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Metabolism and Antifungal Activity of Coumarin and its Derivatives

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Abstract

In the present article, the inhibitory activity against the postharvest fungi *Lasiodiplodia* spp., *Botrytis* spp., and *Fusarium* spp. of coumarin was evaluated. Coumarin exhibited a significant antifungal effect (at concentrations of 1.5 mM and above), with inhibition percentages ranging from 24.8% to 66.4% for *Lasiodiplodia* spp., 55.5% to 78.8% for *Fusarium* spp., and 75.6% to 88.9% for *Botrytis* spp. Additionally, the metabolism of coumarin by phytopathogenic fungi was studied. Coumarin was found to be transformed into two main metabolic products, which were purified by chromatographic techniques and identified by spectroscopic methods (^{1}H and ^{13}C NMR), corresponding to dihydrocoumarin and 3-(2-hydroxyphenyl) propanoic acid. According to the structure of metabolic products, coumarin was hydrogenated at the carbon-carbon double bond and the lactone system was opened, which affects the α,β-unsaturated carbonyl system. Additionally, the effect of different substituents on both the aromatic ring and the α, β-unsaturated carbonyl system in the antifungal activity was evaluated. The results indicate that the presence of substituents such as 3-acetyl and 3-ethyl carboxylate on the double bond moiety significantly enhances the inhibitory effect on the radial growth of postharvest pathogen *Lasiodiplodia* spp.

Keywords: fungitoxicity, metabolic products, *Botrytis* spp., bioreduction, dihydrocoumarin.

1. Introduction

Coumarins constitute a broad group of natural products present in a wide variety of plants. These substances are heterocyclic molecules of the 1,2-benzopyrone type, also known as 2H-chromen-2-ones, and exhibit different biological and pharmacological properties. In particular, various natural coumarins and their derivatives have been found to be active molecules (Mustafa, 2023), especially against fungi and bacteria (Patil *et al*., 2023; Brooker *et al*., 2008; Franco *et al*., 2021; Prusty and Kumar, 2000). Some coumarins have been considered as phytoalexins: low-molecular weight compounds that are biosynthesized *de novo* by plants after exposure to biotic (infection by fungi and bacteria) or abiotic stress (wounds, radiation, freezing) (Stringlis *et al*., 2019; Mansfield, 2000). In the Apiaceae (Umbelliferae) family, coumarin-type phytoalexins such as coumarin, xanthotoxin, isoscopoletin, umbelliferone, isopimpinellin, and bergaptene have been identified (Yang et al., 2022). In the Rutaceae family, particularly Citrus spp., coumarin-type phytoalexins such as scoparone, scopoletin, and xanthyletin, among others, have been reported (Ramírez-Pelayo et al., 2019). The chemical structures of these phytoalexins are shown in Figure 1. For example, accumulation of scopoletin has been directly related to increased resistance against different pathogens (Stassen *et al*., 2021). Otherwise, coumarins can also be constitutive antimicrobial compounds (called phytoanticipins) and act as a first defensive chemical barrier of the plant (Fernandes *et al*., 2022; Stringlis *et al*., 2019). Thus, coumarins with a hydroxyl group in C-7 may present a defensive role against the *Orobanche cernua* parasite, preventing germination, penetration, and connection with the vascular system of the host (Serghini *et al*., 2001).

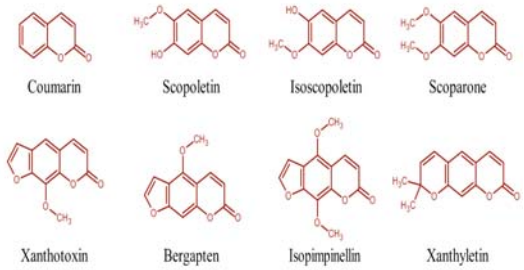

Figure 1. Coumarin-like phytoalexins from Apiaceae and Rutaceae (Yang *et al*., 2022; Ramírez-Pelayo *et al*., 2019)

Due to the antimicrobial activity of defensive compounds found in plants, such as coumarins, expectations have been raised about their potential use. This includes: *i.*) the direct application of phytoalexins or phytoanticipins as antifungal agents in crops, taking advantage of their antibiotic potential; and *ii.*) the design of new antibiotics for plants based on their structural core, which differs from that currently used in fungicides (Song *et al*., 2021; Oros and Kállai, 2019). Unfortunately, in many cases, fungi have adapted through the evolution of

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tissues to tolerate and metabolize the defensive compounds of their hosts using a variety of enzymatic reactions that lead to products innocuous against the fungus (Westrick *et al*., 2021). The elucidation of microbial metabolism and detoxification of plant defensive compounds could provide insight into structural modifications that may be necessary if certain compounds are to be further developed as selective fungal control agents (Pedras and Thapa, 2020; Pedras and Ahiahonu, 2005). Therefore, an environmentally attractive strategy for controlling plant pathogens could be using inhibitors of these fungal detoxifying enzymes or blocking certain functional groups modified by the microorganism to protect the plant against pathogens. In the present study, the antifungal activity of coumarin, as well as its microbial metabolism in three fungi isolated from Tahiti lime (*Citrus latifolia* T.), was evaluated. Additionally, the effect of different substituents on the coumarin nucleus on antifungal efficacy was examined.

