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Phytosterols isolated from endophytic fungus *Colletotrichum gloeosporioides* (Melanconiaceae)

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ABSTRACT

Endophytic fungi are fungi that colonize internal tissues of plants. There are few studies of compounds isolated from endophytic fungi of Amazon plants. Thus, the aim this study was the isolation and structural identification of sitosterol (**1**), stigmasterol (**2**), sitostenone (**3**), squalene (**4**), ergosterol (**5**) and ergosterol peroxide (**6**) from fungus *Colletotrichum gloeosporioides* isolated as endophytic from *Virola michelli*, a typical Amazon plant, used in folk medicine against skin infection. Compounds were isolated by chromatography column on silica and identified by ^{1}H and ^{13}C NMR and MS. The presence of phytosterols in fungi is rare and this is the first report of the isolation of the phytosterols sitosterol, stigmasterol and sitostenone from the genus *Colletotrichum*.

KEYWORDS: Sterols, secondary metabolites, Amazon fungi.

Fotoesteróis isolados do fungo endofítico *Colletotrichum gloeosporioides* (Melanconiaceae)

RESUMO

Fungos endofíticos são fungos que colonizam os tecidos internos das plantas. Existem poucos estudos de compostos isolados de fungos endofíticos de plantas da Amazônia. Assim, o objetivo deste estudo foi o isolamento e identificação estrutural de sitosterol (**1**), estigmasterol (**2**), sitostenona (**3**), esqualeno (**4**), ergosterol (**5**) e peroxido de ergosterol (**6**) do fungo *Colletotrichum gloeosporioides* isolado como endofítico de *Virola michelli*, uma planta típica da Amazônia, usada na medicina popular no combate a infecções de pele. Os compostos foram isolados por cromatografia em coluna de sílica e identificados por RMN ^{1}H e ^{13}C e EM. A presença de fitoesteróis em fungos é rara e este é o primeiro relato do isolamento dos fitoesteróides sitosterol, estigmasterol e sitostenona do gêreno *Colletotrichum*.

PALAVRAS-CHAVE: Esteróides, metabolitos secundários, fungos da Amazônia.

INTRODUCTION

Endophytic fungi are those colonizing the internal tissue of plants without causing apparent damage (Petrini *et al.* 1992). The mechanism of interaction between the endophytic fungi and their host plants has still not been completely understood, mainly from the stand point of chemistry, since it has been known that they live in a neutral or even mutualistic interaction with host plants. Fungi are good producers of secondary metabolites, many with the same biological activity (Rosa *et al.* 2010; Chandra 2012; Pinheiro *et al.* 2013), where some compounds may help the host plant with combating infestation by other fungi, viruses and bacteria (Faeth and Saari 2012; Kusari *et al.* 2013).

There are also reports of a possible "learning" known as "horizontal gene transfer" between fungus and plant, which would be the ability of organisms associated with producing the same class of compounds (Cook *et al.* 2014).

The phytosterols are broadly found in plants. The steroids sitosterol and stigmasterol are present in almost all plants; hence they are so called phytosterols. On the other hand, the steroids ergosterol and ergosterol peroxide are commonly found in fungi, occurrence of phytosterols in fungi is relatively unusual (Fontaine *et al.* 2004). The phytosterols, mainly sitosterol, have the capacity of reducing the blood cholesterol (Awad and Fink 2000), as well as phytosterols can be used to fight of cardiovascular diseases, cancer and inflammatory process (Fernandes and Cabral 2007; Woyengo *et al.* 2009; Poli and Marangoni 2010).

Brazil has about 20% of the world's biodiversity, especially in the Amazon forest, largest tropical and wet tropical forest, and invaluable source of raw materials in various sectors. Despite the huge Amazonian biodiversity, species that compose it and their relationships are not well known, much less its organisms and their interactions with other beings (Souza *et al.* 2004). The vegetal specie *Virola michelli* (Myristicaceae) is a typical Amazon plant known as "ucuúba" for local population and used in folk medicine as a plaster for irritation relief caused by fungi and to treatment of skin infections. Phytochemical and pharmacological studies have showed the anti-inflammatory potential of the crude extracts and pure compounds isolated from *V. michelli* (Santos *et al.* 1996; Santos *et al.* 2007). Thus, according Cook *et al.* (2014), there is a big probability to find compounds with useful biological activities produced by microorganisms.

In this context, we intend to contribute to the knowledge of diversity and biotechnological potential of Amazonian microorganisms; especially of endophytic fungi isolated from plant *Virola michelli*. Then, we describe the isolation and structural identification of sitosterol (**1**), stigmasterol (**2**), sitostenone (**3**), squalene (**4**), ergosterol (**5**) and ergosterol peroxide (**6**) from the endophytic fungus *Colletotrichum gloeosporioides* (Figure 1). The presence of phytosterols in fungi is rare and this is the first report of the isolation of phytosterols sitosterol, stigmasterol and sitostenone from the genus *Colletotrichum*.

