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Analysis of Kojic Acid-Rich AcOET Extract (KaRE) from the Endophytic Fungus *Aspergillus* sp., Isolated from *Hancornia speciosa* and Determination of its Nitric Oxide (NO) Activity

Quantificação do Extrato AcOEt Rico em Ácido Kójico (kaRE) do Fungo Endofítico Aspergillus sp. Isolado de Hancornia speciosa e sua Determinação da Atividade de Eliminação de Óxido Nítrico (NO) In Vitro

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Kojic acid is produced by different types of fungi such as *Apergillus* sp. It has great potential for industrial application, being found in the cosmetic and health industries. The fungus *Aspergillus* sp. isolated from the leaves of *Hancornia speciosa* was grown in liquid medium PDB yielding kojic acid-rich ethyl acetate extract. Analysis of ^{1}H and ^{13}C NMR of the ethyl acetate extract showed the metabolite kojic acid as the main compound, and it was quantified by HPLC-UV. The kojic acid-rich ethyl acetate extract exhibited potent Nitric Oxide (NO) scavenging activity *in vitro* (antioxidant activity), showing the biotechnologic potential of fungus *Aspergillus* sp.

Keywords: Aspergillus sp., kojic acid, Nitric Oxide (NO), scavenging activity, HPLC-UV.

1. Introduction

Kojic acid (**1**), a fungal metabolite produced by various species of *Aspergillus*, *Penicillium*, and *Acetobacter*, was first isolated in 1907 from *Aspergillus* culture.[1–2] It is a polyfunctional heterocyclic, having an oxygen-ring γ-pyrone-type skeleton.[1–3] Interest in kojic acid (**1**) is increasing because of its commercial utility in the chemical industry, food, and cosmetic industry.[5] Kojic acid (**1**) has several modes of action by which it exerts depigmenting properties, as it inactivates tyrosinase, a key enzyme that catalyzes melanin (the skin pigment) production, and is of economic importance in the medical field. Kojic acid (**1**) and its derivatives are also used as anti-inflammatory drugs, antioxidants, and painkillers.[2–4] In addition, in food industry has great potential as an inhibitor of Polyphenoloxidases, also known as tyronases, cresolases, catecholases, diphenolases and phenolases, intracellular metalloenzymes that occur in fungi, plants and animals, that catalyze reactions involved in the enzymatic browning of agricultural products responsible by "off-flavor", the loss of aroma and flavor in foods[1–5]. Many attempts have been made to develop methods of kojic acid (**1**) production. The production of kojic acid by microorganisms through the fermentation of substrates by *Aspergillus* species is considered one of the best techniques used in industry.[2] Being more advantageous to work with the standardized extract rich in kojic acid (**1**), as there is no need to isolate it, which makes the process economically advantageous. Besides, studies of kojic acid-rich extracts obtained of fungi can have biotechnological applications in the industry due to the low cost. Thus, with the aim to find novel compounds and rich extracts with potent inhibitory activity on the production of NO radicals, herein, the fungus *Aspergillus* sp. is grown on potato dextrose (PD) culture medium; after extraction with AcOEt, kojic acid-rich extract (KaRE) is obtained. We report the details of the identification of kojic acid (**1**) in the KaRE. The nitric oxide (NO) scavenging activity, and the antioxidant activity of the KaRE. The commercial kojic acid standard (acquired from Sigma-Aldrich) was also evaluated. The KaRE was standardized using high-performance liquid chromatography (HPLC-UV), and commercial kojic acid was used as analytical standard.

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2. Experimental

1.1. General experimental procedures and reagents

The liquid chromatography system consisted of a Varian chromatograph equipped with a Polaris® 230 delivery module, Polaris® UV/vis detector, and a communications module. Galaxie® software was used to process the data. The analyses were conducted on a Shim-pack ODS column (250 × 4.6 mm i.d., 5 µm; Shimadzu). Acetonitrile (chromatographic grade) was supplied by Mallinkrodt Baker Inc. (Phillipsburg, NJ, USA). Water was purified with a Milli-Q-plus filter system (Millipore, Bedford, MA, USA). The NMR spectra were acquired with a Varian Inova 500 spectrometer (500 MHz for ^{1}H and 125 MHz for ^{13}C). The samples were dissolved in dimethyl sulfoxide (DMSO), and the spectra were calibrated relative to the solvent signals. The kojic acid (**1**) (Figure 1) used as standard was acquired from Sigma-Aldrich (≥98% purity, Darmstadt, Germany).

