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Potentialities of Fermented Cashew Apple Bagasse with *Penicillium roqueforti* ATCC 10110: Total Phenolics, Antifungal Activity and Cytotoxicity

Potencialidades do Fermentado do Bagaço da Maçã do Caju com *Penicillium roqueforti* ATCC 10110: Fenólicos Totais, Atividade Antifúngica e Citotoxicidade

Potencialidades de la Fermentación del Bagazo de la Manzana del anacardo con *Penicillium roqueforti* ATCC 10110: Fenólicos Totales, Actividad Antifúngica y Citotoxicidad

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Abstract
In this study, cashew apple (*Anacardium occidentale* L.) bagasse, a residue from the industrial extraction of the juice, was subjected to a solid-state fermentation process with the fungus *Penicillium roqueforti* ATCC 10110 and the total phenolic content was evaluated, as well as the activity of extracts against strains of dermatophyte fungi of the genera *Trichophyton* and *Microporum*; cytotoxicity was analyzed through hemolytic potential in human erythrocytes. The results showed that the fermented cashew apple bagasse had a higher content of total phenolics compared to the unfermented bagasse, a potent antifungal activity with minimum inhibitory concentrations (MIC) ranging from 256 to 512 µg/mL against the strains: *T. rubrum* (LM-115 and LM-629), *T. mentagrophytes* LM-119, *M. gypseum* LM-512 and *M. canis* (LM-68 e LM-110), with low cytotoxicity. Therefore, the fermented extracts of cashew apple bagasse can be considered as a promising natural non-toxic product for studies in the development of agents against skin diseases, especially for the treatment of dermatophytosis.
Keywords: Dermatophytosis; Solid-state fermentation; Dermatophyte fungi.

Resumo
Neste trabalho, o bagaço da maçã do caju (*Anacardium occidentale* L.), um resíduo da extração industrial do suco foi submetido a um processo de fermentação em estado sólido com o fungo *Penicillium roqueforti* ATCC 10110 e avaliado o teor de fenólicos totais, a atividade antifúngica dos extratos contra cepas dos fungos dermatófitos do gênero *Trichophyton* e *Microporum* e a citotoxicidade através do potencial hemolítico em eritrócitos humanos. Os resultados demonstraram que o bagaço da maçã do caju fermentado apresentou um maior teor de fenólicos totais comparado ao bagaço não fermentado, uma potente atividade antifúngica com concentrações inibitórias mínimas (CIM) que variaram

de 256 a 512 μg/mL contra as cepas: *T. rubrum* (LM-115 e LM-629), *T. mentagrophytes* LM-119, *M. gypseum* LM-512 e *M. canis* (LM-68 e LM-110), com uma baixa citotoxicidade. Dessa forma, os extratos fermentados do bagaço da maçã do caju podem ser considerados como um promissor produto natural não tóxico para estudos no desenvolvimento de agentes contra doenças de pele, especialmente para o tratamento das dermatofitoses.
Palavras-chave: Dermatofitoses; Fermentação no estado sólido; Fungos dermatófitos.

Resumen
En este trabajo se sometió bagazo de la manzana del anacardo (*Anacardium occidentale* L.), residuo de la extracción industrial del jugo, a un proceso de fermentación en estado sólido con el hongo *Penicillium roqueforti* ATCC 10110 y se evaluó el contenido fenólico total, la actividad de extractos contra cepas de hongos dermatofitos del género *Trichophyton* y *Microporum* y citotoxicidad por potencial hemolítico en eritrocitos humanos. Los resultados mostraron que el bagazo de la manzana del anacardo fermentado tenía un mayor contenido de fenoles totales en comparación con el bagazo no fermentado, una potente actividad antifúngica con concentraciones mínimas inhibitorias (CIM) que van desde 256 a 512 μg/mL contra las cepas: *T. rubrum* (LM-115 y LM-629), *T. mentagrophytes* LM-119, *M. gypseum* LM-512 y *M. canis* (LM-68 y LM-110), con baja citotoxicidad. De esta forma, los extractos fermentados del bagazo de la manzana del anacardo pueden considerarse como un producto natural no tóxico prometedor para estudios en el desarrollo de agentes contra enfermedades de la piel, especialmente para el tratamiento de dermatofitoses.
Palabras clave: Dermatofitosis; Fermentación en estado sólido; Hongos dermatofitos.

1. Introduction

Cashew (*Anacardium Occidentale* L.) is a plant of considerable economic importance all over the world as a source of two main products - the cashew nut (botanically representing the true fruit) and the cashew apple (cashew peduncle or pseudofruit) (Brainer, 2021).

The cashew apple corresponds to about 90% of the total weight of the fruit and can be consumed directly or processed as juices, "cajuína" and sweets. The world production of cashew apples in 2019 was 1.32 million tons and Brazil participated with 81.4% of this volume, followed by Mali (12.7%) and Madagascar (5.9%) (FAOSTAT, 2022).