2. Materials and Methods

2.1. Chemical and biological materials

Phytopathogenic fungi were isolated from Tahiti lime fruits (*Citrus latifolia* T.) infected and with evident symptoms of the stem-end rot, grey mold, and root rot diseases, and kept on deposit at the National University of Colombia Medellín (Laboratory of Phytopathology vouchers number: L. Castaño-CL01 (*Lasiodiplodia* spp.), L. Castaño-CL12 (*Botrytis* spp). and L. Castaño-CL14 (*Fusarium* spp). Fungi were maintained at 24±2°C and subcultured monthly in Petri dishes containing Potato Dextrose Agar media (PDA, Merck-KGaA, Darmstadt, Germany). A mycelial dispersion (1 mL) of each fungus was used to inoculate sterile Petri plates (15 cm in diameter) and tests the antifungal activity. The suspension was uniformly spread over the medium using a bent glass rod. Then, the inoculated medium was incubated at 25°C for 48 hours and mycelial disc (6 mm diameter) was used for the fungitoxicity assay. Coumarin (1,2-benzopyrone), umbelliferone, coumarin-3-carboxylic acid, salicylaldehyde, ethyl acetoacetate, malonic ester, and piperidine were purchased from Sigma-Aldrich (St. Louis, MO, USA). Carbendazim (methyl benzimidazol-2-yl carbamate, Colizym®) was from Colinagro S.A. (Bogotá, Colombia). Thymol (2-isopropyl-5-methylphenol) was acquired from Abaquim Ltda. (Medellín, Colombia). Acetic anhydride was from Merck (Darmstadt, Germany). Organic solvents *n*-hexane, CH_2Cl_2, ethyl acetate, and methanol were purchased from Protokimica S.A.S. (Medellín, Colombia). The ingredients of the culture medium for metabolic study were purchased from Oxoid Ltd. (Hampshire, England).

2.2. Equipment and analytical methods

The microbial metabolism of coumarin was monitored using Thin Layer Chromatography (TLC) on Merck Kiesegel 60 F_{254} (0.25 mm thick) plates, with mixtures of *n*-hexane-ethyl acetate as the mobile phases, and High Performance Liquid Chromatography (HPLC system Shimadzu, Kyoto, Japan) equipped with: Prominence Auto Sampler (SIL-20A), reciprocating pumps (LC-20AT), degasser (DGU 20A5), integrator (CBM 20A), diode array detector (SPD-M20A) and LC solution software (1.22 SP1). Reverse phase chromatographic analyses were performed under acetonitrile-water gradient conditions using a C18 guard cartridge (Phenomenex, 4.0 x 3.0 mm) followed by a C18 column (Zorbax Eclipse Plus, 4.6 x 150 mm, 5 µm, Agilent, USA). The gradient program was: 0-5 min, 80% A; 5-10 min, 80-60% A; 10-15 min, 60-40% A; 15-20 min, 40-20% A; and finally, reconditioning the column with 80% A isocratic for 5 min. The flow-rate was 1.0 mL/min. For the purification of the metabolic and synthetic products, different chromatographic techniques were used (column chromatography, CC, and preparative thin layer chromatography). For the CC, silica gel 60 (0.040-0.063 mm, Merck) and Sephadex® LH-20 were used as stationary phases. The compounds were detected in TLC by fluorescence under the UV lamp (254 and 366 nm) or by spraying with oleum reagent followed by heating. For the identification of the metabolic and synthetic products, ^1H (300 MHz) and ^{13}C (75 MHz) Nuclear Magnetic Resonance (NMR) spectroscopy were used in a Bruker AMX 300 spectrometer with deuterochloroform ($CDCl_3$) as solvent. The chemical shifts (δ) were expressed in ppm and the coupling constants (*J*) in Hertz. The attribution of the multiplicities for the carbons was carried out using the DEPT experiments. Notations: s = singlet, d = doublet, t = triplet, m = multiplet, dd = double of doublet, q = quintet.

2.3. Antifungal activity of coumarin and derivatives

The toxicity of coumarin 1 against *Lasiodiplodia* spp., *Botrytis* spp., and *Fusarium* spp. was determined using the Poisoned Food Technique (Velasco *et al*., 2010; Al-Rawashdeh *et al*., 2022). Different concentrations of coumarin (0.5, 1.0, 1.5 and 3.0 mM) dissolved in ethanol (less than 0.2%, v/v) were diluted in Petri dishes with PDA medium. All concentrations were evaluated in triplicate, and the results are shown as mean values of the colony diameters (± standard deviation, SD). Petri dishes without compound were used as a negative control, which contained only ethanol (0.2%, v/v) in the PDA medium. The common fungicide carbendazim (methyl benzimidazol-2-yl carbamate), at a concentration of 0.250 mM, was used as a positive control. The Petri dishes were maintained at room temperature under diffuse light, and the mycelial diameter was measured every 24 h. When the mycelial mass of the control was almost fully grown (*Lasiodiplodia* spp (3.5 days), *Botrytis* spp. (10 days) and *Fusarium* spp (11 days)), the incubation was stopped. The fungitoxicity of 1, in terms of the percentage of inhibition of radial growth, was calculated as (%) = [1- (T / C)] x 100; where, C = diameter of the colony (mm) of the control and T = diameter of the colony (mm) of the treatments (coumarin and derivatives). Additionally, compound 1 was used to determine the IC_{50} according to the methodology of Rivillas-Acevedo and Soriano-Garcia (2007). The antifungal activity evaluation of coumarin derivatives 4-9 was determined at 1 mM, using the fungus *Lasiodiplodia* spp.

