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Phebalosin and its Structural Modifications are Active against the Pathogenic Fungal Causing Paracoccidioidomycosis

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Abstract

The phebalosin was isolated from hexane extract of the *Polygala paniculata* (Polygalaceae). The structural modifications of phebalosin were performed on the epoxy group with different nucleophiles such as H_2O, ethoxy, methoxy, isopropoxy and *n*-butoxy for the corresponding derivatives and with acetic anhydride and acetone to give the diacethyl and cyclic acetal derivatives. The phebalosin and their derivatives were tested against four isolates of the pathogenic fungus *Paracoccidioides brasiliensis*. In this work phebalosin showed promised antifungal activity against isolates of *P. brasiliensis* with MIC value of 31.2 and 62.5 µg/ml. The compound 3 presented the better activity with MIC value of 1.9 µg/ml gainst the isolate *P. brasiliensis* Pb03. Besides, it was used methods of theoretical Chemistry, and chemometrics analysis techniques to perform a SAR study. The compounds activity is due to both types of properties – electronic and structural ones. Overall, these data open new possibilities for the potential use of phebalosin and its structural modifications as antifungal.

Keywords: Phebalosin; Paracoccidioidomycosis; antifungal; *Polygala paniculata;* Molecular modeling; Chemometrics

Introduction

Coumarins are a vast 1,2-benzopyrone group of natural compounds essentially found in green plants. The substitutions into their basic skeleton 1,2-benzopyrone can occur at any of the six available sites providing them extremely variable structures [1]. Although no longer used as food flavouring, coumarin is present in certain tobaccos and alcoholic beverages and is used in various soup, detergent and cosmetic preparations [2].

Hydroxy derivatives of 4-methyl coumarin are important group of coumarin derivatives showing medicinal as well as other biotechnological applications. For example, 5,7-dihydroxy 4-methyl coumarin and 7,8-dihydroxy 4-methyl coumarins are useful precursors to synthesize respective diacetoxy and hydroxyl-amino derivatives of 4-methyl coumarin, which are known to be good antioxidants having excellent radical scavenging properties [3].

Among the various coumarin derivatives, 7-substituted coumarins are important groups showing various kinds of bioactivities and also other applications. For example, 7-hydroxy 4-methyl coumarin is used in fluorometric determination of enzymatic activity, as a starting material for the preparation of insecticides and furano coumarins [4]. Due to their inherent photochemical characteristics, reasonable stability, good solubility and relative ease of synthesis, coumarin derivatives have been extensively investigated for electronic and photonic applications [5].

In this present work we screened the coumarin phebalosin and seven phebalosin -derivatives against four clinical isolates of the pathogenic dimorphic fungus *Paracoccidioides brasiliensis,* the causing agent of Paracoccidioidomycosis (PMC). PCM is the most prevalent systemic endemic mycosis in South America with most reported cases in Brazil [6]. In the absence of drug therapy the disease is usually fatal. The treatment of PCM is usually long, with many patients receiving therapy for one to two years or even more. It appears that the number of drugs active against PCM is scarce [7]. Although azoles and other drugs can arrest the progression of PCM, the fibrosis sequelae persist, probably constituting a source of fungi that could lead to a relapse in the disease following termination of treatment [8,9]. The strong toxicity of amphotericin B makes the effective management of this severe disease difficult [10]. For this reason the discovery of new drugs for the treatment of PMC is very important.

Materials and Methods

Chemistry

^1H and ^{13}C NMR spectra were measured using a Varian 400 MHz spectrometer. A solvent used for NMR measurements was TMS- $CDCl_3$. IR spectra were recorded with a Perkin-Elmer FT 16PC spectrometer (KBr pellets). Mass spectra were taken with a Shimadzu QP 5050A at 70 eV. TLC: Merck Kieselgel 60 F_{254}, spots were visualized under UV at 254 and 360 nm.

Plant material

Polygala paniculata was collected in March 2004 on Daniela beach, Florianópolis, Santa Catarina State, Brazil. The specimen was identified by a botanist, Prof. Dr. Olavo Araújo Guimarães, and the voucher specimen number 26027 was deposited at the Herbarium of the Botany Department of Universidade Federal do Paraná (Curitiba, PR, Brazil).

Extraction and isolation

The dried whole plant (3500 g) was extracted with hexane twice, each for 24 hours at room temperature. The extract was partially concentrated *in vacuum* with controlled temperature observing the

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precipitation of a white amorphous solid. The solid was separated from the supernatant solution followed by successive washing with hexane, yielding 2 g of epoxy coumarin, phebalosin (1).

Structural modification

Epoxy Ring Hydrolysis of the phebalosin (2): For ring epoxy hydrolysis of phebalosin (1), 100 mg was dissolved in dichloromethane (CH_2Cl_2), 10 mlof distilled water, 1 mlof chloridric acid (HCl) and 5 g of silica gel (catalyst). The mixture was subjected to magnetic agitation for 72 h and temperature control (50ºC). After this period, the reaction mixture was extracted with CH_2Cl_2, dried with anhydrous sodium sulfate (Na_2SO_4), filtered and the organic solvent was concentrated. The crude product was purified by chromatography on a flash column (230-400 mesh) with hexane/ethyl acetate 10:90 to yield 19% as a white solid.

MP: 135-136°C; IR (cm^{-1}): 3384, 2965, 1715, 1603, 1494, 1255.

^{1}H NMR ($CDCl_3$, 400 MHz): 1.77 (3H, s, CH_3-5'), 3.97 (3H, s, CH_3O-C7), 4.53 (1H, d, *J* = 8.4 Hz, H-2'), 4.59 (1H, s, H-4'), 4.65 (1H, s, H-4'), 5.31 (1H, d, H-1'), 6.26 (1H, d, *J* = 9.6 Hz, H-3), 6.88 (1H, d, *J* = 8.8 Hz, H-6), 7.40 (1H, d, *J* = 8.8 Hz, H-5), 7.63 (1H, d, *J* = 9.6 Hz, H-4).