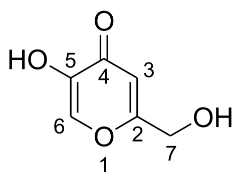

Figure 1. Structure of kojic acid (**1**)

1.2. Preparation of sample solutions, construction of linear analytical curves, and determination of limits of detection (LOD) and quantification (LOQ)

The analytical solutions were prepared by dissolving 1.0 mg of the sample in 1 mL of methanol, followed by filtration through a UNIFLO 25/0.2 PTFE syringe filter (Whatman/Schleicher & Schuell, Maidstone, UK). The metabolite kojic acid was serially diluted (500.0, 300.0, 200.0, 100.0, 80.0, 40.0, and 20.0 µg.mL^{-1}) in methanol. A 20.0 µL aliquot of each solution was injected into the HPLC equipment; the analyses were performed in triplicate. Linear analytical curves were obtained by plotting the peak area for the individual chromatographic standards. The correlation coefficient was calculated using Microsoft Excel®.

The limit of detection (LOD) and limit of quantification (LOQ) were determined from the standard deviation of the response (σ) and the slope of the analytical curve (S), using the expressions LOD = $3.3\sigma/S$ and LOQ = $10\sigma/S$.

1.3. Selectivity

The selectivity of the method was evaluated by comparing UV spectral data in the ascending, upper, and descending regions for the respective peaks of kojic acid (**1**) and the sample in the linear analytical curve. All the spectra matched, confirming that no other metabolites were co-eluted with the target compound.

1.4. Development of the analytical method and quantification of kojic acid in the AcOEt extract (KaRE) of *Aspergillus* sp.

Chromatographic analyses of the kojic acid-rich extract (KaRE) were performed through RP-HPLC-UV using a Shim-pack ODS column (250 mm × 4.6 mm i.d., 5 µm; Shimadzu) and a gradient system consisting of methanol and water with 0.1% acetic acid as the mobile phase (5–100% methanol in 25 min). The flow rate was 1.0 mL.min^{-1}, the injected volume was 20.0 µL, and the detection wavelength was set at 274 nm. The KaRE was diluted in methanol at a concentration of 0.5 mg.mL^{-1}, a 20 µL aliquot was injected into the analytical column, and the measurement was performed in triplicate under the conditions described in the methodology, affording good chromatographic resolution, above 1.5, for the peak corresponding to kojic acid (**1**).

1.5. Plant material

Plant samples of *H. speciosa* leaves were collected in the village of Santa Rita Park, São Cristóvão - SE, Brazil (11º00’53’’ S; 37º12’23’’ W). An exsiccate of the plant species is deposited in the herbarium of the Federal University of Sergipe - UFS. The leaves of *H. speciosa* were processed in the Department of Chemistry, Federal University of Sergipe (UFS).

1.6. Fungal screening

The surface of the leaves was sterilized by dipping the leaves into NaClO (2%) for 5 min, after dipping in 70% ethanol for 1 min. The sample was washed twice in sterile water for 10 min to eliminate epiphytic microorganisms. The sample was then placed for drying with N_2. The endophytic fungi were isolated from fragments of the leaves (3–4 pieces) placed in Petri dishes containing potato dextrose agar (PDA; containing 200 g/L potato, 20 g/L dextrose, and 15 g/L agar, pH 6.0) and antibiotics (gentamicin sulfate 1 mL/250 mL) to prevent bacterial growth.

The fungal growth was monitored daily until each mold colony reached 1–2 cm in diameter. The colonies were successively subcultured to obtain the pure strains based on UV detection. The peaks were continuously monitored until a pure culture of *Aspergillus* sp. was obtained. The strain of *Aspergillus* sp. was filed in the mycology collection of the Chemistry Department at the Federal University of Sergipe. The fungus was identified and classified by Dr. João Basílio Mesquita, professor at the Department of Agronomic Engineering at UFS, and deposited in the library of the Department of Chemistry - UFS with the following deposit code: CMS 002.

1.7. Scale-up and extraction

The *Aspergillus* sp., fungus was cultivated in two

Erlenmeyer flasks (1000 mL) containing 500 mL of potato dextrose broth (PDB) liquid medium by 28 days. After the liquid fermentation period, the broth was separated from the mycelium by filtration and extracted three times with half the volume of the culture using AcOEt. The kojic acid-rich extract (KaRE) (240 mg) was obtained by drying in a rotary evaporator.

1.8. Nitric oxide (NO) scavenging activity *in vitro*

Sodium nitroprusside solution (NPS) was used to evaluate the nitric oxide (NO) scavenging activity *in vitro*.[6–7] NPS solution (5 mM) was diluted in phosphate buffer (0.1 M, pH 7) and mixed with different concentrations of the AcOEt extract of the fungus *Aspergillus* sp. (1.0, 2.5, 7.5, 10.0 mg.mL^{-1}) dissolved in ethanol, and incubated at 24 °C for 150 min. A control experiment was conducted in parallel using pH 7, 0.1 M phosphate buffer and ethanol in place of the samples. At 30 min intervals, 1 mL of the incubated samples was removed and mixed with 1 mL of Griess reagent (1% sulfanilamide in 5% H_3PO_4 and 0.1% naphthylethylenediamine hydrochloride). The absorbance of the chromophore formed during diazotization of nitrite with sulfanilamide and subsequent complexation with naphthylethylenediamine was measured by spectrophotometry at 546 nm.