2.4. Metabolism of coumarin

2.4.1. Preculture:

The methodology described by Castaño *et al*. (2021) was employed with slight modifications. Briefly, the fungi were inoculated in Erlenmeyer flasks containing Czapek-Dox and incubated at room temperature for 72 hours. The

resulting mycelium was filtered, washed with water, and used in the preparative-scale biotransformation and time-course experiments.

2.4.2. Time-course experiments:

The methodology described by Castaño *et al*. (2021) was used with slight modifications. Briefly, the precultured of each fungus was transplanted into Erlenmeyer flasks containing Czapeck-Dox medium (125 mL) and compound 1 at a final concentration of 1.5 mM. The ratios between compound 1 and metabolic products were preliminarily determined based on HPLC peak areas (wavelength: 254nm), and the results were expressed as relative abundances. Controls (without substrate) were carried out to verify the presence of similar compounds in the fungi culture.

2.4.3. Preparative scale metabolism:

The methodology described by Castaño *et al*. (2021) was followed with some modifications. Briefly, mycelia of *Lasiodiplodia* spp. from a 3-day old culture was used, and the final concentration of the compounds 1 in the culture medium was 1.5 mM. A control was performed to verify the presence of similar compounds in fungal cultures without the substrate.

2.5. Isolation and identification of metabolic products

The culture medium was saturated with NaCl and extracted with CH_2Cl_2 (3 x 2.0 L). The organic extracts were combined, dried over anhydrous Na_2SO_4, and concentrated in vacuo. The extract was chromatographed on a column using silica gel as the stationary phase. The elution was performed using a gradient system of *n*-hexane-CH_2Cl_2. The resulting extracts were fractionated to yield six fractions, which were grouped according to their TLC profile. The second fraction (eluted with *n*-hexane-CH_2Cl_2, 10:1) was chromatographed by column on SiO_2 using as eluent *n*-hexane-CH_2Cl_2, 10:1 to give the metabolic product 2. The fourth fraction (eluted with *n*-hexane-CH_2Cl_2, 7:3) was chromatographed by column on SiO_2 using as eluent *n*-hexane-CH_2Cl_2, 8:2 to yield the metabolic product 3. The identification of these metabolites was based on the interpretation of their NMR spectra. Metabolic product 2: it was isolated as an oily liquid. UV (MeOH-H_2O, 1:1) λ_{max} (nm): 273 nm. ^{1}H NMR: δ 7.32-7.22 (m, 2H, H-5, H-7), 7.19-7.10 (m, 2H, H-6, H-8), 3.05-2.90 (t, *J* = 7.2, 2H, H-4), 2.86-2.81 (t, *J* = 7.2, 2H, H-3). ^{13}C NMR: δ 168.9 (C-2), 152.0 (C-8a), 128.3 (C-7), 128.1 (C-5), 127.0 (C-6), 124.5 (C-4a), 117.0 (C-8), 29.3 (C-3), 23.7 (C-4). Metabolic product 3: it was isolated as a crystalline solid. UV (MeOH-H_2O, 1:1) λ_{max} (nm): 269 nm. ^{1}H NMR: δ 7.31-7.11 (m, 2H, H-6', H-4'), 6.94-6.87 (m, 2H, H-5', H-3'), 5.80 (s, 1H, -OH), 2.96 (t, 2H, *J* = 6.9, H-3), 2.81 (t, 2H, *J* = 6.9, H-2). ^{13}C NMR: δ 179.6 (C-1), 154.0 (C-2'), 130.5 (C-6'), 128.0 (C-4'), 126.9 (C-1'), 121.0 (C-5'), 116.6 (C-3'), 34.5 (C-2), 24.7 (C-3).