Epoxy Ring Ethanolysis of phebalosin (3): The same method of (2) was used for ethanolysis with the substitution of the solvent (water by ethanol). The crude product was purified by chromatography on a flash column (230-400 mesh) with hexane/ethyl acetate 40:60 to yield 26% as a white solid.

MP: 130-131°C; IR (cm^{-1}): 3473, 2925, 1728, 1603, 1453, 1251.

^{1}H NMR ($CDCl_3$, 400 MHz): 1.18 (3H, t, CH_3-CH_2O-C2'), 1.69 (3H, s, CH_3-5'), 3.44 (2H, m, CH_3-CH_2-O-C2'), 3.92 (3H, s, CH_3O-C7), 4.63 (1H, s, H-4'), 4.69 (1H, s,H-4'), 4.91 (1H, d, H-2'), 5.14 (1H, d, *J* = 7.2 Hz, H-1'), 6.26 (1H, d, *J* = 9.6 Hz, H-3), 6.86 (1H, d, *J* = 7.0 Hz, H-6), 7.40 (1H, H-5), 7.87 (1H, d, *J* = 9.6 Hz, H-4).

Epoxy ring opening of phebalosin with sodium methoxy (4)

For this reaction, 20 mg of metallic sodium was added to 15 ml of dry methanol. In this solution, 100 mg phebalosin was added and the reaction was terminated after 15 minutes. The reaction medium was acidified with methanol-chloridric acid to pH 2-3. The reaction mixture was extracted with (CH_2Cl_2), dried with anhydrous sodium sulfate (Na_2SO_4), filtered and the organic solvent was concentrated in a vacuum desiccator. The crude product was purified by chromatography on a flash column (230-400 mesh) with hexane/ethyl acetate 50:50 to yield 71% as white solid.

MP: 140-142°C; IR (cm^{-1}): 3474, 2929, 1728, 1605, 1489, 1249.

^{1}H NMR ($CDCl_3$, 400 MHz): 1.59 (3H, s, CH_3-5'), 3.25 (3H, s, CH_3O-C2'), 3.93 (3H, s, CH_3O-C7), 4.51 (1H, d, *J* = 2.0 Hz, H-2'), 4.65 (1H, s, H-1'), 4.87 (1H, s, H-4'), 5.02 (1H, d, H-4'), 6.24 (1H, d, *J* = 9.6 Hz, H-3), 7.05 (1H, d, *J* = 8.8 Hz, H-6), 7.57 (1H, d, *J* = 8.8 Hz, H-5), 7.87 (1H, d, *J* = 9.6 Hz, H-4).

Epoxy ring opening of phebalosin with sodium isopropoxy (5)

For this reaction, the same method of (4) was used with the appropriate nucleophile isoproxy to yield 44% of the product as white solid.

MP: 101-103°C, IR (cm^{-1}): 3557, 2968, 1728, 1604, 1454, 1368.

^{1}H NMR ($CDCl_3$, 400 MHz): 1.01 (3H, d, C2'-OCH-(CH_3)$_2$), 1.24 (3H, d, C2'-CH-(CH_3)$_2$), 1.70 (3H, s, CH_3-5'), 3.54 (1H, m, C2'-O-CH-(CH_3)$_2$), 3.93 (3H, s, CH_3O-C7), 3.86 (1H, s, H-4'), 3.97 (1H, s, H-4'), 4.65 (1H, d, *J* = 8.4 Hz, H-2'), 4.83 (1H, d, *J* = 8.4 Hz, H-1'), 6.26 (1H, d, *J* = 9.6 Hz, H-3), 6.86 (1H, d, *J* = 8.8 Hz, H-6), 7.39 (1H, d, *J* = 8.8 Hz, H-5), 7.62 (1H, d, *J* = 9.6 Hz, H-4).

Epoxy ring opening of phebalosin with sodium n-butoxy (6)

The reaction was carried out using the same procedure as in (4) using t-butoxy a nuccleofilic reagent, to yield 38% of the product like a white solid.

MP: 97-99°C, IR (cm^{-1}): 3435, 2955, 1723, 1605, 1443, 1250.

^{1}H NMR ($CDCl_3$, 400 MHz): 0.83 (3H, t, C2'-O(CH_2)$_3$-CH_3), 1.31 e 1.53 (2H each, m, C2'-OCH_2-(CH_2)$_2$-CH_3), 1.71 (3H, s, CH_3-5'), 3.33 (2H, t, C2'-O-CH_2-(CH_2)$_2$$CH_3$), 3.93 (3H, s, CH_3O-C7), 4.62 (1H, s, H-4'), 4.69 (1H, s, H-4'), 4.92 (1H, d, *J* = 8.8 Hz, H-2'), 5.14 (1H, d, *J* = 8.8 Hz, H-1'), 6.26 (1H, d, *J* = 9.2 Hz, H-3), 6.99 (1H, d, *J* = 8.8 Hz, H-6), 7.45 (1H, d, *J* = 8.8 Hz, H-5), 7.67 (1H, d, *J* = 9.2 Hz, H-4).

Obtaining cyclic acetal of phebalosin with acetone (7)

The acetalization reaction of phebalosin (1) was performed in dry acetone (100 mg) by dissolution of the 100 mg of phebalosin with addition 1 ml of chloridric acid (HCl) and 5 g of silica gel (catalyst). The mixture was subjected to magnetic agitation for 72 h and temperature control (50ºC). After this period, the reaction mixture was extracted with (CH_2Cl_2), dried with anhydrous sodium sulfate (Na_2SO_4), filtered and the organic solvent was concentrated. The crude product was purified by chromatography on a flash column (230-400 mesh) with hexane/ethyl acetate 40:60 to yield 70% as white solid.

MP: 111-112°C; IR (cm^{-1}): 2980, 1724, 1603, 1488, 1375, 1245.

