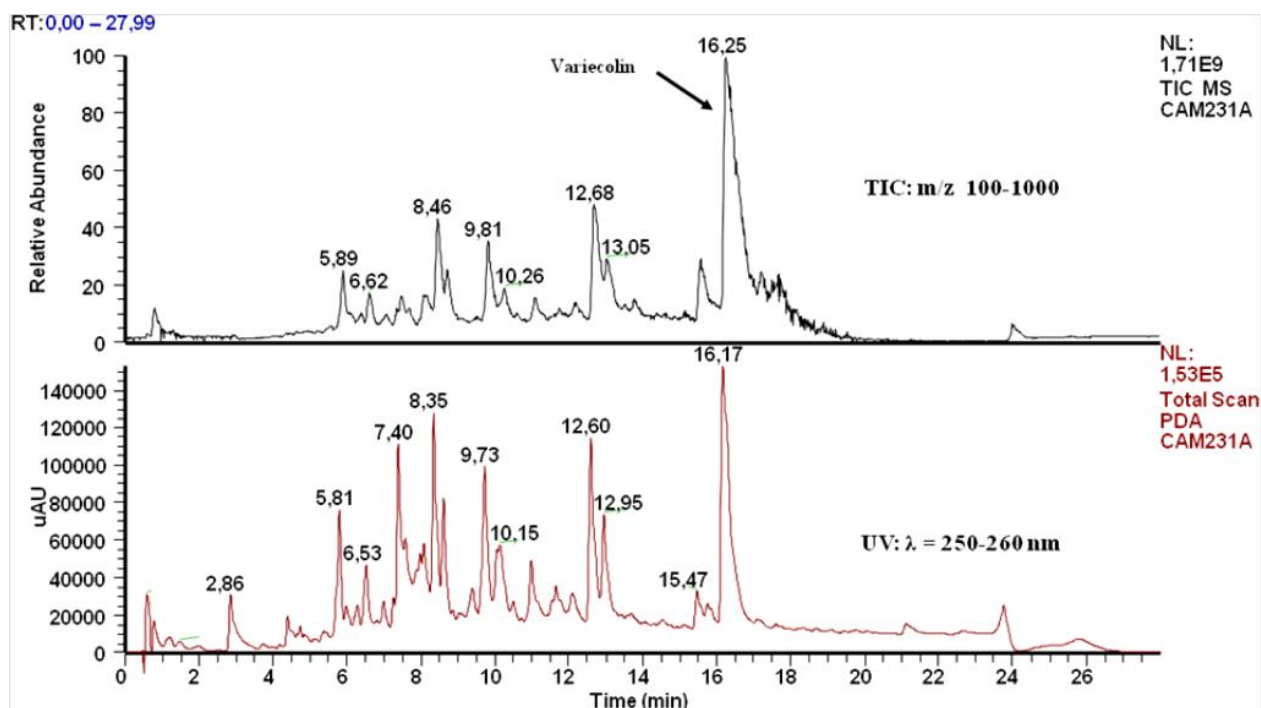


Fig. 1: Total Ion Current (TIC) chromatogram of the crude extract of *Aspergillus japonicus* from solid medium

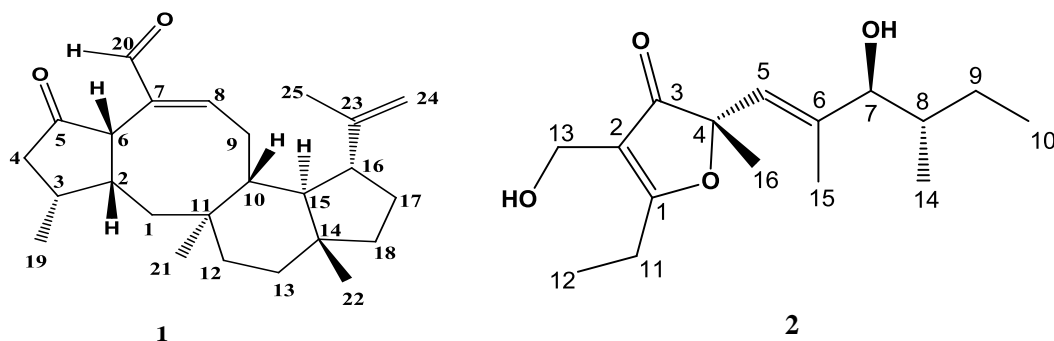


Fig. 2: Chemical structures of isolated compounds from *Aspergillus japonicus*.

Table 1: Diameters of inhibition zones of isolated compounds against Gram-positive and Gram-negative bacteria compared to standard reference (Difloxacin).

| Microorganisms                     | ID (Inhibition diameter) (mm) at 50 µg/mL |         |                     |
|------------------------------------|---|---------|---------------------|
|                                    | 1   | 2       | Difloxacin (5µg/mL) |
| <i>Klebsiella pneumoniae</i>       | 09.0±0.00                                 | -       | 13.5±0.00           |
| <i>Escherichia coli</i>            | 10.0±0.00                                 | -       | 17±0.00             |
| <i>Pseudomonas aeruginosa</i>      | 10.0±0.00                                 | -       | 15±0.00             |
| <i>Aeromonas</i> sp.               | 10.0±0.00                                 | -       | 15±0.00             |
| <i>Streptococcus pneumoniae</i>    | 11.0±0.00                                 | -       | 16±0.00             |
| <i>Serratia</i> sp.                | 11.0±0.00                                 | -       | 18±0.00             |
| <i>Candida albicans</i>            | -   | 10±0.00 | 14±0.00             |
| <i>Staphylococcus aureus</i> ATCC  | 10.50±0.00                                | -       | 16±0.00             |
| <i>Escherichia coli</i> ATCC 25922 | 9.0±0.00                                  | -       | 17±0.00             |

All values are in mm and derived from experiments in triplicates. Values are means of three replication ± SD. (-) No inhibition diameter.

## CONCLUSION

This is the first study to describe the isolation and antibacterial activities of secondary metabolites of an endophytic fungus from *Garcinia preussii*. The results of the investigation are important, in regards to the medical importance of the studied microorganisms. Hence, these data provide evidence that variecolin could be useful for the development of new antibacterial drugs. However, further pharmacological and toxicity studies will be necessary to establish if they could be safely used as topical antibacterial agents.

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