2.6. Preparation of derivatives

3-acetylcoumarin (4): Salicylaldehyde (2-hydroxybenzaldehyde, 2 mmol), ethyl acetoacetate (2 mmol) and a few drops of piperidine were mixed for 5 min at room temperature and without solvent. The reaction was neutralized with HCl (0.1 M) and finally the product was isolated by filtration. Subsequently, the final compound was recrystallized from ethanol. Yield: 95%. Pale yellow solid. ^{1}H NMR: δ 8.55 (s, 1H, H-4), 7.73-7.67 (m, 2H, H-5, H-7), 7.43-7.36 (m, 2H, H-6, H-8), 2.77 (s, 3H, CH_3). ^{13}C NMR: δ 195.6 (-C=O), 160.3 (C-2), 155.4 (C-8a), 147.6 (C-4), 134.5 (C-7), 130.3 (C-5), 125.0 (C-6), 124.5 (C-3), 118.3 (C-4a), 116.7 (C-8), 30.6 (-CH_3). Ethyl coumarin-3-carboxilate (5): Salicylaldehyde (2 mmol), malonic ester (2 mmol) and a few drops of piperidine were mixed for 5 min at room temperature and without solvent. The reaction was neutralized with HCl (0.1 M) and finally the product was isolated by filtration. Subsequently, the final compound was recrystallized from ethanol. Yield: 90%. Pale yellow solid.^{1}H NMR: δ 8.57 (s, 1H, H-4), 7.72-7.64 (m, 2H, H-5, H-7), 7.41-7.31 (m, 2H, H-6, H-8), 4.45 (q, 2H, -CH_2-), 1.45 (t, 3H, -CH_3). ^{13}C NMR: δ 163.1 (-C=O), 156.8 (C-2), 155.2 (C-8a), 148.7 (C-4), 134.4 (C-7), 129.5 (C-5), 124.9 (C-6), 118.3 (C-3), 117.9 (C-4a), 116.8 (C-8), 62.0 (-CH_2-), 14.27 (-CH_3). 7-acetyloxy coumarin (6): A mixture of umbelliferone (1 mmol), acetic anhydride (2 mmol) and a few drops of pyridine were refluxed for 30 min. The reaction was neutralized with NaOH solution (0.1 M) and extracted with CH_2Cl_2. The organic phase was dried with anhydrous Na_2SO_4 and distilled under reduced pressure in a rotoevaporator. Yield: 88%. White crystalline solid. ^{1}H NMR: δ 7.74 (d, *J* = 9.6, 1H, H-4), 7.54 (d, *J* = 8.4, 1H, H-5), 7.16 (d, *J* = 2.3, 1H, H-8), 7.10 (dd, *J* = 8.4, 1H, H-6), 6.45 (d, *J* = 9.6, 1H, H-3), 2.39 (s, 3H). ^{13}C NMR: δ 168.8 (-C=O), 160.4 (C-2), 154.2 (C-8a), 153.2 (C-7), 142.9 (C-4), 128.6 (C-5), 118.5 (C-6), 116.7 (C-4a), 116.1 (C-3), 110.5 (C-8), 21.2 (CH_3). 7-hydroxy-4-methyl coumarin (7): A mixture of resorcinol (1 mmol), ethyl acetoacetate (1 mmol) and a few drops of piperidine was mixed for 5 min at room temperature and without solvent. The reaction was neutralized with HCl solution (0.1 M), and finally the product was isolated by filtration. ^{1}H NMR: δ 7.55 (d, *J* = 8.4, 1H, H-5), 6.99 (d, *J* = 2.1, 1H, H-8), 6.90 (dd, *J* = 8.4, 2.1, 1H, H-6), 6.21 (s, 1H, H-3), 2.47 (s, -CH_3). ^{13}C NMR: δ 162.1 (C-2), 159.5 (C-7), 154.9 (C-8a), 153.1 (C-4), 126.0 (C-5), 114.2 (C-4a), 113.0 (C-3), 111.8 (C-6), 103.4 (C-8), 18.8 (-CH_3). 6-Nitrocoumarin (8): Coumarin (1.1 mmol) was nitrated using a mixture of concentrated sulfuric acid (96%) and nitric acid (65%) and stirring for 1 hour at 25°C. After neutralization with NaOH solution (0.1 M), an extraction with CH_2Cl_2 was carried out. The organic phase was dried with anhydrous Na_2SO_4, filtered, and evaporated to dryness (Yield: 75%). ^{1}H NMR: δ 8.48 (dd, *J* = 2.4, 9.0, 1H, H-7), 8.43 (d, *J* = 2.4, 1H, H-5), 7.85 (d, *J* = 9.6, 1H, H-4), 7.52 (d, *J* = 9.0, 1H, H-8), 6.64 (d, *J* = 9.6, 1H, H-3). ^{13}C NMR: δ 158.9 (C-2), 157.6 (C-8a), 144.1 (C-6), 142.3 (C-4), 126.7 (C-7), 123.8 (C-5), 118.9 (C-4a), 118.8 (C-8), 118.2 (C-3).

2.7. Statistical analysis

The data underwent analysis of variance (ANOVA), and the means were contrasted using the Least Significant Difference test (LSD) with a significance level of $p < 0.05$.

3. Results and Discussion

3.1. Antifungal activity

The fungistatic properties of 1 were analyzed using the technique of mycelial growth inhibition. The inhibitory

