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Antioxidant activity and phenolic compounds of the extract from pigment-producing fungi isolated from Brazilian caves

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ABSTRACT

Few studies have been carried out on the biotechnological potential of microorganisms growing inside caves. Fungi are found in several environments and are sources of metabolites that present biological activities and can be used for industrial purpose. The objective of this study was to evaluate the antioxidant activity and phenolic compounds of extract from pigment-producing fungi isolated from Brazilian caves. The antioxidant activity was evaluated using the free radical scavenging methods 1,1-diphenyl-2-picrylhydrazyl (DPPH) and 2′-azino-bis-3-ethylbenzthiazoline-6-sulphonic acid ($ABTS^{\bullet+}$) assays and the β-carotene-linoleic acid system, while the content of total phenolic compounds was measured using the Folin-Ciocalteu method. Eight fungal isolates were selected based on their pigment production in solid culture media. The ethyl acetate extract of the *Penicillium flavigenum* CML2965 was highlighted among the analyzed extracts, exhibiting 98.2%, 47.1%, and 72.2% of antioxidant activity according to the DPPH and $ABTS^{\bullet+}$ methods and β-carotene system assay, respectively. In addition, the total phenolic compound content in the extract was 201 mg gallic acid equivalent (GAE)/g. The phenolic compounds gallic acid, catechin, chlorogenic acid, caffeic acid, and vanillin were identified and quantified using high-performance liquid chromatography with diode array detection. The high concentration of phenolic compounds, especially gallic acid (11.9 g/L), contributed to the antioxidant activity observed in the extract of *P. flavigenum* (CML2965). Therefore, this fungus shows biotechnological potential for the synthesis of gallic acid, which has several applications in the food and pharmaceutical industries.

1. Introduction

Fungi are found in several environments, and the metabolites produced can contribute to diversification and adaptation in various habitats (Fox and Howlett, 2008). The caves present environmental conditions considered as extreme that could select lineages of microorganisms with specific characteristics, able to produce several substances with bioactive properties (Nakaew et al., 2009). In the Brazilian territory, it is estimated that there are more than 100,000 caves, and, although they are rich in environments, their underground biota is little known (Ferreira et al., 2009). Recently, our research group, studying fungi isolated from caves, identified the colored compounds oosporein, oravactaene, and dihydrotrichodimerol in the extracts of the fungi *Lecanicillium aphanocladii* (CML2970), *Epicoccum nigrum* (CML2971), and *Penicillium flavigenum* (CML2965) (Souza et al., 2016). In addition to their potential as dyes, biological activities can also be associated with pigments produced by fungi (Celestino et al., 2014). Therefore, the synthesis of pigments by fungi may be a way for the selection of lineages with biotechnological potential. Fungi of the *Penicillium* genus synthesize several classes of pigments with potential use as food colorants, and some have biological activities (Mapari et al., 2005). *Penicillium* is widely known for the production of secondary metabolites, and other compounds with antioxidant activity were identified from isolates belonging to this genus (Chen et al., 2008; Lu et al., 2008; Saleem et al., 2007; Sun et al., 2009). Antrovenetin is a yellow pigment isolated from *Penicillium herquei*, which has antioxidant activity and potentiates the activity of tocopherol, an antioxidant, and can be used with food additives (Ishikawa et al., 1991).

Antioxidant compounds have become indispensable as food additives because of their properties of increasing shelf life without adverse effects on the nutritional and sensory quality of food products (Shahidi and Ambigaipalan, 2015). In addition, antioxidants neutralize free radicals resulting from normal biological activities of living organisms. Free radicals in excess can cause serious damages such as cancer, atherosclerosis, immunosuppression, aging, inflammation, ischemic heart disease, diabetes, and neurodegenerative diseases such as

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Parkinson's and Alzheimer's (Oliveira et al., 2008).

Synthetic antioxidants are widely used to prevent lipid oxidation of food and pharmaceutical products such as butyl-hydroxy-toluene (BHT), butyl-hydroxy-anisole (BHA), and propyl (GP) (Kim and Wijesekara, 2010). Studies on the toxicology of synthetic antioxidants have shown that they might exhibit carcinogenic effects (Shahidi, 2015), and some countries even restricted their use. Many natural antioxidants used in foods are plant-derived from the classes of phenolic and polyphenolic compounds, carotenoids and antioxidant vitamins (Shahidi, 2015). Factors such as raw material availability and growing time, which depends on climatic conditions and seasonality, can limit the use of metabolites (Mapari et al., 2005). However, microbial metabolites do not depend on climatic and seasonal factors and can be produced rapidly as their growth is relatively quick. Other advantages are the easy and quick genetic manipulation and production of compounds by fermentative processes. Fungi produce many extracellular enzymes and secondary metabolites such as organic acids, pigments, and other food additives (Akilandeswari and Pradeep, 2016). Among the antioxidant compounds isolated from filamentous fungi, there are carotenoids, flavonoids, phenolic acids, and their derivatives (Akilandeswari and Pradeep, 2016; Huang et al., 2007).

Gallic acid is a phenolic acid that has strong antioxidant activity in emulsions and lipid systems (Madsen and Bertelsen, 1995). It is used in processed foods, cosmetics, and food packaging materials in order to avoid rancidity due to lipid oxidation and deterioration (Yen et al., 2002), and in the pharmaceutical industry for the synthesis of antibiotics, and it also has anti-cancer and anti-inflammatory activities (Kroes et al., 1992; Lekha and Lonsane, 1994; Locatelli et al., 2012). Currently, studies have shown the use of the fermentation of tannins by microorganisms for the synthesis of gallic acid through the enzyme tannase (Aguilar et al., 2007; Banerjee et al., 2007; Govindarajan et al., 2016; Jana et al., 2013; Kumar et al., 2015; Melo et al., 2013;).