MATERIAL AND METHODS

General procedures

The ^{1}H and ^{13}C NMR experiments were recorded on a NMR spectrometer (Mercury 300, Varian, Oxford, Oxfordshire, UK) with $CDCl_3$ as solvent and standard. The MS spectra were carried out in the mass spectrometer using ESI (+) ion mode (Acquity TQD, Waters, Milford, MA, USA).

Microorganism

The fungus *C. gloeosporioides* (Arx, 1957; Melanconiaceae) was obtained from a collection of the "Laboratorio de Bioensaios e Química de Micro-organismos – LaBQuiM / UFPA". This collection contains isolates from *V. michelli*. The fungus was inoculated into a Petri dish containing PDA

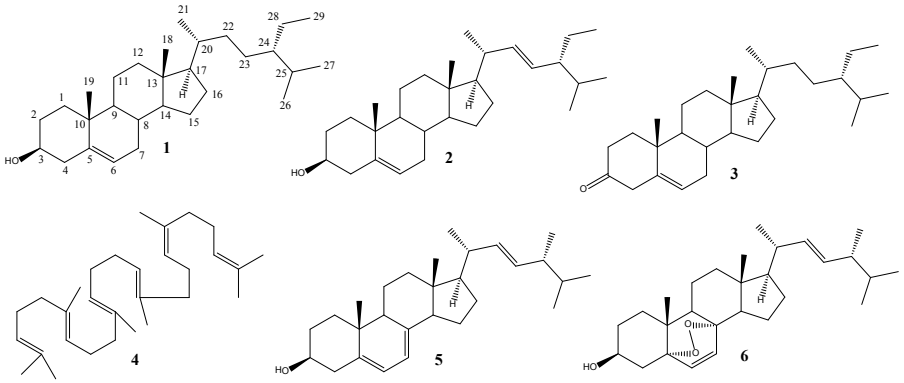

Figure 1. Compounds isolated from Colletotrichum gloeosporioides. Sitosterol (1), stigmasterol (2), sitostenone (3), squalene (4), ergosterol (5) and ergosterol peroxide (6)

culture medium (Potato, Dextrose, and Agar) and incubated at BOD 25 °C for eight days to reactivation. One strain is deposited as a code CGGVM05.

Cultivation of *C. gloeosporioides* CGGVM05 in rice and isolation of the compounds

Twenty Erlenmeyer flasks (1000 mL) containing 200 g of rice ("Uncle's Been®") and 75 mL of water per flasks were autoclaved for 45 min at 121 °C. Small pieces of PDA containing mycelium of *C. gloeosporioides* were added to 18 Erlenmeyer flasks under sterile conditions, then the Erlenmeyer flasks were incubated at 25 °C for 30 days for colony growth, two Erlenmeyer flaks were used as control. Biomass was macerated with hexane, ethyl acetate and methanol, in which the hexane (5.44 g), ethyl acetate (27.09 g) and MeOH (26.70 g) extracts were obtained after evaporation in rotary evaporator of resulting solutions. The hexane extract was fractionated on silica column using a mixture of hexane, ethyl acetate and methanol, in order increasing polarity, as eluent. The resulting fractions were successively chromatographed on silica gel CC by using hexane, ethyl acetate and methanol as mobile phase in a gradient of polarity and monitored by TLC. Following chemical constituents were isolated sitosterol + stigmasterol (**1**+**2**, 18.3 mg), sitostenone (**3**, 5.9 mg), squalene (**4**, 23.9 mg), ergosterol (**5**, 462.2 mg) and ergosterol peroxide (**6**, 113.9 mg).

RESULTS

The chemical study of the fungus *C. gloeosporioides* CGGMV05 lead the isolation of sitosterol (**1**), stigmasterol (**2**), sitostenone (**3**), squalene (**4**), ergosterol (**5**) and ergosterol peroxide (**6**). This is the first report of the isolation of the phytosterols sitosterol, stigmasterol and sitostenone from the genus *Colletotrichum.*

DISCUSSION

The phytosterols are widely spread in the plant kingdom. Normally, they are obtained in mixture of hard separation, because of showing similarity in physical proprieties. The most common are sitosterol and stigmasterol. Mixture containing stigmasterol are identified in ^{1}H NMR spectrum by signals referents to the vinyl hydrogens (H-22 and H-23) that show up as two doublets between δ_H 5.00 and δ_H 5.20. In all cases H-6 show up as a broad doublet in the ^{1}H NMR spectrum at δ_H 5.35 and H-3 hydrogen show as multiple around δ_H 3.50. The phytosterols can be free, glycosylated or esterified.

