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Atividade Citotóxica e Antifúngica de Chalconas Sintetizadas a partir de uma Acetofenona Natural Isolada de *Croton anisodontus*

Resumo: Neste trabalho, oito chalconas foram sintetizadas a partir da 2-hidroxi-3,4,6-trimetoxiacetofenona isolada de *Croton anisodontus* com benzaldeído e seus derivados, bem como avaliadas suas atividades citotóxica e antifúngica. Chalconas foram sintetizadas pela reação de condensação aldólica de Claisen-Schimdt em meio básico e identificadas por RMN de ^{1}H e ^{13}C, IV e EM. O ensaio MTT foi utilizado para determinar a citotoxicidade de todos os compostos sintetizados contra linhas celulares de câncer humano. Os resultados mostraram que % RCV variou de 19,43 ± 1,31 a 75,51 ± 1,84 %. A chalcona (*E*) -3- (4-fluorofenil) -1- (2-hidroxi-3,4,6-trimetoxifenil) prop-2-en-1-ona demonstrou a atividade mais forte contra células HCT-116 (% RCV = 75,51 ± 1,84). O potencial antifúngico *in vitro* das chalconas revelou que as chalconas (*E*)-3-(furan-2-il) -1- (2-hidroxi-3,4,6-trimetoxifenil) prop-2-en-1-ona (CMI 0,625 mg/mL contra *C. albicans* LABMIC 0105) e (*E*) -1- (2-hidroxi-3,4,6- trimetoxifenil) -3- (4-metoxifenil) prop-2-en-1-ona (CMI 0,312 mg / mL contra *C. albicans* LABMIC 0107) foram consideradas como chalconas de melhor inibição fúngica. A avaliação sinérgica *in vitro* mostrou que a união entre a anfotericina B e a chalcona (*E*) -1-(2-hidroxi-3,4,6-trimetoxifenil)-3-(4-metoxifenil) prop-2-en-1-ona mostrou efeito sinérgico contra *C. albicans* LABMIC 0105 (IFIC = 0,124) e efeito indiferente contra *C. albicans* LABMIC 0107 (IFIC = 1,0072), para cinética da morte, apenas o tratamento com a chalcona (*E*)-1- (2-hidroxi -3,4,6-trimetoxifenil)-3-(4-metoxifenil) prop-2-en-1-ona contra LABMIC 0107 foi capaz de promover a redução de células fúngicas entre os períodos de 4 a 8 h e inibição de 100 % a partir daí , semelhante ao mecanismo de ação da anfotericina B.

Palavras-Chave: Síntese, 2-hidroxi-3,4,6trimetoxiacetofenona, atividade antimicrobiana.

Abstract

In this work, eight chalcones were synthesized from the 2-hydroxy-3,4,6trimethoxyacetophenone isolated from *Croton anisodontus* with benzaldehyde and its derivatives as well as evaluated its cytotoxic and antifungal activities. Chalcones were synthesized by the Claisen-Schimdt aldol condensation reaction in basic medium and identified by ^{1}H and ^{13}C NMR, IR and MS. The MTT assay was used to determine the cytotoxicity of all synthetized compounds against human cancer cell lines. The results showed that the % RCV varied from 19.43 ± 1.31 to 75.51 ± 1.84 %. The chalcone (*E*)-3-(4-fluorophenyl)-1-(2-hydroxy-3,4,6-trimethoxyphenyl)prop-2-en-1-one demonstrated the strongest activity against HCT-116 cells (% RCV = 75.51 ± 1.84). The *in vitro* antifungal potential of the chalcones showed that chalcones (*E*)-3-(furan-2-yl)-1-(2-hydroxy-3,4,6-trimethoxyphenyl)prop-2-en-1-one (MIC 0.62 mg/mL against *C. albicans* LABMIC 0105) and (*E*)-1-(2-hydroxy-3,4,6-trimethoxyphenyl)-3-(4-methoxyphenyl)prop-2-en-1-one (MIC 0.31 mg/mL against *C. albicans* LABMIC 0107) were considered as chalcones of better fungal inhibition. The synergistic evaluation *in vitro* showed that the union between Amphotericin B and chalcone (*E*)-1-(2-hydroxy-3,4,6-trimethoxyphenyl)-3-(4-methoxyphenyl)prop-2-en-1-one showed an synergistic effect against *C. albicans* LABMIC 0105 (IFIC = 0.124) and indifferent effect against *C. albicans* LABMIC 0107 (IFIC = 1.0072), for kinetics of fungal death, only treatment with chalcone (*E*)-1-(2-hydroxy-3,4,6-trimethoxyphenyl)-3-(4-methoxyphenyl)prop-2-en-1-one against LABMIC 0107 was able to promote reduction of fungal cells between the periods of 4 to 8 h and 100 % inhibition thereafter, resembling the mechanism of action of Amphotericin B.

Keywords: Synthesis, 2-hydroxy-3,4,6trimethoxyacetophenone, antimicrobial activity.

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Cytotoxic and Antifungal Activity of Chalcones Synthesized from Natural Acetophenone Isolated from *Croton anisodontus*

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1. Introduction

Chalcones are open chain flavonoids that are characterized by two aromatic rings joined by a three-carbon α,β-unsaturated carbonyl system. These compounds can be isolated from natural sources due to their widespread distribution in fruits, vegetables, and tea or they can be synthesized by chemical processes.[1,2] Over the last several years, chalcones have instigated the interest of chemical and pharmacological researchers due to their simple chemical structure and varied biological activities. The chalcone biological activity spectrum includes antinociceptive,[3,4] anti-inflammatory,[5-7] antitumor,[8,9] antibacterial,[10] Anxiolytic,[11] antifungal,[12-13] antileishmanial[14] and antioxidant activity.[15] This wide range of activity is mainly due to the numerous substitution possibilities on the chalcone aromatic rings.[16]