effects of 1 against *Botrytis* spp., *Lasiodiplodia* spp., and *Fusarium* spp. are shown in Figure 2. In general, compound 1 exhibited significant antifungal activity against all three fungi at concentrations of 1.5 mM and above. The results showed that the mycelial growth of these fungi varied according to the amount of compound 1 in the growth medium. At 3.0 mM, the percentages of inhibition of 1 against *Botrytis* spp., *Lasiodiplodia* spp., and *Fusarium* spp. ranged between 88.9 (day 1) and 75.6 (day 9), 66.4 (day 1) and 24.8 (day 3), and 78.8 (day 1) and 55.5% (day 9), respectively. The inhibitory effect of 1 was lower than that shown by the natural antifungal thymol (98-87% inhibition at 1.0 mM) and the synthetic antifungal Carbendazim (all fungi were inhibited 100% at 0.25 mM). In summary, the findings suggest that compound 1 displayed moderate to low antifungal activity against *Botrytis* spp., *Fusarium* spp., and *Lasiodiplodia* spp., with IC_{50} values of 2.19 (on the fourth day), 1.78 (on the third day), and 2.81 mM (on the second day), respectively. Accordingly, the lowest activity of compound 1 was observed against *Lasiodiplodia* spp. The moderate antifungal activity of 1 against *Candida albicans* (3.42 mM), *Aspergillus fumigatus* (6.84 mM) and *Fusarium solani* (3.42 mM) was also reported by Montagner *et al*. (2008). According to Qin *et al*. (2020), the 1,2 benzopyrone skeleton is essential for the antimicrobial activity of 1 and its derivatives. Studies of structure-activity relationship have suggested that the α, β-unsaturated carbonyl system, which is present in the coumarin core, is an important structural feature for exhibiting antifungal activity (El Ouadi *et al*., 2017; Meepagala *et al*., 2003). Compounds having the α, β-unsaturated carbonyl motif can act as Michael acceptors for biological nucleophiles (for example, a cysteine residue) and react irreversibly with biomolecules (for example, proteins) that are relevant to microorganisms, exhibiting the antimicrobial activity attributed to coumarins.

In general, for all fungi, the inhibitory effect of 1 decreased over time, a fact suggesting that the fungus has a detoxification route. To study this metabolic route, the microorganism was incubated with 1 at a concentration of 1.5 mM for 9 days.

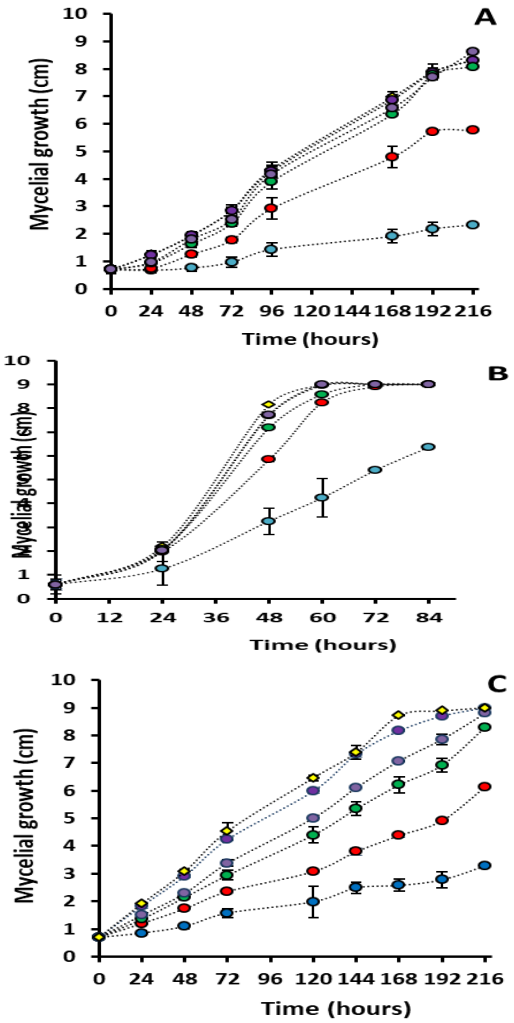

Figure 2. Radial growth of *Botrytis* spp. (**A**), *Lasiodiplodia* spp. (**B**), and *Fusarium* spp. (**C**) without (·····◇·····, Absolute control; ·····●·····, Solvent control) and with coumarin **1** at 0.5 (·····●·····), 1 (·····●·····), 1.5 (·····●·····), and 3 mM (·····●·····).

3.2. Metabolism of compound 1

A comparison of the HPLC-DAD chromatographic profiles obtained from biotransformation is presented in Figure 3. As shown, all fungi metabolized 1 into several metabolic products. *Lasiodiplodia* spp. transformed 1 into approximately 8 metabolic products. The products that are present in greater abundance correspond to the peaks with retention times (RT) of 6.6, 11.5, and 16.6 min. The peak with a retention time of 6.8 min was detected in the controls (data not shown), indicating that it corresponds to a metabolite from the fungus.

The HPLC-DAD chromatograms, obtained of the biotransformation of 1 by *Botrytis* spp. and *Fusarium* spp., exhibited minor similarities showing a single major compound at 9.0 and 19.8 min, respectively. In order to isolate the main metabolic products, a preparative-scale biotransformation of 1 in Czapeck-Dox liquid medium using the fungus *Lasiodiplodia* spp. was performed. Two metabolic products (2 and 3) were isolated and purified by means of column and preparative TLC.