^{1}H NMR ($CDCl_3$, 400 MHz): 1.54 (3H, s, CH_3-8'), 1.70 (3H, s, CH_3-7'), 1.74 (3H, s, CH_3-5'), 3.93 (3H, s, CH_3O-C7), 4.85 (1H, s, H-4'), 4.96 (1H, s, H-4'), 5.02 (1H, d, *J* = 9.6 Hz, H-2'), 5.57 (1H, d, *J* = 9.6 Hz, H-1'), 6.26 (1H, d, *J* = 8.8 Hz, H-3), 6.87 (1H, d, *J* = 8.0 Hz, H-6), 7.41 (1H, d, *J* = 8.0 Hz, H-5), 7.61 (1H, d, *J* = 8.8 Hz, H-4).

Acetilation of phebalosin (8)

The acetylation of phebalosin (1) (100 mg) with acetic anhydride (2 ml), acetic acid (1 ml), catalyzed by 4-(dimethylamino)pyridine (DMAP) was dissolved in ethyl acetate (3 ml). The mixture was submitted to magnetic agitation for 72 h. After the reaction the solvent is evaporated in vacuum desiccator. The crude product was purified by chromatography on a flash column (230-400 mesh) with hexane/ethyl acetate 50:50 to yield 65% as white solid.

MP: 120-121°C; IR (cm^{-1}): 2943, 1741, 1606, 1494, 1244.

^{1}H NMR ($CDCl_3$, 400 MHz): 1.48 (3H, s, CH_3-5'), 1.87 (3H, s, CH_3-CO), 1.92 (3H, s, CH_3-CO), 3.82 (3H, s, CH_3O-C7), 4.57 (1H, s, H-4'), 4.76 (1H, s, H-4'), 5.98 (1H, d, *J* = 12.0 Hz, H-2'), 6.08 (1H, d, *J* = 12.0 Hz, H-1'), 6.57 (1H, d, *J* = 8.0 Hz, H-3), 6.76 (1H, d, *J* = 8.0 Hz, H-6), 7.33 (1H, d, *J* = 8.0 Hz, H-5), 7.54 (1H, d, *J* = 8.0 Hz, H-4).

Paracoccidioides brasiliensis strains

Four clinical isolates of *P. brasiliensis*, Pb18 (from the fungal collection of the Faculty of Medicine of the Universidade de São Paulo, São Paulo, SP, Brazil), Pb03 (clinical isolates from chronic PCM, São Paulo, Brazil– MHH Forjaz / TIE Svidzinski*)*, Pb01 (ATCC- MYA-826) and Pb339 (ATCC 32069), were used in the biological assays.

Inoculum preparation

The strains were maintained by weekly passage in solid Yeast-

Peptone-Dextrose medium, at 37ºC and were used after 7-10 days of fungus growth. Yeast cells in the exponential phase were collected aseptically with a platinum loop and re-suspended in a tube containing 5 ml of sterile saline. If large aggregates existed, they were allowed to settle for several minutes, and the supernatants were collected. The suspensions were then diluted in synthetic RPMI medium (Sigma, St. Louis, MO, USA) with L-glutamine buffered to pH 7.0 with 0.165 morpholine propanesulfonic acid (MOPS, Sigma), and prepared according to the CLSI document M 27-A_2 [11] to obtain a final inoculum size suitable for the strains [12]. After homogenization by vortexing, transmittance was measured at 520 nm and adjusted to 69 to 70% [13].

Susceptibility test

The compounds were dissolved in dimethylsulfoxide (DMSO). Serial dilutions were then performed, using RPMI as a diluent, maintaining a constant volume of 1 ml per tube. The extracts were tested at eight different concentrations that ranged from 500-1.9 µg/ml Volumes of 100 µl of each dilution were distributed in sterile flat-bottom 96-well microplates (Difco Laboratories, Detroit, MI, USA).

Susceptibility was determined by the broth microdilution method. Broth microdilution testing was performed in accordance with the guidelines in the CLSI M27-A_2 document [11] and Nakai *et al.* [12] RPMI medium was used without compounds or solvents as a control for growth and sterility. Solvent DMSO at the same volumes used in the assay was used as control for toxicity. Amphotericin B (Sigma, St Louis, USA) (25 to 0.03 µg/ml and (25 to 0.03 µg/ml in DMSO was included as positive antifungal control with the stock solutions prepared in DMSO and water, respectively. After inoculation of fungal strains the plates were incubated at 37°C for 14 days. The tests were performed in triplicate in at last two independent experiments. The endpoints were determined visually by comparison with the drug-free growth control well. MICs were expressed in µg/ml and defined as the lowest compound concentration for which the well was optically clear. All assays were performed in triplicate and repeated at least once.

Theoretical Chemistry methodology and chemometrics

Structural and electronic properties were obtained from classical mechanics [14] calculations (AMBER force field, at 298 K, in a vacuum) implemented in HYPERCHEM 7.5 molecular modelling program [15] and HF/6-31G** electronic structure calculations (*ab initio* method of Quantum Chemistry [16], 298 K, in a vacuum), implemented in GAUSSIAN program [17]. The obtained data were compared and correlated with antifungal activity by using chemometrics analysis [18]: Hierarchical Clustering Analysis (HCA), Partial Least Squares (PLS) and Principal Component Analysis (PCA) methods by using MINITAB 15® program [19].

Results

Whole *P. paniculata* plants were extracted with hexane to afford the coumarin phebalosin as a white amorphous solid, m.p. 124°C (Lit. 121-123°C); Rf=0.39 (eluent 60:40 hexane/ethyl acetate) and was identified by spectroscopic analysis and comparison with literature data [20]. A study of the reactivity of phebalosin (1) was planned, preparing a series of derivatives involving the opening epoxide ring by nucleophilic substitution reactions using different reagents, acetylation and formation of the cyclic acetal as shown in Figure 1.