The industrial process of cashew juice production generates approximately 20 to 25% of residual fiber (bagasse), which is still inappropriately discarded in the environment or only used as a food supplement (Barros et al., 2017).

Some studies have demonstrated more efficient uses for the cashew apple bagasse (CAB). Some authors have already investigated its use as a substrate for the production of cellulosic ethanol (Padilha et al., 2020), biohydrogen (Silva et al., 2018), etc. However, we found no record in the literature on the antifungal potential of the fermented extract of this residue.

In a recent publication, we investigated the presence of total phenolics and antioxidant activity in CAB using Centroid-Simplex mixture design and Three-Level Factorial design, combined with Response Surface methodology (RSM). The results showed that the CAB extract can still be considered a potential source of phenolic compounds with antioxidant activity and thus, an important alternative of cheap natural biomass for use in bioprocesses (Felix et al, 2018).

Therefore, this study aims to evaluate the potential of the fermented extract of cashew apple bagasse (FCAB) with *Penicillium roqueforti* ATCC 10110 on the total phenolic content, the *in vitro* antifungal activity against dermatophytes of the genera *Trichophyton* and *Microporum* and the cytotoxicity through human erythrocytes, in order to verify its viability for use as a non-toxic antifungal agent.

2. Materials and Methods

2.1 Chemicals and reagents

Gallic acid monohydrate (≥99%) (Sigma-Aldrich, São Paulo, SP, Brazil), sodium carbonate PA (Sigma-Aldrich, São Paulo, SP, Brazil), phenol reagent according to Folin-Ciocalteu (Sigma- Aldrich, São Paulo, SP, Brazil), Trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid) (Sigma-Aldrich, São Paulo, SP, Brazil), DPPH (1,1- diphenyl-2-picrylhydrazyl) (Sigma-Aldrich, São Paulo, SP, Brazil), ABTS (2,2'-azino-bis-3-ethylbenzothiazoline-6-sulfonic acid) (Sigma-Aldrich, São

Paulo, SP, Brazil), potassium persulfate PA (Sigma-Aldrich, São Paulo, SP, Brazil), Triton X-100 (Sigma-Aldrich, São Paulo, SP, Brazil), chloramphenicol (Sigma-Aldrich, São Paulo, SP, Brazil), ketoconazole (Sigma-Aldrich, São Paulo, SP, Brazil), Sodium Resazurin (Sigma-Aldrich, São Paulo, SP, Brazil), Acetone PA (Vetec, Rio de Janeiro, RJ, Brazil), Calcium Chloride PA (Vetec, Rio de Janeiro, RJ, Brazil), Tween 80 (Sigma-Aldrich, São Paulo, SP, Brazil), dimethylsulfoxide (Sigma-Aldrich, São Paulo, SP, Brazil), and 95% ethanol PA (Vetec, Rio de Janeiro, RJ, Brazil), 99.8% Methanol PA (Chemis, São Paulo, SP, Brazil) and Potato Dextrose Agar (PDA) (Himedia, Mumbai, India).

2.2 Microorganism and inoculum

The *Penicillium roqueforti* ATCC 10110 strain used in this study was acquired from the collection of microorganisms of INCQS/Fiocruz (Manguinhos, Rio de Janeiro, Brazil), under registration number 40074. For the preparation of the inoculum, the sporulated culture of the fungus with 7 days incubation in PDA medium at 30 °C was suspended in Tween 80 (0.01% v/v) and the number of spores was counted in a binocular microscope (Bioval L1000, São Paulo, Brazil), using a Neubauer chamber, according to Ferreira et al. (2017). The inoculum concentrations used were $1x10^7$ spores/g of solid medium.

2.3 Cashew apple bagasse (CAB)

CAB was kindly provided by the fruit pulp industry Frutisol (Jequié-Bahia-Brazil). The residues were dried in an oven (Solab SL 102, Piracicaba, Brazil) at 50 °C for 72 hours and then ground in a knife mill (Wiley, ACB Labor, São Paulo, Brazil) with a 20-mesh sieve until reaching the size of 2 mm granules. The powder obtained was placed in a hermetically sealed glass container and stored at room temperature until further use.

2.4 Solid-state fermentation (SSF)

10 g of CAB were autoclaved (121 °C/15 min) in 125 mL Erlenmeyer flasks. After cooling, the sterile substrate was inoculated with 10^7 spores/g and moistened with sterile distilled water until reaching a moisture content of 60% and a water activity (aw) of 0.98. Fermentations took place in bacteriological ovens (BOD SL200/90, Solab, Piracicaba, São Paulo, Brazil) and the independent variables incubation temperature (T, °C) and fermentation time (t, h) were evaluated according to the Doehlert design.