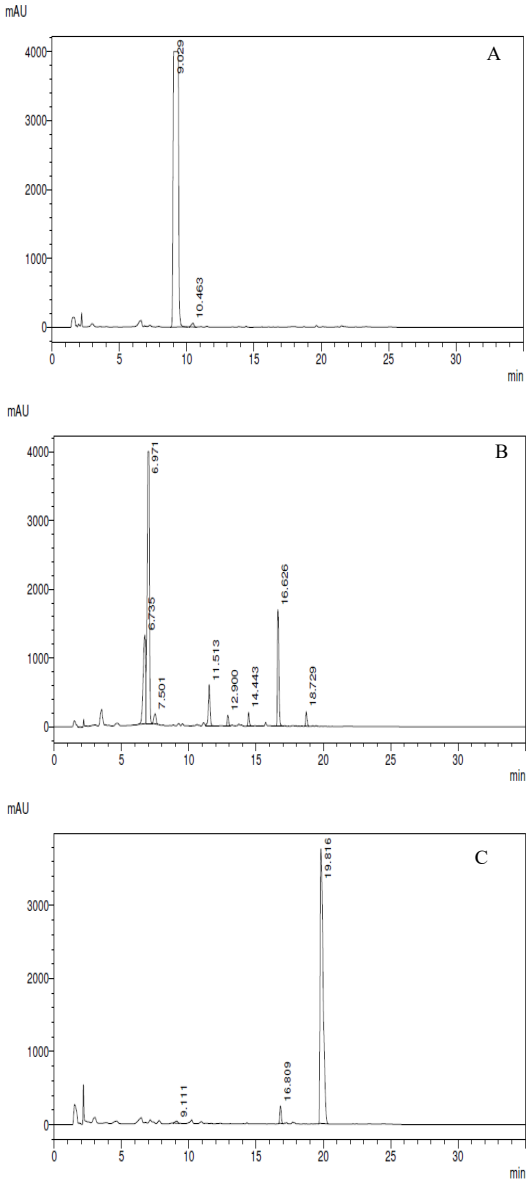

Figure 3. HPLC-DAD profile of the metabolism of **1**, after 6 days of fermentation, by *Fusarium* spp (A), *Lasiodiplodia* spp (B), and *Botrytis* spp (C). Wavelength: 254nm.

The structure of the isolated compounds was elucidated through interpretation of the spectroscopic data. Compound 2: The ^{1}H NMR spectrum showed signals for eight protons. Two upfield signals (at δ 2.86-2.81 and 3.05-2.90 ppm) that integrate each one for two protons. This indicates a reduction in the double bond of coumarin. At downfield, two multiple signals were observed (δ 7.32-7.22 and 7.19-7.10 ppm), corresponding to the four aromatic protons. In the ^{13}C NMR spectrum, nine signals corresponding to nine carbons were found. According to the DEPT-135 spectrum, the upfield signals, δ 29.3 and 23.7 ppm, correspond to two methylenic carbons. Additionally, it is observed that there are six carbon signals at downfield, consisting of four CH carbons at δ 117.0, 124.5, 128.1 and 128.3 ppm, and two quaternary carbons at δ 152.0, 124.5 ppm, belonging of the aromatic ring. There is also one quaternary carbon signal of the ester group at δ 168.9 ppm. From the spectroscopic data, the metabolic product 2 was identified as dihydrocoumarin (RT = 11.5 minutes).

Compound 3: Signals for nine protons were observed in the ^{1}H NMR spectrum. Two upfield signals were observed at δ 2.81 and 2.96 ppm, each integrating for two protons. A broad singlet signal was observed near 5.8 ppm, which corresponds to the hydroxyl group. At the downfield, two multiple signals were observed (δ 7.31-7.11 and 6.94-6.87 ppm), corresponding to the four aromatic protons. The two upfield signals allow us to infer that there was a reduction in the double bond of coumarin, while the presence of the broad singlet suggests that the lactone system was opened. In the ^{13}C NMR spectrum, nine signals corresponding to nine carbons are observed. According to the DEPT-135 spectrum, the low field signals, δ 24.7 and 34.5 ppm, correspond to two methylenic carbons. At lower fields, four CH carbons (δ 116.6, 121.0, 128.0, 130.5 ppm) of the aromatic ring are observed. In addition, 3 signals are observed at δ 179.6, 154.0 and 126.9 ppm, corresponding to the quaternary carbons C-1 (carbonyl group of carboxylic acid), C-2', C-1', respectively. From the spectroscopic data, the metabolic product (3) was identified as 3-(2-hydroxyphenyl) propanoic acid (RT = 6.6 minutes).

The metabolism of compound 1 with *Lasiodiplodia* spp., was monitored for 12 days by taking samples every 1, 3, 6, 9 and 12 days (Figure 4). The relationship between substrate 1 and the metabolic products 2 and 3 in the course of time was determined based on the area of the HPLC peaks.