The phebalosin (1) was treated with water and silica gel under

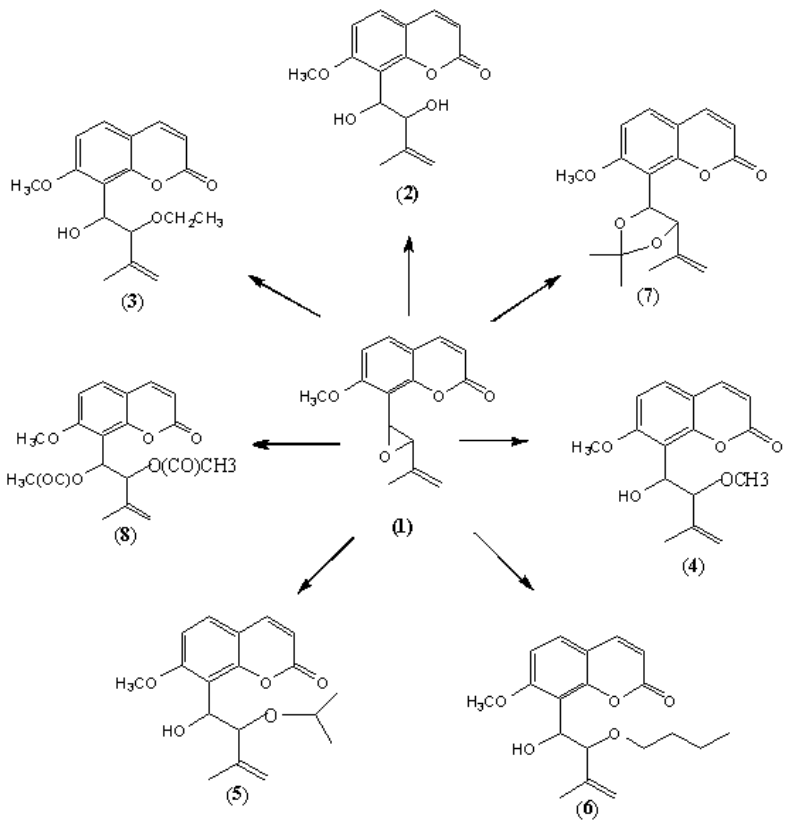

Figure 1: Structural modifications of phebalosin (1), reaction of phebalosin with H_2O (2), ethanol (3), methoxy sodium (4), isopropoxy sodium (5) and *n*-butoxy sodium (6), acetone (7), and acetic anhydride (8).

agitation being partially converted to a dihydroxy derivative 3 (19%) which presented itself in the form of a white solid with MP 135-136°C (Lit. 135-136°C; [18] Rf=0.38 (eluent ethyl acetate 100%). The spectrum in region IR in KBr pellets showed the appearance of intense hydroxyl absorption at υ 3384 cm^{-1}, showing the opening of the epoxide ring, while it maintained the other absorption characteristics of coumarin. The ^1H NMR spectrum of the reaction product kept the absorption characteristics of the coumarin nucleus: Halfway prenyl, the displacement of hydrogen atoms H-1' and H-2' of 3.92 ppm and 3.99 ppm (epoxy) to 5.31 ppm (1H, d) and 4.53 ppm (1H, d, J = 8.4 Hz) respectively was observed due to the formation of a diol derivative.

In the treatment with ethanol and silica gel under agitation, phebalosin was converted to a 1'-hydroxy-2'-ethoxy derivative (26%) which was presented in the form of white solid, with MP 130-131°C, with Rf=0.32 (eluent hexane/ethyl acetate 50:50). The spectrum in region IR in KBr pellets showed the appearance of intense hydroxyl absorption at υ 3473 cm^{-1}, showing the opening of the epoxide ring, and the other absorption characteristics of coumarin. The ^1H NMR spectrum of the reaction product kept the absorption characteristics of the coumarin nucleus. Halfway prenyl, we observed the displacement of hydrogen atoms H-1' and H-2' of 3.92 and 3.99 ppm (epoxide) to 5.14 ppm (1H, d, J = 7.20 Hz) and 4.91 ppm (1H, d) respectively, due to the formation of the ethoxy derivative; furthermore was observed an additional spin system to ethoxy group at 1.18 ppm (3H, t) and 3.44 ppm (2H, m).

The phebalosin react quickly with sodium methoxy to give 1'-hydroxy-2'-methoxy derivative 4 (71%) as a white solid, with MP 140-142°C, and Rf=0.24 (eluent hexane/ethyl acetate 50:50). The spectrum in the IR region in KBr pellets showed the appearance of intense hydroxyl absorption at υ 3476 cm^{-1}, showing the opening of the epoxide ring, keeping the other absorption characteristics of coumarin. The ^1H NMR spectrum of the reaction product kept the absorption characteristics of the coumarin nucleus. Halfway prenyl, the displacement of hydrogen atoms H-1' and H-2' of 3.92 and 3.99 ppm (epoxide) to 4.65 ppm (1H, d, J = 2.0 Hz) and 4.51 ppm (1H, d, J = 2.0 Hz) respectively was observed, due to the formation of the methoxy derivative. Additionally, the ^1H NMR spectrum recorded the appearance of a absorption at 3.25 ppm (3H, s) to additional methoxy group.

The reaction with sodium isopropoxy afforded 1'-hydroxy-2'-isopropoxy derivative 5 (44%) presented in the form of a white solid, with MP 101-103°C, and Rf=0.48 (eluent hexane/ethyl acetate 50:50). The spectrum in the IR region in KBr pellets showed the appearance of hydroxyl absorption at υ 3557 cm^{-1}, showing the opening of the epoxide ring, keeping other absorption characteristics of coumarin. The ^1H NMR spectrum of the reaction product kept the absorption characteristics of the coumarin nucleus. Halfway prenyl, we observed the displacement of hydrogen atoms H-1' and H-2' of 3.92 and 3.99 ppm (epoxide) to 4.83 ppm (1H, d, J = 8.4 Hz) and 4.65 ppm (1H, d, J = 8.4 Hz) respectively, due to the opening of the ring and the appearance an additional spin system at 1.01 ppm (3H, d), 1.24 ppm (3H, d) and 3.54 ppm (1H, m) to isopropoxy group inserted.