The antifungal properties of chalcones is due to the presence of a reactive α,β-unsaturated keto function in chalcones. Chalcones inhibit β(1,3)glucan and chitin synthases, enzymes, that catalyze the biosynthesis of β(1,3)glucan and chitin polymers of the fungal cell wall, respectively. The studies on the antifungal ability chalcones can perform have received considerable attention from the pharmaceutical sector due the alarming increase of mycotic infections caused in individuals immunocompromised by species of *Candida* spp., mainly *Candida albicans*, in hospital environments as well as by the fungal resistance and the small arsenal of conventional drugs used. Alterations in the antifungal properties of chalcones is related to position of the substituents present on the aromatic rings. Usually chalcones in which electron-releasing groups such as methoxy and hydroxyl are present exhibit higher antifungal activity, as can be seen in the chalcones synthesized from 2-hydroxy-4,6-dimethoxyacetophenone.[17-19]

In addition, the antimicrobial and modulatory activity of 2-hydroxy-3,4,6-trimethoxyacetophenone isolated from *Croton anisodontus* towards *Escherichia coli, Pseudomonas aeruginosa* and *Staphylococcus aureus* strains was reported. The results showed to be significant towards *P. aeruginosa* and *S. aureus* 358, with $p < 0.001$ in association with amikacin and indicate that acetophenone isolated from *C. anisodontus* (Figure 1) may be a starting compound for the synthesis of chalcones with antimicrobial activity.[10,20]

There is a continuous scientific effort that has been expended on the design and development of anticancer chalcones that has resulted in many novels and chemically diverse chalcones with therapeutic potential against different types of cancer.[21,9] In this context, chalcones are able to induce cell cycle arrest and apoptosis in various human cancer cell lines. These abilities can be attributed, in part, by tubulin polymerization inhibition and/or the capacity to bind at the DNA minor groove of neoplastic cells. The cytotoxic potential against human cancer cell lines are related to chalcones containing electron donating/withdrawing groups.[9] In this context, the present study reports the cytotoxic and antifungal activity of chalcones synthesized from the 2-hydroxy-3,4,6-trimethoxyacetophenone isolated from *C. anisodontus*.

2. Material and Methods

2.1. Plant material

C. anisodontus samples were collected in the city of Itapiúna, Ceará state, Brazil, in March,

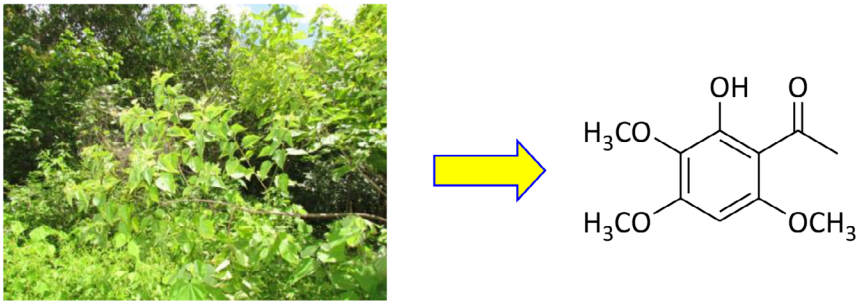


Figure 1. Aerial parts of *C. Anisodontus* (a) 2-hydroxy-3,4,6-trimethoxyacetophenone (b)

2011. Botanical identification was performed by Professor Edson Nunes from Universidade Federal do Ceará. An exsiccate was deposited at Herbário Prisco Bezerra-UFC under registry number 48964.[20]

2.2. Extraction and isolation

C. anisodontus stem bark (1462.6 g) was dried at room temperature, followed by trituration and cold extraction with hexane for three days. The resulting solution was distilled under reduced pressure to give the hexane extract (23.25 g), which was adsorbed on silica gel and sent to the chromatographic column, using the following eluents: hexane, chloroform, ethyl acetate and ethanol. The chloroform fraction (9.77 g) was reintroduced in the column, now with the following eluents: hexane (F 1-6), hexane/ethyl acetate (8:2 F 7-26), hexane/ ethyl acetate (7:3 F 27-40), ethyl acetate (F 41-60) and methanol (F 61-66). Fractions 12-16 revealed a yellow crystalline solid, named 2-hydroxy-3,4,6trimethoxyacetophenone, which, after recrystallization with hexane and Thin Layer Chromatography analysis, was found to be pure.[20]

2.3. General procedures

The chemical reagents were from Sigma-Aldrich. ^{1}H and ^{13}C NMR spectra were obtained using a Bruker Spectrometer, model Avance DPX-300 and model Avance DRX- 500 operating at a frequency of 300 MHz and 500 MHz for hydrogen, 75 MHz and 125 MHz for carbon respectively. The spectra were measured in $CDCl_3$ solvents and chemical shifts are reported as δ values in parts per million (ppm) relative to tetramethylsilane (δ 0.00) as internal standard. MS for the analysis of the volatile constituents was carried out on a Hewlett-Packard Model 5971 GC/MS using a non-polar DB-5 fused silica capillary column (30 mm x 0.25 mm i.d., 0.25m film thickness); carrier gas helium, flow rate 1 mL/min and with split ratio 1:1. The injector temperature and detector temperature were 250º C and 200º C, respectively. The column temperature was programmed from 35 C to 180º C at 4º C/min and then 180 C to 250º C at 10º C/min. Infrared spectra were determined on a Perkin Elmer FT-IR 1000 spectrophotometer and are reported in wave number (cm^{-1}). The melting point was done in the apparatus MQAPF- 302 (microchemistry) with heating rate 3.0 °C min^{-1}.