1.9. Diphenyl-2-picrilhhydrazyl (DPPH) free radical scavenging assay

The DPPH free radical scavenging was determined by the method described by Blois (1958), with some modifications. A 15 μL aliquot of the sample was added to 2 mL of methanolic solution of DPPH (90 mM). The absorbance of the mixture at 515 nm was read on a spectrophotometer. The DPPH scavenging activity was calculated using the formula: DPPH scavenging activity (%) = (ADPPH − Sample)/ADPPH × 100. Butylhydroxytyene (BHT) and epigalatocatechin (1.0 mg mL^{-1}) were used as the standards.

1.10. Determination of antioxidant activity by analyzing reactive oxygen species with thiobarbiturate acid /H_2O_2 *in vitro*

The antioxidant ability of the extract was determined according to the method of Polydoro et al. (2004)[8] using egg yolk and 5% saline solution as a lipid source. The reaction was carried out in an acid medium (20% acetic acid) in the presence of 10% sodium dodecyl sulfate (SDS), 1% thiobarbituric acid (TBA), and 10 mM hydrogen peroxide (H_2O_2) by incubating for 40 min at 900 °C in a water-bath. The product of this reaction was extracted with butyl alcohol, and the absorbance at 532 nm was read using a spectrophotometer. Butylhydroxytyene (BHT) (1 mg mL^{-1}) was used as a standard.

2. Results and Discussion

2.1. Identification and quantification of kojic acid (1) in KaRE

The ^{1}H NMR spectrum of the KaRE showed signals of one major substance (Figures S1, S2, and Table S1), as confirmed by the HPLC chromatogram (according to the methodology described in section 2.4 and comparison with the literature data).[9]

After obtaining the KaRE, its kojic acid (**1**) content was determined using the developed reverse-phase HPLC analytical method. A linear calibration curve was obtained by plotting the peak area of kojic acid (**1**) against the standard concentrations, and a correlation coefficient of 0.99 was achieved. The linear regression equation (y = 669.254516x + 18.9875) and the LOD (0.0008248 mg mL^{-1}) and LOQ (0.002749 mg mL^{-1}) indicate that the developed method is very sensitive and adequate for determining the kojic acid (**1**) content in KaRE. Figure 2 presents a comparison between the typical HPLC-PDA chromatograms obtained for the sample (KaRE) and for the analytical standard kojic acid.

The obtained concentration of kojic acid in the sample was 0.35 mg mL^{-1}, which corresponds to 70.83% of kojic acid (**1**) in KaRE, thus confirming the previously reported high content of this compound produced by different species of *Aspergillus*. The relative standard deviation (RSD) for the kojic acid (**1**) concentration was below 5% and the means obtained on different days did not differ statistically ($p < 0.05$).

In general, glucose and yeast extract are the sources of carbon and nitrogen respectively used by various fungal strains. Several published articles report that after optimizing the carbon source, nitrogen source, pH and cultivation method, kojic acid (**1**) production increases enormously at levels suitable for industrial application[10,11]. However here we report that the *Apergillus* sp. strain isolated from *H. speciosa* without any optimization produces a significant amount of kojic acid. Initially kojic acid (**1**) was quantified in the crude ethyl acetate extract. When the fungal extract is obtained through the liquid-liquid partition with ethyl acetate, losses can occur. Thus, we can conclude that when kojic acid is quantified directly from the fermented broth, the concentration obtained will be higher[10,11].

These results show that the fungus *Aspergillus* sp. is very promising to produce kojic acid (**1**) on a commercial scale, considering that it was grown in a medium without optimizing the conditions for cultivation. Such optimizations can lead to a significant increase in the production of kojic acid (**1**), which has a great economic potential, being the active component of several cosmetics.[5] In addition, there are advantages when working with the standardized extract,