The reaction with sodium n-buthoxy gives the 1'-hydroxy-2'-n-buthoxy derivative 6 (38%). The compound was presented in the form of yellow solid, with MP 97-99°C, and Rf=0.52 (eluent hexane/ethyl acetate 50:50). The spectrum in the IR region in KBr pellets showed the appearance of hydroxyl absorption at υ 3435 cm^{-1}, showing the opening of the epoxide ring, keeping other absorption characteristics of coumarin. The ^1H NMR spectrum of the reaction product kept the absorption characteristics of the coumarin nucleus. Halfway prenyl, the displacement of hydrogen atoms H-1' and H-2' of 3.92 and 3.99 ppm (epoxide) to 5.14 ppm (1H, d, J = 8.8 Hz) and 4.92 ppm (1H, d, J = 8.8 Hz) was observed respectively. We also observed the appearance of the signals 0.82 ppm (3H, t), 1.31 ppm (2H, m), 1.53 ppm (2H, m) and 3.33 ppm (2H, t) of the n-butoxy group inserted.

The treatment of phebalosin with acetone and silica gel under agitation lead to formation of the respective cyclic derivative (20%) as a colorless crystal, with MP 111-112°C, with Rf=0.58 (eluent hexane/ethyl acetate 50:50). The spectrum in the IR region in KBr pellets showed the appearance of intense hydroxyl absorption at υ 2980 cm^{-1}, showing the opening of the epoxide ring, while it maintained the other absorption characteristics of coumarin. The NMR spectrum of the reaction product kept the absorption characteristics of the coumarin nucleus. Halfway prenyl, a pair of doublet absorbing at $\delta_{H1'}$ 5.57 and $\delta_{H2'}$ 5.02 ppm (1H each, d, J = 9.60 Hz) was displaced in relation to epoxy moiety. The singlets that absorb at 1.70 ppm and 1.74 ppm, with integration for three hydrogen atoms each, were assigned to the two methyl H-8' and H-7' of the acetal group.

Under acetylation with acetic acid and acetic anhydride under agitation, the phebalosin was partially converted to a diacetyl derivative 8 (65%) which presented itself in the form of solid white, with MP 120-121°C, with Rf=0.40 (eluent hexane/ethyl acetate 50:50). The spectrum in the IR region in KBr pellets showed no absorption of hydroxyl and retained all other characteristic absorptions of coumarin. The ^1H NMR spectrum of the reaction product kept the absorption characteristics of the coumarin nucleus. Halfway prenyl, we observed the displacement of hydrogen atoms H-1' and H-2' of 3.92 and 3.99 ppm (epoxide) to 6.08 ppm (1H, d, J = 12.0 Hz) and 5.99 ppm (1H, d, J = 12.0) respectively, due to the formation of acetyl derivative. The acetylation is confirmed by the presence of signals 1.87 ppm (3H, s) and 1.92 ppm (3H, s) relative to the acetyl groups.

Antifungal activity

In this work, the phebalosin was active against all isolates of *P. brasiliensis* tested (Table 1). The MIC values ranged from 31.2 to 500 µg/ml In relation to the phebalosin-derivative compounds, an increase of antifungal activity was observed for compounds 3, 5 and 6 against isolate Pb03 of *P. brasiliensis*. However, Pb18 did not present significant susceptibility against the compounds 7, 8 and 4. Isolate Pb03 was more susceptive to drugs tested but not for trimethoprim-sulfamethoxazole (300 µg/ml) in the tested conditions.

Molecular modelling and chemometrics

The coumarin phebalosin compound and its seven phebalosin derivatives had the optimization of their geometries realized through Classical Mechanics (AMBER force field, vacuum, 298 K) aimed at obtaining the structure with a minimum of energy. Then used the PM3 level of theory [15] (semiempirical quantum mechanics) at 298 K, vacuum, in order to determine the physico-chemical properties of interest (descriptors). All protocol for obtaining descriptors was held in the programmes HyperChem Professional 7 5 and GAUSSIAN 09. In this work, the following descriptors were determined through the protocol described in the previous section (Table 2): enthalpy of formation (ΔH_f^o), variation of energy between the frontier orbitals (the highest occupied energy - HOMO and lowest unoccupied energy - LUMO), $\Delta E_{HOMO\text{-}LUMO}$, lipophilicity parameter (log P), dipole moment (µ), polarizability, molecular volume, solvent accessible area (SA) and system energy (Hartree Fock energy, at 6-31G** level of theory). The values of activity were converted into log(1/a) from each antifungal analysis, where a = experimental activity.

Initially it was applied a hierarchical method agglomerative (HCA)

Compound	Pb18		Pb03		Pb01		Pb339	
	µg/mL	µmol/L	µg/mL	µmol/L	µg/mL	µmol/L	µg/mL	µmol/L
1	32.2	120	31.2	120.0	-	-	-	-
2	62.5	248.0	31.2	127.0	125.0	504.0	250.0	120.0
3	62.5	214.0	1.9	6.5	125.0	428.0	125.0	428.0
4	500	1582.0	250.0	791.0	-	-	-	-
5	≥ 500	≥ 2049.0	31.2	127.8	-	-	-	-
6	500	1724.0	62.5	215.5	-	-	-	-
7	62.5	196.5	7.8	24.5	125.0	393.0	125.0	393.0
8	31.2	93.9	15.6	46.9	125.0	376.0	62.5	188.2
Amphotericin B	0.06	0.06	0.02	0.02	0.06	0.06	0.06	0.06
Trimethoprim/ sulfamethoxazole	300	987.10/ 172.20	300.0	987.10/ 172.20	75.0	246.4/ 43.0	75.0	246.4/ 43.0

-: not determined;

Table 1: Antifungal activity of phebalosin and compounds derivatives against clinical isolates of *Paracoccidioides*.