Studies reporting the biotechnological potential of cave fungi are still scarce, and we know about the importance of these fungi mainly in the production of pigments (Souza et al., 2016) and enzymes (Melo et al., 2013, 2014). In this context, the objective of this study was to evaluate the antioxidant activity and total phenolic compounds of pigment-producing fungi extract isolated from caves.

2. Material and methods

2.1. Fungal isolates and inoculum preparation

The filamentous fungi used in this study were selected based on the production of pigments in solid culture media. The fungi used were part of the collection of the Laboratório de Bioprospecção e Genética de Fungos Filamentosos (Biogen) at the Universidade Federal de Lavras, Brazil. The fungi of the genus *Penicillium*, *Aspergillus*, *Lecanicillium* and *Epicoccum* were isolated from Brazilian caves (Fig. 1) and deposited in the Coleç ão Micológica de Lavras (CML) at the Departamento de Fitopatologia at the Universidade Federal de Lavras, Brazil. The fungi were grown in potato dextrose agar (PDA) culture medium (200 g/L potato, 20 g/L dextrose, and 15 g/L agar) and were incubated at 25 °C for 7 days prior to use.

2.2. Extraction of fungal cultures

The methodology used to obtain the fungal extracts was carried out according to the protocol described by Souza et al. (2016), with modifications. Disks of approximately 9 mm diameter from the fungal colonies were transferred to 1 L of PD broth, which was incubated at 30 °C in the dark on a shaker at 150 rpm for 7 days. The cultures were filtered and extracted twice by liquid-liquid partition with half part of ethyl acetate (EtOAc) for one part of extract. The extracts were concentrated in a rotary evaporator (RV10 digital, IKA).

2.3. Determination of antioxidant activity

2.3.1. 1,1-diphenyl-2-picrylhydrazyl (DPPH) and 2′-azino-bis-3-ethylbenzthiazoline-6-sulphonic acid (ABTS) assays

The method of scavenging the DPPH radical was performed according to the protocol described by Santos et al. (2011), with modifications. The extracts at the concentrations of 500, 250, and 125 µg/mL were diluted in ethyl alcohol. In a dark environment, 100 µL of each dilution of the extracts was transferred to the test tube, and 900 µL of the DPPH solution was added at a concentration of 0.004%. The negative control used was ethyl alcohol, while the antioxidant Trolox 0.05% was used as positive control. The samples were incubated for 30 min at room temperature (22 °C at 25 °C) in total absence of light. The readings were performed using a spectrophotometer at 517 nm. The experiment was carried out in triplicate.

The scavenging method of the $ABTS^{\bullet+}$ radical was performed according to the method described by Rufino et al. (2007), with modifications. The $ABTS^{\bullet+}$ radical was prepared from the reaction of 5 mL of the 7 mM ABTS reagent solution with 88 µL of the 140 mM potassium persulfate solution, which was kept in the dark for 16 h. Then, 1 mL of this reaction was diluted in ethyl alcohol until it reached an absorbance of 0.7 nm ± 0.05 nm at 734 nm. The extracts at the concentrations of 500, 250, and 125 µg/mL were diluted with ethyl alcohol. In a dark environment, 30 µL of each dilution of the extract was transferred to test tubes with 3 mL of the $ABTS^{\bullet+}$ radical and homogenized in a tube shaker. For the negative control, ethyl alcohol was used, and, for the positive control, the antioxidant Trolox 2 mM was used. After 6 min in the dark, the reading was carried out at 734 nm.

The percentage of inhibition (I%) of the DPPH and $ABTS^{\bullet+}$ free radicals was calculated using the expression below (Eq. (1)).

$$I\% = [(A_{blank} - A_{sample})/A_{blank}] \times 100 \quad (1)$$

where A_{blank} is the absorbance of the negative control, and A_{sample} is the absorbance of the tested extracts. The results were also presented as 50% effective concentration (EC_{50}), which expressed the minimum antioxidant concentration in reducing 50% of the initial concentration of the DPPH and $ABTS^{\bullet+}$ radicals. A curve with the values of I% plotted on the y-axis and the concentration of the extracts (mg/mL) on the x-axis was obtained, and the equation of the line (Eq. (2)) was determined.

$$y = ax + b \quad (2)$$

where y is the percentage inhibition of the DPPH and $ABTS^{\bullet+}$ radicals, and x is the EC_{50} (mg/mL).

2.3.2. β-Carotene-linoleic acid assay

The methodology used in this assay was proposed by Rufino et al. (2006), with modifications. The β-carotene solution was prepared in a 2 mL light-shielded tube, adding 20 mg of β-carotene and 1 mL of chloroform. For the β-carotene-linoleic acid solution, 40 µL of linoleic acid, 530 µL of Tween 40, 50 µL of β-carotene solution, and 1 mL of chloroform was added in a light-Erlenmeyer flask. The system solution was homogenized, and the chloroform was evaporated. Water treated with oxygen was added until absorbance ranged from 0.6 to 0.7 nm at 470 nm. The extracts at the concentrations of 500, 250, and 125 µg/mL were diluted in ethyl alcohol. In a dark environment, 0.4 mL of each dilution of the extract was mixed with 5 mL of the system solution in test tubes, homogenized in a shaker, and run at zero time at 470 nm. The tubes were kept in a water bath at 40 °C for 2 h and run at final time at 470 nm. The spectrophotometer was calibrated with water. The blank was read as the β-carotene/linoleic acid system without extracts, and the positive control was Trolox (200 mg/mL).

The antioxidant activity in the β-carotene bleaching model in percentage (A%) was calculated according to the following equation (Eq. (3)):