2.5 Doehlert design

A Doehlert matrix was used to evaluate the influence of incubation temperature (T) and fermentation time (t) on the total phenolic content produced by *P. roqueforti* ATCC 10110 in the solid-state fermentation of CAB. The matrix consisted of nine experiments (Table 1), including three replications at the central point to estimate the experimental error, evaluate quadratic, linear and interaction effects of the variables and adjust a mathematical model for the experimental procedure. From the experimental values obtained, a second order polynomial model was built according to equation 1, to evaluate the effects of the independent variables on the response, where: Y is the response (total phenolic compounds content), β0 is the term constant; β1, β2 and β3 are the coefficient of linear terms; β11, β22 and β33 are the coefficients of the quadratic terms; β12, β13 and β23 are the coefficient of the interaction terms.

$$Y = \beta0 + \beta1A + \beta2B + \beta3C + \beta11A2 + \beta22B2 + \beta33C2 + \beta12AB + \beta13AC + \beta23BC \quad (1)$$

The level of statistical significance of the generated model was evaluated by analysis of variance (ANOVA, $p < 0.05$), regression coefficients were evaluated from the Pareto chart (based on t-Student, $p < 0.05$); and model quality was evaluated by the coefficient of determination (R^2), by the adjusted coefficient of determination (R^2-adj) and by the lack of fit test. The experiments

were performed in triplicate and the results were presented as mean ± standard deviation (M ± SD). Statistica software version 7.0.0 (Statsoft Inc., USA) was used to analyze the results and plot the graphs.

2.6 Determination of total phenolics

Total phenolics in the CAB were extracted through a sequential process using 60% water, 30% ethanol and 30% acetone for 2 hours at 34 °C, according to Felix et al. (2018). The total phenolic content was determined by the adapted Folin-Ciocalteu method (Rebaya et al., 2015). The extracts (0.5 mL) were mixed with 2.5 mL of Folin-Ciocalteu reagent (1:10) and 2 mL of sodium carbonate solution (4%). The mixture was stirred and kept at room temperature for 2 hours in the dark. The absorbance of the sample was measured in a spectrophotometer (UV-340G, Gehaka, São Paulo, Brazil) at 750 nm against a blank. Gallic acid solutions were used as a standard and its calibration curve was used to calculate the content of total phenolics present in the sample. Results were expressed in milligrams of gallic acid equivalents per 100 grams of residue (mg GAE/100 g). All measurements were performed in triplicate.

2.7 Antifungal activity assay

2.7.1 Tested products

Unfermented (UCAB) and fermented (FCAB) cashew apple bagasse extracts were solubilized in dimethyl sulfoxide (DMSO) at a proportion of 10% and Tween 80 at 0.02%. The final volume was then made up with sterile distilled water (*q.s.p.* 3 mL) to obtain an emulsion at an initial concentration of 1024 µg/mL and subsequent dilutions to 4 µg/mL (Hadacek & Greger, 2000). The mixtures were shaken for 3 minutes in a Vortex apparatus (251, Fanem, Guarulhos, São Paulo, Brazil). The emulsions of the products used in the antifungal assays were prepared at the time of the tests. For antifungal control, Ketoconazole (Sigma-Aldrich, São Paulo, SP, Brazil) was used as the standard drug.

2.7.2 Culture media

The culture media used in the assays to evaluate the antifungal activity were: Brain Heart Infusion (BHI) and Sabouraud Dextrose Agar (SDA) (Difco Laboratories Ltd, Detroit, USA) for strain maintenance, and BHI and RPMI 1640 medium with L-glutamine without bicarbonate (Sigma-Aldrich, St. Louis, Missouri, USA) for assays of biological activity against fungi. The media were solubilized in distilled water and sterilized in an autoclave at 121 ºC for 15 minutes, according to the manufacturers' instructions.

2.7.3 Microorganisms and inoculum

For the antifungal activity assay, the following strains were used: *Trichophyton rubrum* (LM-115 and LM-629), *Trichophyton mentagrophytes* LM-119, *Microsporum gypseum* LM-512, *Microsporum canis* (LM-68 and LM-110), kindly donated by MICOTECA of the Mycology Laboratory of the Department of Pharmaceutical Sciences (DCF) of Universidade Federal da Paraíba (UFPB). All strains were maintained in ASD/BHI at 4°C, which were prepared according to the manufacturers' descriptions. 24-48 hour repeats in BHI and ASD were used for assays incubated at 35 ± 2 °C.

For the preparation of the inoculum, colonies obtained from cultures of the fungal strains maintained in ASD were suspended in sterile saline NaCl solution (0.85% w/v) and adjusted according to the 0.5 tube of the standard McFarland scale to obtain 106 colony forming units per mL (CFU/mL) (Antunes, 2006). Inoculum quantification was confirmed by plating 0.01 mL of the suspensions in ASD. The plates were incubated at 28 ºC and examined daily for colony counting, determining the number of CFU/mL.