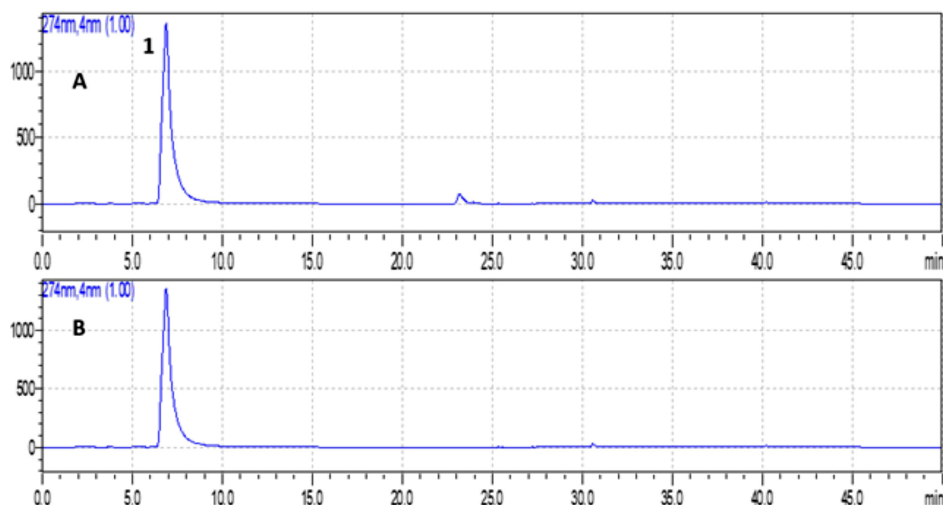

Figure 2. HPLC-PDA chromatograms showing (**A**) kojic acid-rich extract (KaRE) of *Aspergillus* sp., and (**B**) the analytical standard kojic acid peaks, obtained in the established analytical conditions (a gradient system consisting of methanol and water with 0.1% acetic acid as mobile phase 5% to 100% of methanol in 25 minutes. The flow rate was 1.0 mL min^{-1}, the injected volume was 20.0 µL and the detection wavelength was set at 274 nm)

since no need for isolation, making the process economically advantageous.[12]

2.2. Evaluation of DPPH antioxidant activity and nitric oxide (NO) scavenging activity

The antioxidant activity of the KaRE and kojic acid (**1**) (standard acquired from Sigma Aldrich) were tested under the same conditions. Kojic acid (**1**) as well as the KaRE showed a low free radical scavenging capacity in the DPPH test (Table 1). Thiobarbituric acid (TBA) analysis was also performed *in vitro* by the hydrogen peroxide (H_2O_2) method. Both samples showed no significant scavenging activity (%) in relation to the control group (ethanol) based on the student t-test at $p < 0.05$.

Although the DPPH method is widely used to assessment of the antioxidant capacity, the antioxidant assessment should not be based solely on in a single methodology, being necessary other methods to full characterization of a compound as antioxidant, because some of the methods can present negative results as in the results presented here.[13]

The nitric oxide (NO) scavenging activity of the KaRE and kojic acid (**1**) (positive standard) was evaluated using NPS (Table 2). Both showed strong NO scavenging activity, evidenced by the IC_{50} values of 150 and 40 µg.mL^{-1} respectively.

Table 1. DPPH free radicals scavenging potential

Conc. (mg mL^{-1})	KaRE Activity (%)	kojic acid (1) Activity (%)	BHT* Activity (%)	Epigalatocatechin* Activity (%)
1.0	9.0	10.0	86.5	92.3
2.5	18.0	11.0	-	-
7.5	16.0	12.0	-	-
10.0	18.0	12.0	-	-

* standard

Table 2. Nitric Oxide (NO) scavenging activity

KaRE Conc.*	KaRE Activity (%)	IC_{50}	Kojic acid (1) Conc.*	kojic acid (1)# Activity (%)	IC_{50}
1.0	37.0	150 µg mL^{-1}	0.10	36.0	40 µg mL^{-1}
2.5	47.0		0.25	37.0	
5.0	55.0		0.50	41.0	
7.5	55.0		0.75	41.0	
10.0	50.0		1.0	50.0	

*mg mL^{-1}; # standard

Nitric oxide is a signaling molecule that is implicated in a variety of inflammatory conditions,[14] where the excessive production of inorganic, free NO has detrimental effects on organ systems of the body, leading to tissue damage, and even leading to septic shock.[15–16] Agents that scavenge NO might be beneficial for the treatment of inflammatory responses and several diseases. The NO scavenging activity may be beneficial in treating pathological conditions induced by NO.[17] Notably, a NO scavenging activity of 20% may be important in the treatment of diseases such as pre-eclampsia, fetal hypoxia, and/or reperfusion ischemia, where NO functions as a vasodilator.[18]

3. Conclusion

The KaRE and kojic acid (**1**) showed strong nitric oxide (NO) scavenging activity, indicating that the endophytic fungus *Aspergillus* sp. from *H. speciosa* has great biotechnological potential. Although the developed protocol was not subjected to any optimization so far, the endophytic fungus Aspergillus sp. From H. speciosa produced a significant amount of kojic acid, which was recovered in the AcOEt extract (KaRE).[10,11] In addition, due to its richness in kojic acid, the KaRE can potentially be useful develop extracts rich in kojic acid that can be used in various biotechnological applications after proper standardization.

Supplementary Material

Supplementary material related to this article can be found in https://rvq.sbq.org.br/.

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