The mixture of compounds **1** + **2** was analyzed by mass spectrometry ESI (+) and peaks *m/z* 415 $[M+H]^+$ and *m/z* 413 $[M+H]^+$ were observed, allowing propose the molecular formulas $C_{29}H_{50}O$ to **1** and $C_{29}H_{48}O$ to **2**. Analysis of the ^{1}H NMR spectrum of the fraction studied showed that it had a mixture of two compounds, coded **1** and **2**. The spectrum indicated signals characteristic to sterols, such as olefinic hydrogens δ_H 5.35 (*d*, *J* = 6.0 Hz, H-6) and carbinolic hydrogen δ_H 3.50 (*m*, H-3), including the accumulation of signals in the region between δ_H 0.60 and δ_H 2.40 referent to many groups of hydrogens such as methyl, methylene and methine, which characterize the sitosterol (**1**). The double doubet at δ_H 5.13 (*J* = 15.5 and 8.0 Hz, H-22) and δ_H 5.01 (*J* = 15.5 and 8.0 Hz, H-23) are referent to the olefinic hydrogens of double bond with stereochemistry *trans* in the side chain of a stigmastane. These double doublet combined to others signals discussed to sitoterol characterize the compound stigmasterol (**2**). Through the strength of the signals relating to the olefinic protons H-22, H-23 and H-6 was also observed that sitosterol is in a higher proportion in the mixture. The ^{13}C NMR spectrum of the mixture of **1** and **2** showed characteristic signs of sitosterol and stigmasterol δ_C 121.7 (C-6); δ_C 140.6 (C-5); δ_C129.3 (C-23) and δ_C 138.3 (C-22) confirming identification (Goulart *et al.* 1993).

The compound **3** was analyzed by mass spectrometry ESI (+) and peak *m/z* 413 $[M+H]^+$ was observed, allowing propose the molecular formula $C_{29}H_{48}O$ to **3**. The ^{1}H NMR spectrum of compound **3** showed the same pattern signals for **1** and **2** with the exception of the signal at δ_H 3.50 referring to the carbinolic hydrogen H-3. In the ^{13}C NMR spectrum was observed an additional signal at δ_C 199.3 (C-3) referent to carbonilic carbon, thus the compound **3** was identified as sitostenone (Prachayasittikul *et al.* 2009).

The compound **4** was isolated from the nonpolar fractions of extract in the form of oil. In its ^{1}H NMR spectrum verified the presence of olefinic hydrogens in the region between δ_H 5.07 and δ_H 5.14. Signals assigned to methylene hydrogens neighboring double bonds were observed at δ_H 2.00. In δ_H 1.60 and δ_H 1.68 are signs commonly attributed to hydrogens of methyl attached to carbon sp^2. In the ^{13}C NMR spectrum showed six signs related to olefinic carbons (δ_C 135.1; 134.9; 131.2; 124.4; 124.3; 124.2) signals for six methylene carbons (δ_C 39.7 2x ; 29.7; 28.3; 26.7 and 26.6) and four signals related to methyl carbons (δ_C 25.7; 17.7; 16.0; 15.9). A comparison of the NMR data of the compound **4** with the literature enabled us to identify as squalene (Barreto *et al.* 2013).

The compounds **5** and **6** are commonly isolated from fungi and were identified through analysis of ^{1}H and ^{13}C NMR spectra and compared to the literature data and presented total similarity to ergosterol and ergosterol peroxide (Marinho *et al.* 2009).

To confirm that the phytosterols were produced by the fungus *C. gloeosporioides* the control extract (only the rice culture medium) was analyzed by direct-infusion electrospray mass spectrometry in positive ion mode ESI(+), where the

spectrum obtained showed the total absence of peaks for phytosterols.

Phytosterols have presented important biological activities are broadly found in plants. Although isolation of phytosterols in fungi to be relatively unusual, some studies have demonstrated the isolation of these compounds from some fungi species (Ling *et al.* 2007; Yan *et al.* 2010; Tarawneh *et al.* 2013) which opens up new possibilities to the isolation and obtaining this important class of compounds. This is the first report of the isolation of phytosterols sitosterol, stigmasterol and sitostenone from the genus *Colletotrichum*. The isolation of phytosterols from the fungus *C. gloeosporioides* corroborates with the theory of transfer of skills between endophytic fungus and host plant for the production of secondary metabolites, also contribute to knowledge secondary metabolites from Amazon fungi.

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