2.4. General procedure for synthesis of chalcones (1-8)

The description of the procedure of the synthesis of the eight chalcones are shown in Figure 1. The chalcones **(1-8)** were synthesized by a Claisen-Schmidt condensation reaction in basic medium.[22] An ethanol solution of 2-hydroxy-3,4,6trimethoxyacetophenone (2 mmol) isolated from *C. anisodontus*[20] was added to a solution of benzaldehyde and the derivatives (2 mmol), followed by the addition of 10 drops of 50 % w/v aq. NaOH with stirring for 48 h at room temperature (Scheme 1). The solid that formed was filtered under reduced pressure, washed with cold water and analyzed by TLC. The chalcones obtained were considered sufficiently pure to be used in tests pharmacological.

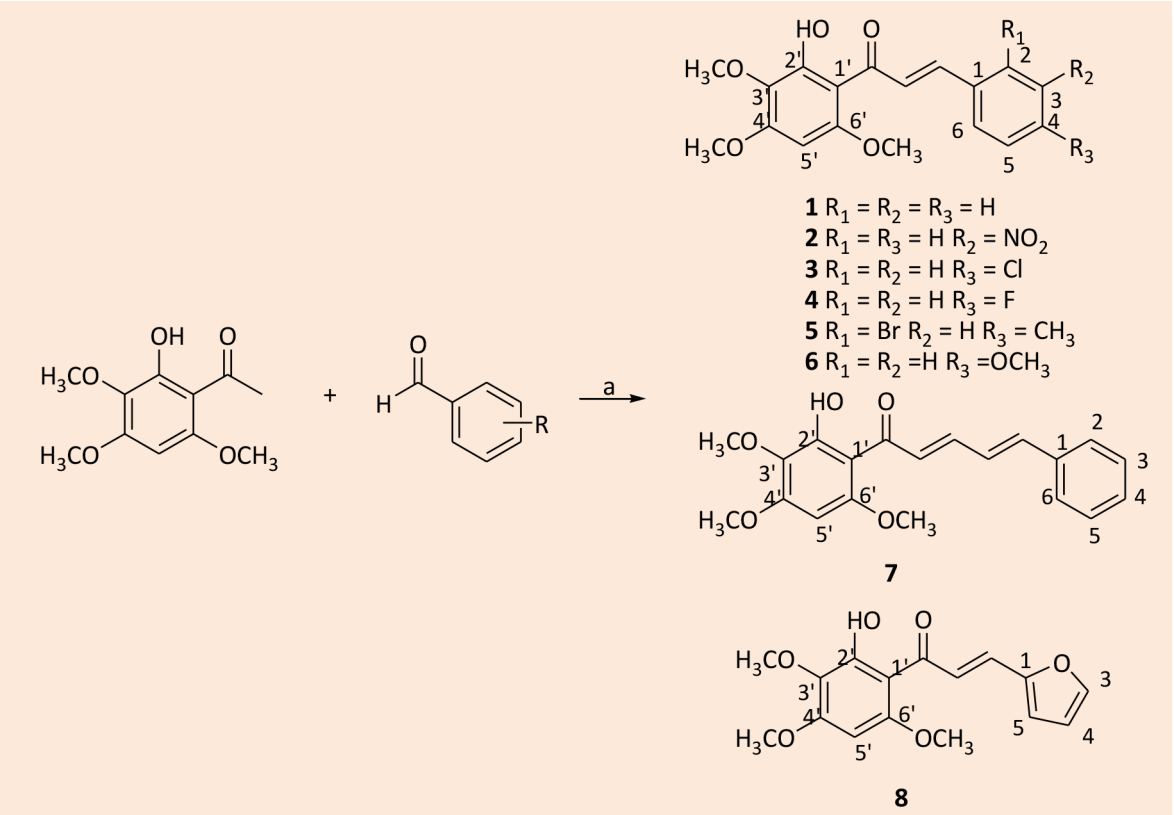
2.5. Analytical data of chalcones

(*E*)-1-(2-hydroxy-3,4,6-trimethoxyphenyl)-3-phenylprop-2-en-1-one (1)

Yellow solid (Yield: 38.0 %), R_F = 0.55 (hexane/ethyl acetate, 80:20), m.p. 135.4 – 136.1 ºC; FT-IR (KBr, ν_{cm}^{-1}): 1634, 1600, 1588, 1575, 1480, 1167. ^{1}H RMN ($CDCl_3$, ppm): 3.96 (s, MeO-3'); 3.96 (s, MeO-4'); 3.85(s, MeO-6'); 6.01 (s, H-4'); 7.42 (m, H-4); 7.43 (m, H-3/5); 7.60 (dd, H-2/6, J = 7,53; 1,77 Hz); 7.79 (d, Hα, J = 15.6 Hz); 7.88 (d, Hβ, J = 15.6 Hz).^{13}C RMN ($CDCl_3$, ppm): 193.5 (C=O); 60.9 (MeO-3'); 56.3 (MeO-4'); 56.2(MeO-6'); 107.1 (C-1'); 158.7 (C-2'); 131.2 (C-3'); 159.5 (C-4'); 87.3 (C-5'); 158.8 (C-6'); 135.7 (C-1); 128.6 (C-2/6); 129.1 (C-3/5); 130.3 (C-4); 127.7 (Cα); 142.8 (Cβ). MS-EI *m/z* (%) = 314.

(*E*)-1-(2-hydroxy-3,4,6-trimethoxyphenyl)-3-(3-nitrophenyl)prop-2-en-1-one (2)

Yellow solid (Yield: 59.0 %), R_F = 0.49 (hexane/ethyl acetate, 80:20), m.p. 181.2 - 182.6; FT-IR (KBr, ν_{cm}^{-1}): 1634, 1600, 1588, 1575, 1480, 1167. ^{1}H RMN ($CDCl_3$, ppm): 3.98 (s, MeO-3'); 3.97 (s, MeO-4'); 3.84 (s, MeO-6'); 6.02 (H-4'); 8.45 (s, H-2); 8.22 (d, H-4, J = 8.13 Hz), 7.59 (t, H-5); 7.86 (d, J = 7.6 Hz); 7.75 (d, Hα, J = 15,6 Hz); 7.94 (d, Hβ, J = 15,6 Hz). ^{13}C RMN ($CDCl_3$, ppm): 192.7 (C=O); 60.9 (MeO-3'); 56.3 (MeO-4'); 56.2(MeO-6'); 106.9 (C-1'); 158.9 (C-2'); 131.1 (C-3'); 159.6 (C-4'); 87.2 (C-5'); 159.2 (C-6'); 137.5 (C-1); 122.3 (C-2); 148.9 (C-3); 124.4 (C-4); 130.1 (C-5), 134.5 (C-6); 130.6 (Cα); 139.6 (Cβ). MS-EI *m/z* (%) = 359.