2.7.4 Assessment of the minimum inhibitory concentration (MIC)

Antifungal activity assays were performed according to the protocols of Cleland & Squires (1991) and NCCLS (2000). The MIC of the products on the fungal strains was determined using broth microdilution in a cell culture plate (TPP/Switzerland/Europe) containing 96 wells. Initially, 100 µL of doubly concentrated RPMI/BHI were dispensed into the wells of the microdilution plates. Subsequently, 100 µL of the emulsion of the prepared double-concentrated products were dispensed into the wells of the first row of the plate and concentrations of 1024 µg/mL up to 4 µg/mL were obtainedby a serial dilution at a ratio of two. Finally, 10 µL of the dermatophyte strain suspensions were added to the wells, where each column of the plate specifically refers to a strain. In parallel, controls were obtained: microorganisms, culture medium and standard antifungal for dermatophytes. Assays were performed in triplicate. The prepared and aseptically sealed plates were subjected to incubation for 7-10 days/room temperature (28-30°C).

After the appropriate incubation time, the assay was read to evaluate the MIC for each product tested. The MIC was defined as the lowest concentration of the product capable of visually inhibiting fungal growth when compared to control growth.

The antifungal activity of UCAB and FCAB were interpreted and considered as active or inactive, according to the following criteria: 50-500 µg/mL = strong/optimal activity; 600-1500 µg/mL = moderate activity; > above 1500 µg/mL = weak activity or inactive product (Holetz et al., 2002; Houghton et al., 2011).

2.8 Cytotoxicity assessment

After approval by the research ethics committee (Protocol No. 2.373.249/Centro Universitário de Patos - UNIFIP), the cytotoxicity of UCAB and FCAB extracts was evaluated using human erythrocytes (groups A, B, O), obtained from healthy volunteers (25-30 years old), according to the method by Verma & Raval (1991), with modifications. Intravenous blood samples collected in EDTA vials were diluted with normal saline (0.9% NaCl) and centrifuged at 1000 g for 10 minutes. This procedure was repeated three times and the sediment was finally collected and diluted with saline solution to have a cell density of $2x10^4$ cells/mL.

2.8.1 Assessment of hemolytic potential.

The UCAB and FCAB extracts were solubilized in dimethyl sulfoxide (DMSO) at a proportion of 10% and Tween 80 at 0.02%. The final volumes were then completed with sterile distilled water (*q.s.p.* 3 mL) for the obtention of an emulsion at different concentrations (1000, 500, 100, 50 µg/mL). The emulsions were preserved in amber glass vials and kept under refrigeration at 18 ºC until testing.

UCAB and FCAB samples at different concentrations were added to 2 mL of the erythrocyte suspension for a final volume of 2.5 mL. An erythrocyte suspension was used as a negative control (0% hemolysis) and an erythrocyte suspension plus 1% Triton X-100, as a positive control (100% hemolysis). Subsequently, the samples were incubated for 1 hour at 22 ± 2 °C under slow and constant stirring. The samples were then centrifuged at 2500 g for 5 minutes and hemolysis was quantified by spectrophotometry at a wavelength of 540 nm (Rangel et al., 1997). All experiments were performed in triplicate.

2.9 Statistical analysis

Statistical analysis was performed using One Way ANOVA and Student t test. Values were considered statistically significant when they presented $p<0.05$. Graphs were plotted using the software GraphPad Prism5.0®.

3. Results and Discussion

3.1 Fermentation effect on total phenolic content in CAB

The values of total phenolic in UCAB and FCAB are shown in Table 1. There was a significant increase ($p<0.05$) during the fermentation process. However, some variations in the values were observed when different levels of the variables were used. To mathematically evaluate these variations, the results were submitted to regression analysis for the construction of a second-order polynomial prediction model, according to equation 2.

Table 1. Doehlert matrix for the calculation of total phenolics in CAB with coded and real values (in parentheses) for the independent variables fermentation time (t) and incubation temperature (T).

Run	t (h)	T (ºC)	Total phenolics UCAB (mg GAE/100 g)	Total phenolics FCAB (mg GAE/100 g)
1	+1 (120)	0 (30)	256.06 ± 22.32	382.12 ± 9.50
2	+0.5 (96)	+0.866 (40)	287.92 ± 6.15	393.34 ± 13.50
3	-1 (24)	0 (30)	266.31± 7.47	277.23 ± 8.20
4	-0.5 (48)	-0.866 (20)	244.06 ± 12.65	347.05 ± 7.80
5	+0.5 (96)	-0.866 (20)	280.35 ± 3.73	368.06 ± 5.30
6	-0.5 (48)	+0.866 (40)	267.06 ± 22.32	305.20 ± 7.30
7	0 (72)	0 (30)	259.34 ± 4.90	387.08 ± 11.20
8	0 (72)	0 (30)	230.30 ± 22.28	407.32 ± 13.20
9	0 (72)	0 (30)	266.31 ± 14.15	398.16 ± 10.50

Source: Authors (2022).