compound	log ($1/c_{Pb18}$)	log ($1/c_{Pb03}$)	log ($1/c_{Pb01}$)	log ($1/c_{Pb339}$)	$\Delta E_{HOMO-LUMO}{}^{+}$ /eV	µ/D	LogP	polarizability $Å^3$	volume/ $Å^3$	SA/ $Å^2$	ΔHf kcal/mol
1	-1.494	-1.494	-1.796	-1.796	8.64	4.27	2.17	27.01	749.17	378.21	-53.39
2	-1.796	-1.494	-2.097	-2.398	8.47	8.59	1.45	28.33	788.71	389.21	-135.69
3	-1.796	-0.279	-2.097	-2.097	8.45	7.19	2.17	32.09	868.97	427.31	-135.74
4	-2.699	-1.796	-	-	8.30	7.42	1.73	30.17	844.10	422.51	-129.55
5	-1.796	-0.892	-2.097	-2.097	8.42	7.06	2.58	33.93	901.74	447.00	-135.16
6	-1.494	-1.193	-2.097	-1.796	8.30	7.47	2.93	35.67	1006.37	532.34	-143.46
7	-2.699	-2.398	-	-	8.39	6.99	2.75	33.06	897.09	447.40	-130.97
8	-2.699	-1.494	-	-	8.33	9.40	1.59	34.57	987.55	477.63	-201.72

* dipole moment
** Surface Area

Table 2: Antifungal activity (µg/ml) of phebalosin and their compounds derivatives against clinical isolates of *Paracoccidioides brasiliensis* and descriptors obtained from HF/6-31G** electronic structure calculations (*ab initio* method of Quantum Chemistry, 298 K, in a vacuum).

with all submissions, each forming its own grouping and by similarity, the final grouping of the clusters (also called final partition) which must, as expected, identify groups whose comments or variables have characteristics in common was determined. PCA and PLS techniques were performed in order to separate the compound as function of their antifungal activities.

Despite a few numbers of compounds and a low range of activity values observed, a chemometric analysis was performed: in a general sense, it seems that polarizability is the most important descriptor for antifungal activity against Pb03, Pb339, Pb18 and Pb01, respectively.

The results from PCA technique are showed in Figures 2-5. For Pb339 (Figure 2) the second PCA component (PC2) was responsible for compounds separation as function for antifungal activity (Cumulative eigenvalues: 75.8%, 97.5%):

PC1 = 0.378 Log($1/c_{Pb339}$) + 0.508 polarizability + 0.537 SA + 0.558 LogP

Minimal values of PC2 are corresponding to the best antifungal activities that mean: lower polarizability and SA values and higher lipophilicity character.

The first PCA component (PC1) separated the compounds (activity) because of two properties, the enthalpy of formation (ΔH_f^o) and the variation of energy between the frontier orbitals ($\Delta E_{HOMO-LUMO}$) for Pb01 (Figure 3). Higher values of these properties lead to more effective compounds against Pb01 (Cumulative eigenvalues: 93.9%; 100.0%):

PC1 = 0.583 Log($1/c_{Pb01}$) + 0.591 ΔH_f^o + 0.558 $\Delta E_{HOMO-LUMO;}$

PC2 = -0.490 Log($1/c_{Pb01}$) + -0.292 ΔH_f^o + 0.821 $\Delta E_{HOMO-LUMO;}$

For Pb18 the results are not conclusive (Figure 4), but they pointed to three descriptors: volume, surface area (SA) and polarizability (Cumulative eigenvalues 73.8%; 98.0%):

PC1 = -0.152 Log($1/c_{Pb18}$) + 0.579 volume + 0.570 polarizability + 0.563 SA;

PC2 = 0.981 Log($1/c_{Pb18}$) + 0.022 volume + 0.056 polarizability + 0.185 SA.

The PC2 component is responsible for the compounds separation. When those three values increases, the activity increases, either.

Finally, for the Pb03 fungus, whose compounds revealed the best activities, the second component (PC2) showed a good separation as function of activity (Figure 5):

The properties which describe adequately the antifungal activity are polarizability, volume and the variation of energy between the frontier orbitals ($\Delta E_{HOMO-LUMO}$). Cumulative eigenvalues 66.4%; 93.0%:

PC1 = 0.040 Log($1/c_{Pb03}$) - 0.538 $\Delta E_{HOMO-LUMO}$ + 0.590 polarizability + 0.600volume;

PC2 = 0.959 Log($1/c_{Pb03}$) + 0.252 $\Delta E_{HOMO-LUMO}$ + 0.122 polarizability + 0.042volume

In this case, the antifungal activity should be increased by increasing the values of the cited properties.

Discussion

Currently, there are few works on the search for new drugs against *P. brasiliensis*. San-Blas *et al.* [21] was one of the prime works with a natural product against this fungus. Recently, Thomaz *et al.* [22]

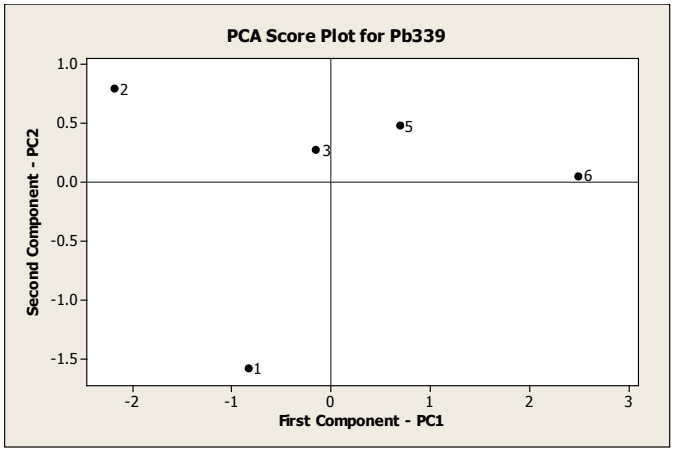

Figure 2: PCA technique showing the compounds separation as function of antifungal activity (Score plot) and the mainly descriptors (Biplot) for Pb339 fungus.