Scheme 1. Preparation of chalconeS a) NaOH 50 % w/v, ethanol, r.t., 48 h

(*E*)-3-(4-chlorophenyl)-1-(2-hydroxy-3,4,6-trimethoxyphenyl)prop-2-en-1-one (3)

Yellow solid (Yield: 39.0 %), R_F = 0.45 (hexane/ethyl acetate, 80:20), m.p. 94.3 - 96.8 ºC; FT-IR (KBr, ν_{cm}^{-1}): 1634, 1600, 1588, 1575, 1480, 1167. ^{1}H RMN ($CDCl_3$, ppm): 3.96 (s, MeO-3'); 3.96 (s, MeO-4'); 3.94 (s, MeO-6'); 6.02 (s, H-5'); 7.38 (d, J = 8.35, H-2/6); 7.53 (d, J = 8.35, H-3/5); 7.79 (d, Hα, J = 15.6 Hz); 7.83 (d, Hβ, J = 15.6 Hz). ^{13}C RMN ($CDCl_3$, ppm): 193.5 (C=O); 60.9 (MeO-3'); 60.9 (MeO-4'); 60.9 (MeO-6'); 106.5 (C-1'); 159.0 (C-2'); 129.7 (C-3'); 159.6 (C-4'); 87.4 (C-5'); 159.2 (C-6'); 134.2 (C-1); 131.2 (C-2/6); 131.7 (C-3/5); 136.2 (C-4); 128.2 (Cα); 141.3 (Cβ). MS-EI *m/z* (%) = 348.

(*E*)-3-(4-fluorophenyl)-1-(2-hydroxy-3,4,6-trimethoxyphenyl)prop-2-en-1-one (4)

Yellow solid (Yield: 61.0 %), R_F = 0.42 (hexane/ethyl acetate, 80:20), m.p. 144.3 - 144.8 ºC; FT-IR (KBr, ν_{cm}^{-1}): 1634, 1600, 1588, 1575, 1480, 1167. ^{1}H RMN ($CDCl_3$, ppm): 3.96 (s, MeO-3'); 3.96 (s, MeO-4'); 3.84 (s, MeO-6'); 6.07 (s, H-5'); 7.63 (d, J = 8.58, H-2/6); 7.65 (d, J = 8.58, H-3/5); 7.82 (d, Hα, J = 15,6 Hz); 7.83 (d, Hβ, J = 15.6 Hz). ^{13}C RMN ($CDCl_3$, ppm): 193.2 (C=O); 60.9 (MeO-3'); 56.3 (MeO-4'); 56.2 (MeO-6'); 107.1 (C-1'); 158.7 (C-2'); 130.4 (C-3'); 159.6 (C-4'); 87.4 (C-5'); 158.7 (C-6'); 131.9 (C-1); 131.2 (C-2/6); 131.9 (C-3/5); 141.5 (C-4); 127.4 (Cα); 141.5 (Cβ). MS-EI *m/z* (%) = 322.

(*E*)-3-(2-bromo-4-methylphenyl)-1-(2-hydroxy-3,4,6-trimethoxyphenyl)prop-2-en-1-one (5)

Yellow solid (Yield: 56.1 %), R_F = 0.44 (hexane/ethyl acetate, 80:20), m.p. 155.5 - 156.3 ºC; FT-IR (KBr, ν_{cm}^{-1}): 1634, 1600, 1588, 1575, 1480, 1167.^{1}H RMN ($CDCl_3$, ppm): 3.96 (s, MeO-3'); 3.94 (s, MeO-4'); 3.85 (s, MeO-6'); 6.01 (s, H-5'); 7.47 (s, H-3); 7.58 (d, J = 8,00, H-5); 7.15 (d, J = 7.85, H-5); 7.78 (d, Hα, J = 15.5 Hz); 8.11 (d, Hβ, J = 15.5 Hz); 2.26 (CH_3). ^{13}C RMN ($CDCl_3$, ppm): 193.2 (C=O); 60.9 (MeO-3'); 56.3 (MeO-4'); 56.2 (MeO-6'); 107.1 (C-1'); 158.7 (C-2'); 131.2 (C-3'); 159.6 (C-4'); 87.3 (C-5'); 158.8 (C-6'); 131.2 (C-1); 142.0 (C-2); 134.2 (C-3); 131.4 (C-4); 127.8 (C-5); 128.8 (C-6); 129.3 (Cα); 141.0 (Cβ); 21.2 (CH_3). MS-EI *m/z* (%) = 407.

(*E*)-1-(2-hydroxy-3,4,6-trimethoxyphenyl)-3-(4-methoxyphenyl)prop-2-en-1-one (6)

Yellow solid (Yield: 44.2 %), R_F = 0.4 (hexane/ethyl acetate, 80:20), m.p. 94.7 - 94.8 ºC; FT-IR (KBr, ν_{cm}^{-1}): 1634, 1600, 1588, 1575, 1480, 1167.