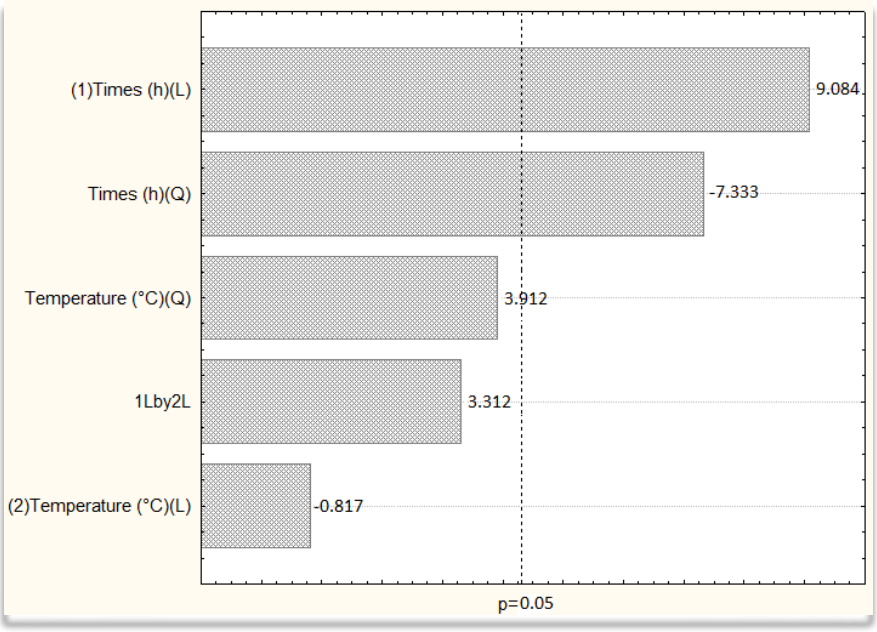
$$Y = 84.29 + 3.24\ t - 0.29\ t^2 + 10.83\ T - 0.27\ T^2 + 0.069\ t*T \quad (2)$$

Where, Y = is the total phenolic content, t representes fermentation time (24 to 120 h) and T is the incubation temperature (20 to 40 ºC).

The statistical significance of linear, quadratic and interaction terms (linear x linear) of equation 2 was plotted as a Pareto chart (Figure 1). In this type of graph, the absolute value of the standardized effect amplitude (relation between the effect and the normal error) of each of the coefficients is plotted in descending order and compared with the magnitude of a statistically significant factor (Novaes et al., 2017; Novaes et al., 2018). Assuming a confidence level of 95%, represented by the vertical line plotted on the graph, it was observed that only terms t and t^2 were significant. The significant effect of these linear (t) and quadratic (t^2) terms suggests that fermentation time is important for the formation of phenolic compounds, indicating that small changes in its values significantly affect the total phenolic content in the process.

On the other hand, the linear and quadratic terms of the variable incubation temperature (T and T^2), as well as the interaction term (linear x linear) between the investigated variables (t*T), were not significant at 95% confidence. These results suggest that the effect of temperature variation (within the investigated experimental domain) on the CAB fermentation process does not significantly affect the total phenolic content.

Figure 1. Pareto chart demonstrating the influence of fermentation time (t) and temperature (T) and their interactions on the extraction of total phenolics from CAB during solid-state fermentation by *P. roqueforti* ATCC 10110.



Source: Authors (2022).

The validity of experimental data obtained through the Doehlert design was analyzed by analysis of variance (ANOVA) (Table 2). From the ANOVA data ($p<0.05$), it can be inferred that the model is well adjusted and does not lack fit ($F_{calculated}>F_{tabled}$), demonstrating that the results were not random. The obtained R^2 (0.99) indicated a high correlation between the predicted values and the experimental results, meaning that 99% of the results can be explained by the applied experimental design.

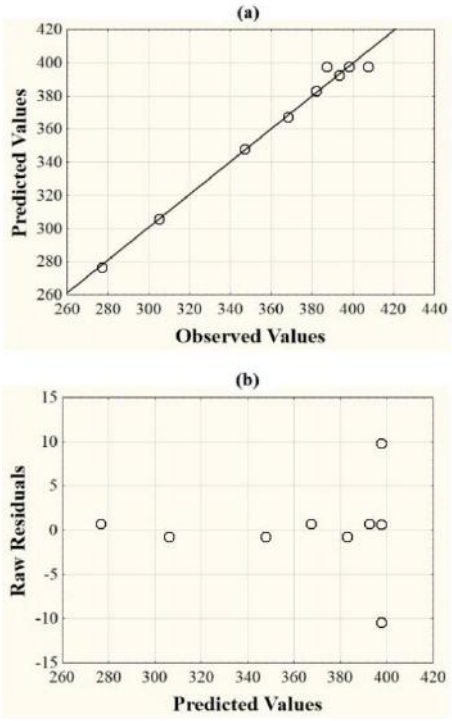
Table 2. Analysis of variance (ANOVA) for the model adjusted from the experimental results for the total phenolic content in FCAB.