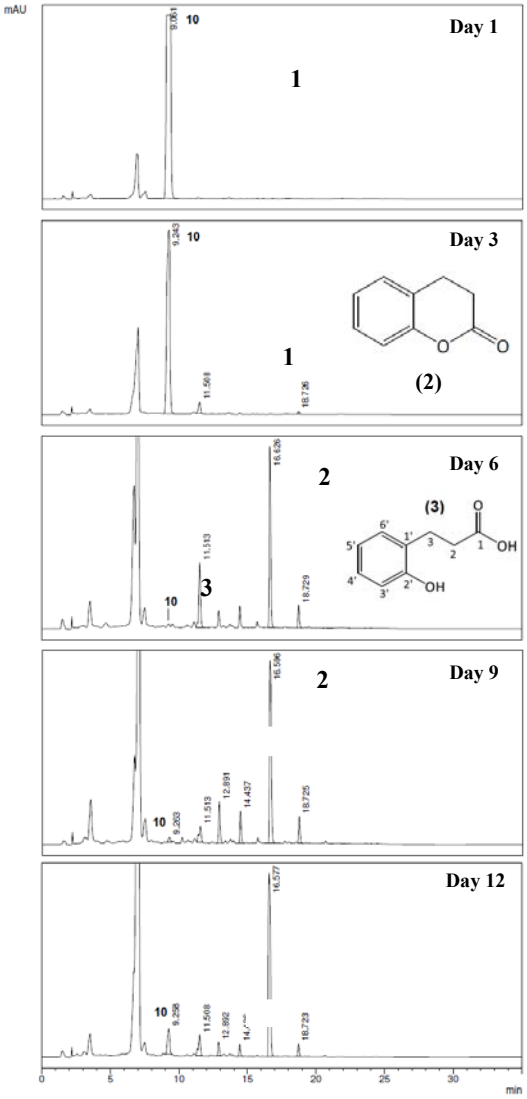

Figure 4. Time-course study for the metabolism of **1** by *Lasiodiplodia* spp. Wavelength: 254 nm.

The results, expressed as relative abundances, are shown in Figure 5. The fungus *Lasiodiplodia* spp. rapidly consumed the compound 1. After 6 days, about 94% of 1 was modified by *Lasiodiplodia* spp., being converted mainly to compound 3. The relative abundance of 2 and 3 was always less than 42%; possibly, these products are further transformed during metabolic biotransformation to generate other compounds.

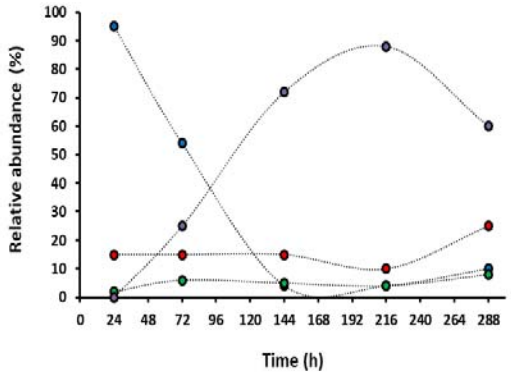

Figure 5. Relative abundance over time for microbial transformation of coumarin (**1**, ·····●·····) by *Lasiodiplodia* spp. Microbial products: compound (**2**, ·····●·····), dihydrocoumarin; compound (**3**, ·····●·····), 3-(2-hydroxyphenyl)propanoic acid; and others (·····●·····). Analysis wavelength: 254 nm.

The conversion of 1 is probably carried out by the action of ene-reductases, which are enzymes capable of reducing the activated C-C double bond to obtain 2. Subsequently, lipase-type hydrolases could act to open the lactone ring and produce 3 (Serra *et al*., 2019). The production of 2 from 1 was also reported by different yeasts and filamentous fungi; the authors found that all the tested strains were able to selectively reduce the compound 1, but with different conversion rates and sensitivity to substrate concentration (Serra *et al*., 2019). According to the structures of metabolic products 2 and 3, it is possible to affirm that the conversion of 1 initially affected the α, β-unsaturated carbonyl system, which is related to its antimicrobial activity. This would suggest a detoxification mechanism of 1 by *Lasiodiplodia* spp. through the action of ene-reductase enzymes. In fact, compound 1 is considered a toxic compound, while compound 2 is classified as a substance safe or GRAS, indicating lower toxicity to living organisms (Serra *et al*., 2019). It seems that the lower antifungal activity of 1 against *Lasiodiplodia* spp., compared to the other two microorganisms, may be a result of its greater ability to convert 1 to other metabolic products. *Lasiodiplodia* spp. modified almost all of 1 in only 6 days (Figure 4 and 5). Therefore, the ene-reductase enzymes could be important metabolic targets for developing new antifungal agents.

Unfortunately, it was not possible to purify and identify other metabolic products, which reached a maximum abundance around 90% for day 9 under the given conditions. According to Aguirre-Pranzoni *et al*. (2011), the biotransformation of 1 by *A. ochraceus* and *A. flavus*, afforded the compound 2 and 5-hydroxycoumarin, respectively. Furthermore, when *A. niger* was used, a mixture of compounds including 2, 3, 6-hydroxy-3,4-dihydrochromen-2-one, 3-(2-hydroxyphenyl) propanal, and 2-(3-hydroxypropyl)phenol was obtained.

3.3. Antifungal activity of coumarin derivatives

The microbial transformation of 1 showed significant structural changes produced by *Lasiodiplodia* spp.; consequently, the antifungal activity of synthetic coumarin derivatives was evaluated to obtain information about the relationship between structure and activity (Figure 6 and 7).

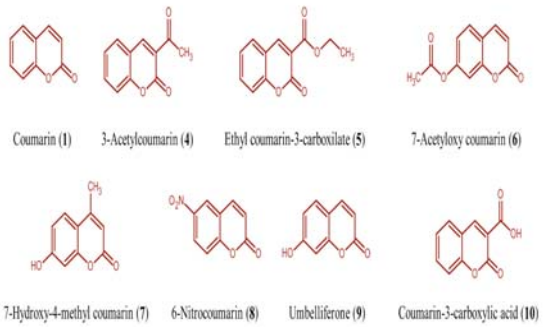

Figure 6. Coumarin derivatives employed to determine the antifungal activity against *Lasiodiplodia* spp.