^{1}H RMN ($CDCl_3$, ppm): 3.91 (s, MeO-3'); 3.90 (s, MeO-4'); 3.86 (s, MeO-6'); 3.78 (s, MeO-4); 5.93 (s, H-4'); 7.89 (m, H-3/5, J = 8.4); 7.53 (d, H-2/6, J = 8.4 Hz); 7.24 (d, Hα, J = 14.6 Hz); 7.75 (d, Hβ, J = 14.6 Hz). ^{13}C RMN ($CDCl_3$, ppm): 193.3 (C=O); 60.9 (MeO-3'); 56.2 (MeO-4'); 55.8 (MeO-6'); 55.5 (MeO-4); 107.1 (C-1'); 158.7 (C-2'); 130.3 (C-3'); 159.5 (C-4'); 87.3 (C-5'); 158.6 (C-6'); 130.7 (C-1); 125.2 (C-2/6); 130.3 (C-3/5); 128.4 (Cα); 142.9 (Cβ). MS-EI *m/z* (%) = 344.

(2*E*,4*E*)-1-(2-hydroxy-3,4,6-trimethoxyphenyl)-5-phenylpenta-2,4-dien-1-one (7)

Yellow solid (Yield: 35.4 %), R_F = 0.5 (hexane/ethyl acetate, 80:20), m.p. 144.8 - 145.2 ºC; FT-IR (KBr, ν_{cm}^{-1}): 1634, 1600, 1588, 1575, 1480, 1167. ^{1}H RMN ($CDCl_3$, ppm): 3.84 (s, MeO-3'); 3.93 (s, MeO-4'); 3.95(s, MeO-6'); 6.99 (s, H-5'); 7.44 (m, H-4); 7.38 (d, H-3/5, J = 7.1 Hz); 7.51 (d, H-2/6, J = 7.2 Hz); 7.62 (d, Hα, J = 14.6 Hz); 7.64 (d, Hβ, J = 14.6 Hz); 7.40 (d, Hα, J = 14.6 Hz); 7.43 (d, Hβ, J = 14.6 Hz). ^{13}C RMN ($CDCl_3$, ppm): 193.2 (C=O); 60.9 (MeO-3'); 56.1 (MeO-4'); 56.2 (MeO-6'); 107.0 (C-1'); 158.7 (C-2'); 131.0 (C-3'); 159.6 (C-4'); 87.2 (C-5'); 158.5 (C-6'); 136.5 (C-1); 127.7 (C-2/6); 129.1 (C-3/5); 131.0 (C-4); 129.2 (Cα); 143.5 (Cβ); 127.0 (Cα); 141.8 (Cβ). MS-EI *m/z* (%) = 340.

(*E*)-3-(furan-2-yl)-1-(2-hydroxy-3,4,6-trimethoxyphenyl)prop-2-en-1-one (8)

Yellow solid (Yield: 78.1 %), R_F = 0.42 (hexane/ethyl acetate, 80:20), m.p. 129.6 – 130.2 ºC; FT-IR (KBr, ν_{cm}^{-1}): 1634, 1600, 1588, 1575, 1480, 1167. ^{1}H RMN ($CDCl_3$, ppm): 3.81 (s, MeO-3'); 3.92 (s, MeO-4'); 3.92 (s, MeO-6'); 5.97 (s, H-5'); 6.65 (d, J = 2,9 Hz, H-4); 6.47 (s, H-3); 7.48 (s, H-5); 7.65 (d, Hα, J = 15,4 Hz); 7.73 (d, Hβ, J = 15,4 Hz). ^{13}C RMN ($CDCl_3$, ppm): 193.1 (C=O); 60.9 (MeO-3'); 56.2 (MeO-4'); 56.1 (MeO-6'); 107.0 (C-1'); 158.8 (C-2'); 131.0 (C-3'); 159.5 (C-4'); 87.3 (C-5'); 158.5 (C-6'); 152.3 (C-1); 144.9 (C-5); 115.9 (C-4); 112.8 (C-3); 125.1 (Cα); 131.0 (Cβ). MS-EI *m/z* (%) = 304.

2.6. Cytotoxic activity

The cytotoxicity of the compounds was determined using the MTT assay.[26] Briefly, cells were seeded in 96-well plates 7.0 x 10^3 cells/well for adherent cells and 3.0 x 10^4 cells/well for suspended cells, respectively. The compounds dissolved in DMSO were added to each well using HTS (High-Throughput Screening) with a Biomek 3000 (Beckman Coulter, Inc. Fullerton, California, USA) and incubated for three days (72 h). The control group received the same amount of vehicle, and the final concentration of DMSO in the culture media was constant (below 0.5 %). After incubation, the supernatant was replaced by fresh medium containing MTT (0.5 mg.mL^{-1}). Three hours later, the MTT formazan product was dissolved in 150 µL of DMSO, and the absorbance was measured at 595 nm (DTX 880 Multimode Detector, Beckman Coulter, Inc. Fullerton, California, USA).To carry out a preliminary structure–activity relationship study, all compounds were tested against HCT-116 cells at single concentration (10 µM) to determine the percentage of reduction in cell viability (% RCV) after 72 h of incubation. In this study, doxorubicin (Sigma-Aldrich) was used as positive control. The data were obtained from at least two independent experiments performed in triplicate and calculated by GraphPad Prism *Software* (version 6).

2.7. Fungal strains

Strains belonging to the fungal collection of *Candida* spp. (*C. albicans* LABMIC 0105/ LABMIC0107 and *C. tropicalis* LABMIC 0110/0111) were considered, which belongs to patients cases from the holy house of mercy from Sobral and the collection of fungal culture of the Department of Mycology of the University Mycology of Recife (URM) under the number of opinion 644.365 and report date 04/23/2014.

2.8. Inoculum preparation for antifungal susceptibility tests

Considering the microdilution methodology in broth used, the standard inoculum for *Candida* spp. (2.5 to 5 x 10^3 CFU mL^{-1}) were prepared according to turbidity indication of the McFarland scale. Initially, *Candida* spp. was cultivated in Sabouraud Dextrose Agar (SDA) at room temperature and the growth of both could be observed after 48 h after the peaking. For the inoculum, 9 mL of sterile saline solution (0.85 %) was added to the cultures and, afterwards, a light smear was carried out with the aid of the chromium-nickel handle, aiming at the release of blastoconidia of *Candida* spp.The suspensions were diluted 1:2000 for *Candida* spp. in Roswell Park Memorial Institute 1640 (RPMI 1640) to allow inoculation of 2.5 to 5 x 10^3 CFU/mL^{-1}.