Source of variation	SS	DF	MS	$F_{calculated}$	F_{tabled}
Regression	15835.0652	5	3167.013	45.576	9.01
Residue	208.4678	3	69.489		
Lack of fit	3.0246	1	3.025	0.029	18.51
Pure error	205.4432	2	102.722		
Total SS	16043,5330	8			
R^2	0.99				

SS = Sum of squares; DF = Degrees of freedom; MS = Mean Square; R^2 = Determination coefficient; *F* = Fisher test.
Source: Authors (2022).

The value of lack of fit was insignificant ($p<0.05$), as can be observed from the graph of actual and predicted values and the residual plot represented in Figure 2. The model presented the expected values very close to the experimental values, with the residues being randomly distributed with relatively low values (<2.7%); these characteristics made the model represented by Equation 2 valid for the construction of the response surface graph and the prediction of the desired optimal regions.

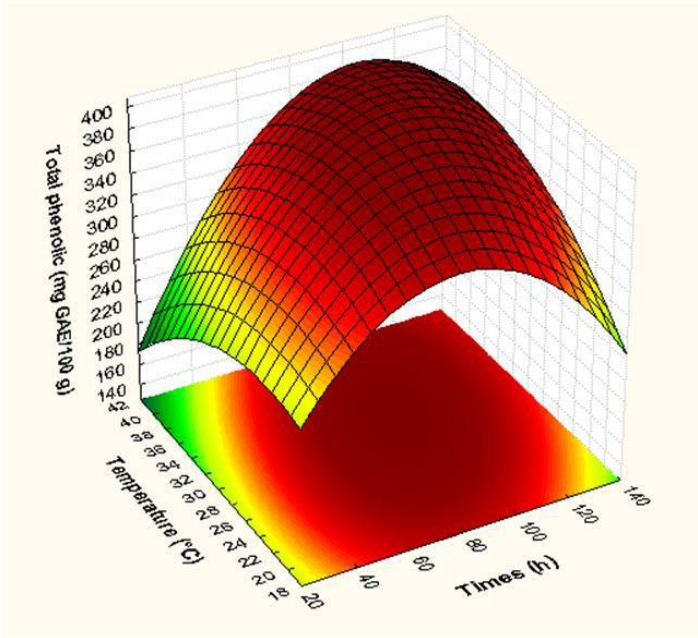
Figure 2. (a) Parity plot showing correlation between predicted and experimental values. (b) Residual plot for the fitted model.



Source: Authors (2022).

From Table 1, the response surface graph was plotted (Figure 3), relating the effect of time (h) and temperature (ºC) and their interactions on the total phenolic content in FCAB. From this graph, a region of maximum total phenolic content was observed when the fermentation time and temperature were maintained between 72 and 100 hours and between 28 and 36 °C, respectively. Thus, the conditions predicted for the maximum production of total phenolic contents were: t = 93 h and T = 32 °C; under these conditions, the predicted total phenolic content was 408.81 mg of GAE/100 g. The predicted value was confirmed and validated by carrying out an experiment (in triplicate) using the optimized conditions, obtaining an experimental value of 406.59 ± 9.61 mg of GAE/100 g, very close to the predicted value (absolute error of 0.54%). The value found for total phenolic content was 63% higher than that found for UCAB (249.10 ± 34.76 mg GAE/100 g).

Figure 3. Response surface graph representing the interactions between fermentation time (h) and temperature (ºC) on the total phenolic content of FCAB.



Source: Authors (2022).

The total phenolic content present in FCAB residues of this study can be considered a rich source of total phenolics compared to the values found in other studies, such as: Barretto et al. (2015) using enzymatic extraction processes with *Aspergillus awamori* and hydroethanolic extraction to obtain phenolic extracts enriched in CAB, found total phenolic values lower than 50 and 35 mg of GAE/100 g, respectively; Broinizi et al. (2007) evaluating the total phenolics in cashew apple bagasse, found values of 2.8 and 10.4 mg of GAE/g for bagasse and its concentrated crude extract, respectively. In addition, compared to other fruit residues such as banana peel (total phenolics = 380 mg GAE/100g) (Babbar et al., 2011) and orange peel (total phenolics = 275.8 mg GAE/100 g) (Khan et al., 2010), the values of total phenolics found in FCAB are also higher.