A series of coumarin derivatives was prepared by standard chemical methods and tested against the fungus *Lasiodiplodia* spp. at a concentration of 1 mM. The percentages of inhibition of radial growth are shown in Figure. 7.

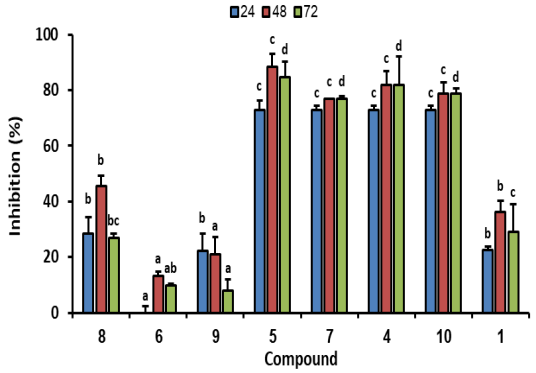

Figure 7. Inhibition (%) of radial growth of *Lasiodiplodia* spp. with **1** and derivatives. Different letters for the same time correspond to significant differences at a confidence level of 95%.

Results showed that substitution in the aromatic ring to afford 8 (with an electron-withdrawing group, -NO_2 in C6), 6 (with a *O*-acetyl group in C7) and 9 (with a hydroxyl group in C7) only slightly affected the antifungal activity with respect to 1. Compound 8 exhibited growth inhibition values for *Lasiodiplodia* spp similar to 1, while compounds 6 and 9 displayed a decrease in antifungal activity.

Remarkably, compounds 4, 5 and 10, which are substituted at C3 of the coumarin, showed a significant increase in fungal inhibition at 48 (~1.8-fold) and 72 h (~2.5-fold). Since the C-C double bond of 4, 5 and 10 is affected by two carbonyl systems (two electron-withdrawing groups), these compounds are better Michael acceptors than 1, and the β carbon (C4) is more electron deficient and therefore more electrophilic. The presence of these structural features would increase the reactivity of compounds 4, 5 and 10 towards biological nucleophiles, making them more active against the fungus. In addition, the presence of substituents on the α,β-unsaturated carbonyl system can change the selectivity or kinetics of the ene-reductase enzymes and, consequently, the ability of the microorganism to detoxify the compounds. Substituents can prevent the reduction of the C-C double bond by ene-reductase enzymes, enhancing the biological activity of compounds 4, 5 and 10 because the pathogenic fungus does not easily detoxify them. The ene-reductase enzymes are flavin-dependent, and the mechanism involves hydride transfer from flavin (cofactor) to the β-carbon of the C-C double bond; then, a tyrosine residue donates a proton on the opposite side of the α carbon, producing an anti-addition. Therefore, steric factors could affect the reduction of the C-C double bond (Vaz *et al*., 1995; Hult and Berglund, 2007).

Similarly, derivative 7, which is substituted at C4 and C7 with a methyl and a hydroxyl group, respectively, exhibited greater antifungal activity than 6 and 9 (substituted only in C7) and comparable fungistatic properties than 4, 5, and 10. The presence of a methyl substituent at the β carbon (C4) of the α,β-unsaturated carbonyl system significantly increased the inhibition of the fungus, as demonstrated by the comparison between compounds 7 and 9. The substituent at C3 or C4 of the coumarin core could contribute to the antifungal activity by decreasing the detoxification by the microorganism. This is possibly due to it steric hindrance, which hinders the transfer of hydride from flavin. Guerra *et al*. (2018) found that by introducing the acetate group in C4 of the coumarin, a derivative with promising antifungal activity was obtained; specifically, the derivative inhibited the mycelial growth and spore germination of *A. fumigatus* and *A. flavus*, affecting the cell structure and virulence of the fungus. According to Sahoo *et al*. (2021), substituents at C3 and C4 of coumarins are appreciated for the development of new antimicrobial agents. It is suggested that modifications at the C3 and C4 positions of coumarin could restrict or block metabolic conversions, thus improving antifungal activity.

The fact that compounds 4, 5 ,7, and 10 maintained high levels of inhibition with slight variations suggests that the toxic effects of these compounds were not immediately mitigated or reduced over time (24 to 72 h). The slow metabolism may be due to the of the fungus´s inability to reduce substituted coumarins at the α o β positions of the activated double bond.

4. Conclusions

Coumarin exhibited moderate antifungal activity against *Botrytis* spp., *Lasiodiplodia* spp., and *Fusarium* spp., which decreased over time. The evaluation of coumarin metabolism shows that fungi rapidly transform the compound into a species- and time-dependent form. In particular, *Lasiodiplodia* spp. affects the α,β-unsaturated carbonyl system, producing dihydrocoumarin and 3-(2-hydroxyphenyl)propanoic acid through reduction and hydrolysis reactions. Structural modification of coumarin at C3 and C4 increases antifungal activity against *Lasiodiplodia* spp. It is suggested that some electronic (improving character as acceptors Michael) and steric characteristics (blocking the double bond) can increase antifungal activity and reduce detoxification of coumarin derivatives by the microorganism.

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