2.9. Antifungal susceptibility testing by the broth microdiluition broth

For this assay, the minimum inhibitory concentration (MIC) for *Candida* spp. according to standards established and described by M27-A3 of the Clinical and Laboratory Standards Institute (CLSI). It was weighed 10 mg of each sample in analytical balance and 1 mL of dimethylsufoxide (DMSO) was added to each eppendorf with chalcone followed by vortex mixer homogenization for ten minutes. The standard drug used is Amphotericin B (AMB) with concentration ranging from 0,5 to 16 µg/mL for *Candida* spp. The sensitivity test was performed in a 96-well microplate, where the graded growth had concentrations ranging from 0.002 to 2.5 mg/mL, the standard drug in the last column was serially diluted causing its concentration to range from 0.5 to 16 mg/mL for AMB, positive control evaluated the quality of the culture medium and negative control evaluated the possible contamination of the plaque or not. The microplates were incubated in oven at 37 °C and read after 48h. The MIC was established as the lowest concentration the sample could assume in order to block microbial development, while the minimum fungicidal concentration (MFC) was established as the lowest concentration of the sample in which it results in no microbial growth.[23]

2.10. Microdilution checkerboard assay

The tests performed with *Candida* spp. were followed by the chessboard technique to determine the combined effect between chalcone and a standard antifungal. To obtain these necessary results, first, consider the values found for the Minimum Inhibitory Concentration (MIC) corresponding to chalcone, isolated and combined with antifungal, and antifungal, isolated and combined with chalcone. Then, the Fungicide Inhibitory Concentration (FIC) is measured through the quotient between isolated MIC and combined MIC of chalcone and antifungal.

At where

A = Chalcone
B = AMB

FIC (A) = MIC of agent (A) in combination/MIC of agent (A) alone

FIC (B) = MIC of agent (B) in combination/MIC of agent (B) alone

The IFIC, in turn, is defined according to the addition of the values corresponding to the Fungicide Inhibitory Concentration (FIC) of each of the compounds considered in the assay.

$$IFIC = FIC (A) + FIC (B)$$

The turbidity of the fungal suspensions was adjusted to McFarland standard (10^5 CFU/mL). In the solutions, the samples tested were used in the concentrations of their respective MICs. Initially, 50 µl RPMI 1640 medium was added to all 96 wells of the microdilution plate. Then 50 µL chalcone was added to the first column, where serial dilutions were performed on the plate to the eighth column, with sample concentrations varying from 0.002 to 2.5 mg/mL. In the vertical lines, 50 µL of the standard AMB was placed in concentrations ranging from 0.5 to 16 µg/mL and finally, 100 µL of inoculum was added to all wells. RPMI 1640 medium with inoculum was used as positive control, while wells containing only RPMI 1640 were considered as negative control. The microplates were incubated at 37 ºC and read visually after 48h.[24]

2.11. Time-kill assay

Cells of *C. albicans* were inoculated in sterile saline solution (0.85 %) and then a 5 µL aliquot was added to RPMI 1640 culture medium. A treatment containing chalcone 2x MIC was prepared on the microplate according to the treatment containing AMB and a third control without treatment were selected in which all three mentioned had the presence of RPMI 1640 and fungal inoculum (~ $1x10^5$ UFC mL^1). Aliquots of 100 µL were withdrawn from both treatments and untreated at predetermined times (0, 2, 4, 6, 8, 10, 12 and 24h), and serially in eppendorfs containing 900 µL of sterile saline solution to obtain dilutions 10^{-1}, 10^{-2} and 10^{-3}. Finally, aliquots of 10 µL were withdrawn from the dilutions of each treatment and not treated for their respective Petri dishes containing Saboraud Dextrose Agar (SDA) and induced to fungal incubator at 34 °C for 24h. [25]

3. Results and Discussion

A series of chalcones were easily synthetized by the condensation reaction of the Claisen-Schmidt in basic medium,[22] being eight chalcones, between acetophenone derivatives and appropriate aryl aldehyde (Scheme 1). According to the product formed, the base acting as a catalyst removes an α hydrogen from the 2-hydroxy-3,4,6trimethoxyacetophenone, transforming it into a nucleophile, which then reacts with the carbonyl carbon of the aldehyde that acts as an electrophile. The reaction precedes the release of a water molecule and formation of the chalcone with the α, β-unsaturated conjugate system. The reaction yield ranges from 35.4 - 78.1 %. The variation in yield is dependent on the nature of the group attached to ring B. The presence of electron-withdrawing groups leads to greater yield. The presence of electron donor groups in the ring produces a lower yield.

The structures were determined by NMR, IR and mass spectra. The ^{1}H NMR spectra of the chalcones synthesized showed signals between δ_H 13.94 - 13.99 ppm referring to the chemical displacements of the hydrogens of each OH group, and other three signals between δ_H 3.84 - 3.96, 3.96 - 3.97 and 3.84 - 3.98 ppm relative to the hydrogens of methoxy groups MeO-3, MeO-4 and MeO-6, respectively. The signals between δ_H 7.75 and 7.94 ppm (J = 15.6 Hz) were attributed to doublets referring to α,β unsaturated hydrogens, whose coupling constant (J) confirms the stereochemistry *E*. The singlets observed between δ_H 6.01 - 6.07 ppm refer to hydrogen attached to the carbon 5'-carbon of ring A. In the ^{13}C-NMR spectrum of the chalcones synthesized it was possible to observe a signal concerning α, β unsaturated at δ_C 192.7 to 193.5 ppm. The ketone absorbs at δ_C 203.8 ppm, however, the presence of α, β unsaturation causes a displacement to high field and the probable cause is the delocalization of charge by the benzene ring or by the double bond that makes carbonyl carbon less electron deficient. The olefinic carbons α and β are observed between 127.4 and 142.8 ppm, respectively. The signals between δ_C 56.2 - 60.90 ppm refer to the carbons of the methoxy groups.