However, the polarity of the solvents involved in the extraction process, the type of treatment given to the sample (different drying systems) etc, are also important parameters that may have affected the extraction yield of total phenolics (Felix et al., 2018) and possibly contributed to the differences in the results compared in this study. Still, it is observed that solid-state fermentation with *P. roqueforti* ATCC 10110 proved to be efficient for increasing the total phenolic content in FCAB.

It is already well established in the scientific literature that phenolic compounds may have antioxidant, antibacterial, anti-inflammatory and anticancer activities (Dinica et al., 2015; Chang et al., 2016; Jiang-ke et al., 2016; Mojzer et al., 2016); thus, FCAB appears as an alternative biomass candidate for use in biological tests.

3.2 Effect of fermentation on the antifungal activity of CAB

Antifungal studies that determine minimum inhibitory concentrations (MIC) provide significant information about the potential of a given product to be clinically used as an antifungal. The results obtained may guide other studies aimed at the pharmacological use of this product. It is essential that a natural product, as a candidate to be used as an antifungal, obtain relevant results in this *in vitro* study to justify its continuation.

Therefore, the antifungal potential of UCAB and FCAB was tested against different strains of dermatophyte fungi and compared with a commercial antifungal agent indicated for this type of treatment (Table 3).

All fungal strains were able to grow in the presence of the UCAB extract, in the culture media (microorganism control) and in the emulsifying agents used to dilute the extracts (DMSO and Tween 80), confirming that the impediment to microbial growth is the presence in the medium of the FCAB extract, thus confirming its antifungal capacity. A sterility control was carried out to certify that the culture media used in the tests were not contaminated with microorganisms, which would make the tests unfeasible if there were any microbial growth.

The MIC of FCAB ranged from 256 to 512 µg/mL for the tested strains. It was observed that FCAB showed its greater inhibition for *M. canis* strains (LM-68 and LM-110), compared to Ketoconazole. For the strain *M. canis* LM-68, the FCAB presented the lowest MIC value (256 µg/mL). This value was better compared to that found for ketoconazole which, at any concentration tested for *M. canis* strains (LM-68 and LM-110), did not prevent microbial growth. Furthermore, for the strain *T. rubrum* LM-115, the value found for the MIC of FCAB (512 µg/mL) was also better compared to the MIC of Ketoconazole (1024 µg/mL). For the strains *T. rubrum* LM-629 and *T. mentagrophytes* LM-119, the value found for MIC of FCAB (512 µg/mL) was equal to the value found for Ketoconazole. Thus, according to the criteria adopted by the specialized literature, the FCAB extract showed an antifungal activity that can be classified between medium and strong.

Table 3. Evaluation of the antifungal activity of CAB against dermatophyte fungal strains.

Dermatophyte fungi	UCAB (µg/mL) MIC	FCAB (µg/mL) MIC	Tween 80 and DMSO	Sterility control	Microorganism control	ketoconazole (µg/mL) MIC
T. rubrum LM-115	+	512	+	-	+	1024
T. rubrum LM-629	+	512	+	-	+	512
T. mentagrophytes LM-119	+	512	+	-	+	512
M. gypseum LM-512	+	512	+	-	+	256
M. canis LM-68	+	256	+	-	+	+
M. canis LM-110	+	512	+	-	+	+

(+) Microbial growth. (-) Absence of microbial growth. Source: Authors (2022).

The result presented for FCAB is important, since the genera *Trichophyton* and *Microsporum* are the main causative agents of dermatophytosis in humans and animals, and it is considered one of the most diagnosed infectious diseases worldwide (Pereira et al., 2020). Research aimed at finding new products with antifungal activity is necessary to overcome the difficulty encountered in the treatment of these mycoses.

In addition, FCAB is as an antifungal candidate and an alternative to synthetic antifungals, especially chlorine-based compounds, since many of these products have carcinogenic and teratogenic properties, in addition to residual toxicity (Santos et al., 2017; Chandra et al., 2020).