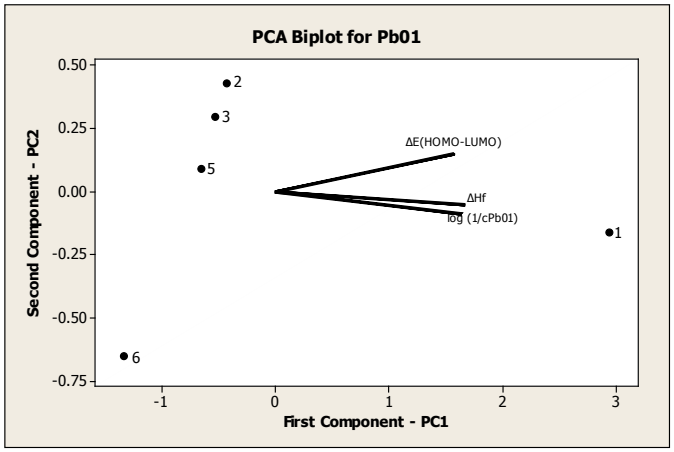

Figure 3: PCA technique showing the compounds separation as function of antifungal activity (Score plot) and the mainly descriptors (Biplot) for Pb01 fungus.

showed that alone from garlic therapy was effective in Balb/c mice infected intra tracheally with the virulent isolate *P. brasiliensis* Pb18. Pelegrini *et al.* [23] have tested peptides from *Passiflora edulis* against this fungus, but they did not find any effective activity. Marques *et al.* [24] studied a peptide vaccine with P10, derived from gp43 (major diagnostic antigen), as an adjuvant to chemotherapy, which reduced treatment time, and prevented relapsing disease. In another work, Martins *et al.* [25], studied the antifungal activity of curcumin, against 23 fungal strains and observed that *P. brasiliensis* were the most susceptive to curcumin. In present work, the phebalosin was active against all isolates of *P. brasiliensis* tested. Due to the great relationship between the coumarins and the biological activities mentioned above, a series of modifications were made to the majority (phebalosin) of *Polygala paniculata* for the evaluation of a possible improvement in antifungal activity displayed by these compounds. The compound of reaction of phebalosin with ethanol (3) presented strong activity against isolate Pb03 of *P. brasiliensis* with a MIC values of the 1.9 µg/ml (6.5 µmol/L), sixteen times more active than phebalosine. The isopropoxy (5) and n-butoxy (6) derivatives were respectively four times and twice more active than phebalosine against Pb03 (Table 1). These experimental results suggest important hydrophobic and steric interaction simultaneously with alkyl groups in the moiety isoprenyl of the phebalosin. This result indicates an activity intermediate to the two drugs used in the treatment of PCM, and the most used in Brazil by the National Health Care System (SUS) is trimethoprim-sulfamethoxazole [25]. Other authors also tested the antifungal activity of amphotericin B and trimethoprim-sulfamethoxazole against several isolates of

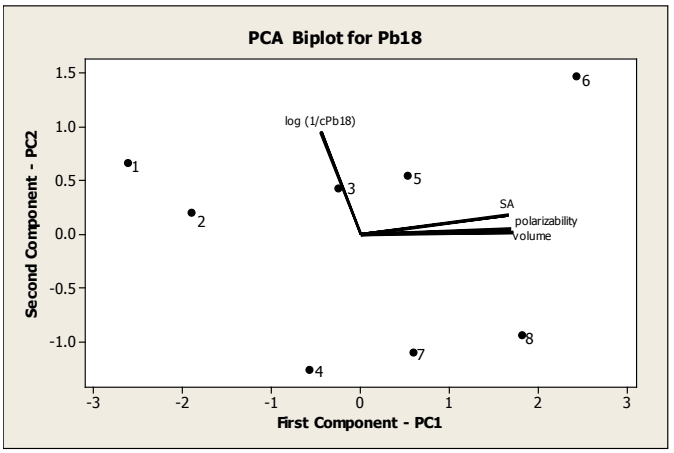

Figure 4: PCA technique showing the compounds separation as function of antifungal activity (Score plot) and the mainly descriptors (Biplot) for Pb18 fungus.

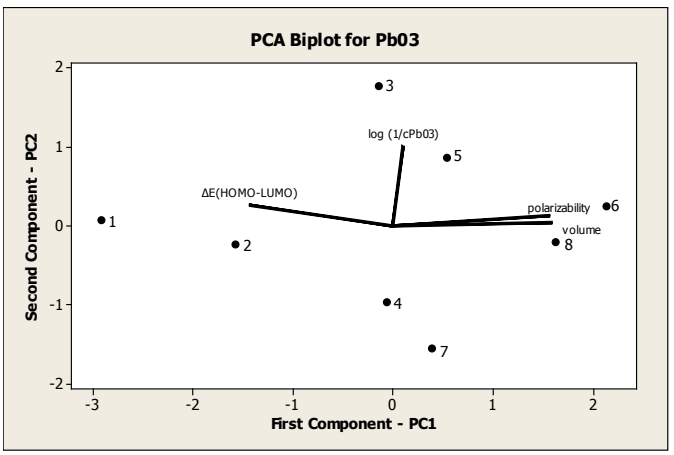

Figure 5: PCA technique showing the compounds separation as function of antifungal activity (Score plot) and the mainly descriptors (Biplot) for Pb03 fungus.

Paracoccidioides, with MIC values close to those found in present work, using RPMI medium, range 0.25-2 μg/ml and 300-75 μg/ml for amphotericin B and trimethoprim-sulfamethoxazole, respectively [25].

The chemometric results, derived from quantum chemistry calculations, revealed the same tendency observed from experimental data: structural properties are responsible for the antifungal activity. Polarizability, surface area and volume showed a good correlation with activity. In spite of all the observations, it's important to remark that the variation of energy between the frontier orbitals ($\Delta E_{HOMO\text{-}LUMO}$) is an important property related to activity, either.