The structures of the synthesized chalcones were also confirmed through the analysis of the mass spectra in a fragmentation proposal, whose ions formed come from an α segmentation, which from there, there is the loss of a CO molecule by inductive segmentation, generating a second fragment ion. The mass spectra revealed peaks of the M^+ molecular ion. Justifying the molecular formulas of the synthesized chalcones, in addition to the base peak characteristic of the general process of fragmentation of chalcones. The data were compared with the literature of chalcones of similar structures (Figure 2).

The figure 3 shows the % RCV of the investigated compounds whose data were used for a preliminary structure–activity relationship analysis. The results showed that the % RCV varied from 19.43 ± 1.31 to 75.51 ± 1.84 %, revealing that small modifications to the chalcone chemical structure were able to alter the biological activity. Careful analysis of the acquired cytotoxicity data revealed that compound **4** demonstrated the strongest activity against HCT-116 cells (% RCV = 75.51 ± 1.84). The strong cytotoxic effect of **4**

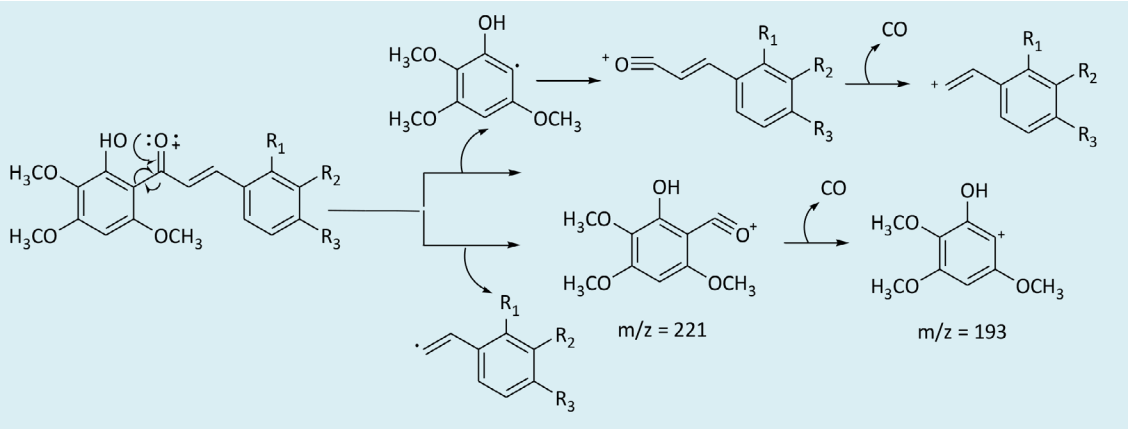


Figure 2. General fragmentation mechanism justifying the fundamental peaks of the synthesized chalcones

could be attributed to the combined effect of the presence in ring A of electron donor methoxy and hydroxy groups and the electron withdrawing fluorine atom present in para position of ring B. The presence of an extended conjugated system reduces the cytotoxic effect which can be observed by the comparison between compounds **1** (% RCV = 54.10 ± 0·20) and **7** (% RCV = 41.08 ± 0·20)· Another important structural feature that promoted the increase of cytotoxic activity was the presence of electronic withdrawal groups in ring B and the substitution of ring B by a heterocyclic moiety of the compounds demonstrated by the moderate activity of compounds **2** (% RCV = 44.83 ± 2·60), **3** (% RCV = 47.76 ± 2·60), **5** (% RCV = 62.94 ± 2·60) and **6** (% RCV = 19.43 ± 2·60)· On the other hand, the presence of electron donor methoxy groups in ring B promoted the reduction of cytotoxic activity demonstrated by the weak activity of compound **8** (% RCV = 57.26 ± 2·60)·

The *in vitro* antifungal potential of the chalcones is detailed in Table 1. The results showed that chalcone **8** (MIC 0,625 mg/mL against *C. albicans* LABMIC 0105) and **6** (MIC 0,312 mg/mL against *C. albicans* LABMIC 0107) were considered as chalcones of better fungal inhibition, whereas Amphotericin B showed MIC (1 µg/mL) of inhibition previously expected for all tests.

The synergistic evaluation *in vitro* (Table 2) showed that the union between

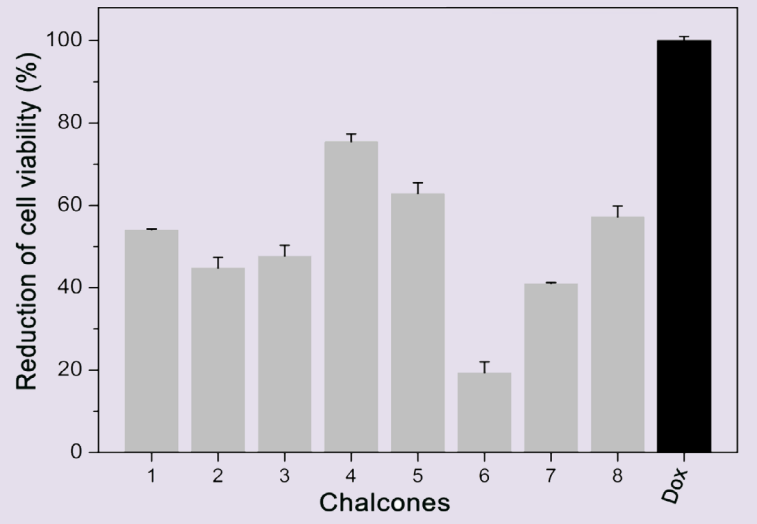


Figure 3. Cytotoxicity of the compounds (1-8) at a single concentration (10 µM) against HCT-116 (human colon carcinoma) cells after 72 h of incubation using MTT assay. Data are presented as percentage of Reduction of cell viability (%) RCV ± SEM) obtained from at least two independent experiments performed in triplicate. Doxorubicin (Dox, 1 µM) was used as the positive control