3.3 FCAB Cytotoxicity assessment

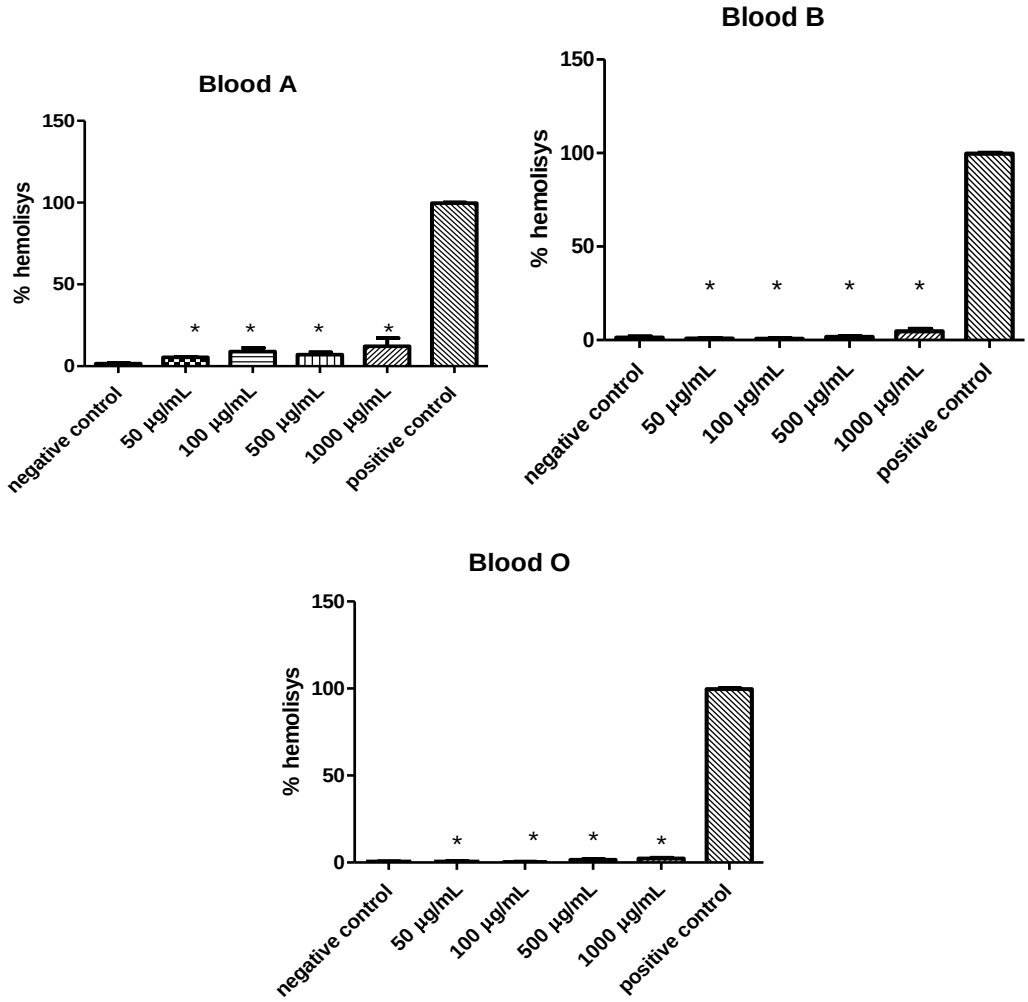
The evaluation of cytotoxicity against human blood cells (erythrocytes) was performed since, despite having antidermatophytic activity for the studied strains, FCAB may not be useful for pharmacological preparations if it has a toxic effect. Therefore, the importance of evaluating the balance between pharmacological activity *versus* toxicity is evident, in order to verify whether the therapeutic applicability of the FCAB extract is promising.

One of the experimental models used for *in vitro* toxicity assessment is the cytotoxicity assay using blood suspensions with human erythrocytes (A, B and O). This test is used as a screening method for the toxicity of new drugs in order to estimate

the harm they can produce *in vivo*. The evaluation of cytotoxicity has the advantages of reducing systemic effects, lower drug and time expenditure, reduction in animal tests, in addition to allowing studies in human cells (Sharopov et al., 2016).

The hemolytic activity of FCAB is shown in Figure 4. Each concentration shows the average percentage of hemolysis repeated in three experiments. According to Rangel et al. (1997), a percentage of hemolysis between 0 and 40% can be considered low; from 40 to 80%, moderate and, above 80%, high. Even testing on different blood types, the FCAB samples at the highest concentration did not reach 40% hemolysis; therefore, it can be considered of low cytotoxicity, compared to the positive control.

Figure 4. Evaluation of FCAB cytotoxicity against type A, B and O erythrocytes.



Source: Authors (2022).

4. Conclusion

The results observed in this study demonstrated the potential that FCAB with *P. roqueforti* ATCC 10110 has to generate a higher content of total phenolics and to be a promising antifungal against strains of dermatophyte fungi of the genera *Trichophyton* and *Microporum*.

The cytotoxicity results revealed that FCAB showed a low percentage of hemolysis for human erythrocytes of the ABO system and, therefore, it can be an important natural raw material or an alternative therapeutic agent in combination with other products for *in vivo* tests in the treatment of dermatophytosis, since the use of antifungal agents from natural products is globally accepted by the specialized scientific community. However, auxiliary studies are necessary to better elucidate the mechanisms of action of this natural product.

Thus, the results found here encourage us to continue the studies in the search to elucidate the phenolic potentials responsible for the strong antifungal activity demonstrated by the extracts, together with new in vivo tests.

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