Despite the chemometric analysis is not completely conclusive, PCA technique was able to separate the compounds as function of antifungal activity. In order to increase the antifungal activity, it can be suggested that structural modifications of phebalosin should have the following properties: greater values for polarizability, volume and the variation of energy between the frontier orbitals ($\Delta E_{HOMO\text{-}LUMO}$). It pointed to steric influence (volume), lipophilicity character (polaizability and volume) and compound stability during the interaction (electronic influence, $\Delta E_{HOMO\text{-}LUMO}$) for the substituint alkyl groups.

In conclusion, phebalosin showed promised antifungal activity against isolates of *P. brasiliensis* tested. The structural modifications of phebalosin with different nucleophiles yielded seven compounds, four being new compounds (8, 4, 5, 6). Overall, these data open new possibilities for the potential use of phebalosin and its structural modifications as possible antifungal drugs against *P. brasiliensis*. However, new studies are necessary to characterize the mechanism of action of these compounds and produce more conclusive chemometric data.

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References

1. Sajora M, Santhi R, Annapoorani S (2012) Wound healing activity of flavonoid fraction of Cynodondactylon in Swiss albino mice. Int Res J Pharm 3: 230-231.
2. MacKay D, Miller AL (2003) Nutritional support for wound healing. See comment in PubMed Commons below Altern Med Rev 8: 359-377.
3. Guo S, Dipietro LA (2010) Factors affecting wound healing. See comment in PubMed Commons below J Dent Res 89: 219-229.
4. Tonnesen MG, Feng X, Clark RA (2000) Angiogenesis in wound healing. See comment in PubMed Commons below J Investig Dermatol Symp Proc 5: 40-46.
5. Edwards R, Harding KG (2004) Bacteria and wound healing. See comment in PubMed Commons below Curr Opin Infect Dis 17: 91-96.
6. Davis SC, Ricotti C, Cazzaniga A, Welsh E, Eaglstein WH, et al. (2008) Microscopic and physiologic evidence for biofilm-associated wound colonization in vivo. See comment in PubMed Commons below Wound Repair Regen 16: 23-29.
7. Sasidharan S, Nilawatyi R, Xavier R, Latha LY, Amala R (2010) Wound healing potential of Elaeis guineensis Jacq leaves in an infected albino rat model. See comment in PubMed Commons below Molecules 15: 3186-3199.
8. Okafo JC, Myrianthus arboreus P. Beauv PROTA 2 (2004) Vegetables/ Légumes PROTA, Wageningen Netherlands.
9. Burkill HM (1997) The useful plants of West Tropical Africa, 2nd ed., volume 2, Families E– I. Royal Botanic Gardens, Kew, Richmond, United Kingdom.
10. Agyare C, Asase A, Lechtenberg M, Niehues M, Deters A, et al. (2009) An ethnopharmacological survey and in vitro confirmation of ethnopharmacological use of medicinal plants used for wound healing in Bosomtwi-Atwima-Kwanwoma area, Ghana. See comment in PubMed Commons below J Ethnopharmacol 125: 393-403.
11. Mavar-Mangar H, Lejoly J, Quetin-Leclercq J, Schelzer GH (2007) Alchornea cordifolia (Schumach. & Thonn.) Müll. Arg. PROTA, Wageningen, Netherlands.
12. Evans WC (2009) Trease and Evans' Pharmacognosy. 16th ed. Elsevier Health Sciences, United Kingdom.
13. Sofowora A (1993) Medicinal plants and traditional medicine in Africa, Spectrum Books, Ibadan.
14. Harborne JA (1988) Phytochemical methods: A guide to modern techniques of plant analysis. Plant Sciences 14: 302.
15. Agyare C, Koffuor GA, Boakye YD, Mensah KB (2013) Antimicrobial and anti-inflammatory properties of Funtumia elastica. See comment in PubMed Commons below Pharm Biol 51: 418-425.
16. Agyare C, Koffuor GA, Boamah VE, Adu F, Mensah KB, et al. (2012) Antimicrobial and Anti-Inflammatory Activities of Pterygota macrocarpa and Cola gigantea (Sterculiaceae). See comment in PubMed Commons below Evid Based Complement Alternat Med 2012: 902394.
17. Eloff JN (1998) A sensitive and quick microplate method to determine the minimal inhibitory concentration of plant extracts for bacteria. See comment in PubMed Commons below Planta Med 64: 711-713.
18. British Pharmacopoeia. Stationary Office, London, United Kingdom, 2007.
19. Ugboduma AO, Adu F, Agyare C, Annan K, Osei-Asante S (2013) Phytochemical screening and antimicrobial activity of Entandrophragma angolense. J Pharm Nutr Sci 3: 241-249.
20. Chizzola R, Michitsch H, Franz C (2008) Antioxidative properties of Thymus vulgaris leaves: comparison of different extracts and essential oil chemotypes. See comment in PubMed Commons below J Agric Food Chem 56: 6897-6904.
21. Ebi GC (2001) Antimicrobial activities of Alchornea cordifolia. See comment in PubMed Commons below Fitoterapia 72: 69-72.
22. Biapa PC, Agbor GA, Oben JE, Ngogang JY (2007) Phytochemical studies and antioxidant properties of four medicinal plants used in Cameroon. See comment in PubMed Commons below Afr J Tradit Complement Altern Med 4: 495-500.
23. Fabry W, Okemo PO, Ansorg R (1998) Antibacterial activity of East African medicinal plants. See comment in PubMed Commons below J Ethnopharmacol 60: 79-84.
24. Nayak BS (2006) Cecropia peltata L(Cecropiaceae) has wound-healing potential: a preclinical study in a Sprague Dawley rat model. See comment in PubMed Commons below Int J Low Extrem Wounds 5: 20-26.
25. Houghton PJ, Hylands PJ, Mensah AY, Hensel A, Deters AM (2005) In vitro tests and ethnopharmacological investigations: wound healing as an example. See comment in PubMed Commons below J Ethnopharmacol 100: 100-107.

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