Table 1. MIC and MFC of chalcones against C. albicans (LABMIC 0105/0107) and C. tropicalis (LABMIC 0110/0111)

Chalcones	*C. albicans* LABMIC 0105		*C. albicans* LABMIC 0107		*C. tropicalis* LABMIC 0110		*C. tropicalis* LABMIC 0111	
	MIC[a]	MFC[b]	MIC	MFC	MIC	MFC	MIC	MFC
1	2.5	>2.5	2.5	>2.5	2.5	>2.5	1.25	2.5
2	1.25	2.5	1.25	2.5	2.5	>2.5	1.25	2.5
3	2.5	>2.5	2.5	>2.5	1.25	2.5	1.25	2.5
4	1.25	2.5	1.25	2.5	2.5	>2.5	1.25	2.5
5	1.25	2.5	2.5	>2.5	1.25	2.5	2.5	>2.5
6	0.62	1.25	1.25	1.25	1.25	2.5	1.25	2.5
7	2.5	>2.5	2.5	>2.5	1.25	2.5	2.5	>2.5
8	1.25	2.5	0.31	0.62	2.5	>2.5	1.25	2.5
Amphotericin B MIC (µg/mL)	1	1	1	1				

[a]mg/mL; [b]µg/mL

Amphotericin B and chalcone **6** showed an synergistic effect against *C. albicans* LABMIC 0105 (IFIC = 0.124) and indifferent effect against *C. albicans* LABMIC 0107 (IFIC = 1.0072), on the other hand the chalcone **8** showed an indifferent effect against *C. albicans* LABMIC 0105 (IFIC = 2) and indifferent effect against *C. albicans* LABMIC 0107 (IFIC = 1).

The fungal death curve showed that chalcones **6** were tested at 2x MIC concentration. For chalcone **6** the scope of substantial effect was possible because the compound had a fungicidal effect partially similar to the Amphotericin B, both slowly reducing the number of fungal CFUs of the *C. albicans* strain LABMIC 0105 in the first four hours of treatment with subsequent reduction between the period of 4 and 8 hours and about 100 % of effectiveness from this time (Figure 4).

4. Conclusions

Eight chalcones were synthesized from natural isolated acetophenone from *Croton anisodontus*. Regarding the cytotoxic activity the chalcone (*E*)-3-(4-fluorophenyl)-1-(2-hydroxy-3,4,6-trimethoxyphenyl)prop-2-en-1-one (**4**) containing the methoxy and hydroxy groups on ring A and a fluorine atom on ring B showed strong activity against HCT-116 cells tumor cell. The *in vitro* antifungal potential of the chalcones showed that chalcones (*E*)-3-(furan-2-yl)-1-(2-hydroxy-3,4,6-trimethoxyphenyl) prop-2-en-1-one (**8**) (*E*)-1-(2-hydroxy-3,4,6-trimethoxyphenyl)-3-(4-methoxyphenyl)prop-2-en-1-one (**6**) were considered as chalcones of better fungal inhibition against *C. albicans* LABMIC 0105 and LABMIC 0107, respectively. The synergistic evaluation *in vitro* showed that the union between Amphotericin B and chalcone **6** showed an synergistic effect against *C. albicans* LABMIC 0105 (IFIC = 0.124) and indifferent effect against *C. albicans* LABMIC 0107 (IFIC = 1.0072), for kinetics of fungal death only treatment with chalcone **6** was able to promote reduction of fungal cells between the periods of 4 to 8 h and 100 % inhibition thereafter, resembling the mechanism of action of Amphotericin B.

Table 2. Synergistic potential of chalcones 6 and 8 against strains of C. albicans (LABMIC 0105/ LABMIC 0107)

Combinations	*C. albicans* LABMIC 0105			*C. albicans* LABMIC 0107		
	MIC (mg/mL)		IFIC	MIC (mg/mL)		IFIC
	Individual	Combined		Individual	Combined	
Chalcone 6	0.62	0.077	0.124	1.25	0.009	1.0072
Amphotericin B	2	1		2	0.05	
Chalcone 8	1.25	2.5	2	0.31	0.31	1
Amphotericin B	1	0.5		1	1	

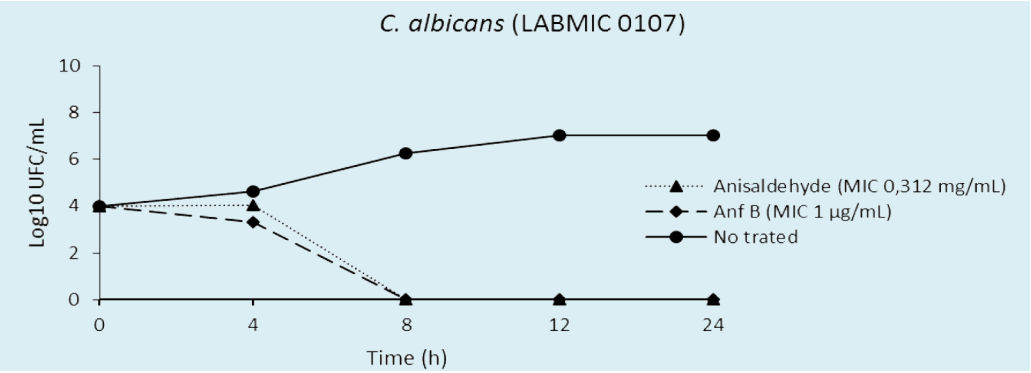


Figure 4. Time curve of *C. albicans* LABMIC 0107 treated with chalcone 6 and Amphotericin B MICs

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