Current Trends in Biotechnology and Pharmacy
Vol. 17(3) 1020 - 1029, JUly 2023, ISSN 0973-8916 (Print), 2230-7303 (Online)
DOI: 10.5530/ctbp.2023.3.40

Antifungal Activity of Crude Extract from the Seeds of *Inga laurina* (Fabaceae)

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Abstract

This study aimed to evaluate the antimicrobial activity of crude extract from the seeds of *Inga laurina* against bacterium and fungi and develop a topical preparation with antimicrobial activity. The *I. laurina* seed flour was extracted with phosphate buffer (pH 7.6) to obtain saline crude extract (SCE). The antimicrobial activity was determined by the broth microdilution method. Carbopol gel containing different concentrations of extract (1, 5, 10, 15, and 20% w/w) was prepared and stored (40 °C ± 2 °C) during selected intervals for up to 30 days. Homogeneity, odor, color, pH, and microbiological activity of the gel were analized. We report that SCE was active at 4 mg/mL, showing specificity against fungi and no signs of acute toxicity in the *Galleria mellonella* model. The MIC values ranged between 31.25 to 1000 µg/mL. The Carbopol gel + SCE (5%) showed an inhibition zone of 14.66 ± 1.22 mm at day zero. However, there was a loss of antifungal activity after 21 days. This is the first report that proposes the development of a preparation containing extract from the seeds of *Inga laurina*, which has demonstrated potential application in the treatment of topical infections associated with *Candida sp*.

Keywords: *Candida albicans*; Carbopol gel; stability study; semi-solid formulation; saline crude extract.

Introduction

Microbial resistance to traditional therapeutic antibiotics has attracted attention to discovering new compounds with biological activities. In recent years, resistant bacteria have outperformed even the most promising antibiotics, making it challenging to treat complex infections. Therefore, it is urgent and necessary to develop more effective and less toxic ways to treat these infectious diseases (Nathan 2020).

Plants, sources of promising biologically active molecules for the treatment of various diseases, account for 50-70% of all agents in clinical use (Pye et al. 2017). Among the molecules of natural origin that have potential biological action are protease inhibitors (PIs), proteins or peptides produced by all living organisms.

PIs are potential molecules for therapeutic use due to their broad-spectrum antibacterial activity, greater efficacy at low concentrations, target specificity, low propensity to resistance, low impact on the environment, and synergistic action with classical antibiotics (Da Cunha et al. 2017).

Inga laurina (Sw.) Willd. (Fabaceae), is a tree species widely distributed in the Brazilian flora, occurring in the Cerrado, Amazon, Caatinga and Atlantic Forest. Our research group reported several biological activities associated with *Inga laurina* trypsin inhibitor (ILTI) extracted from its seeds, including antimicrobial and insecticidal activity against insect pests.

This work aimed to evaluate the antimicrobial activity of protein extracts obtained from *Inga laurina* seeds and to develop an antimicrobial semi-solid preparation for topical use containing the extract.

Methods

Plant ,aterial

About 300 g of *Inga laurina* (Fabaceae) seeds were dried at room temperature and then crushed to obtain flour. The delipidation of the flour was carried out with the organic solvent hexane (1:2 w/v).

Preparation of crude saline extract

The saline crude extract (SCE) was extracted with 0.1 M phosphate buffer, pH 7.6 (1:10, w/v) for 2 h followed by centrifugation at 10,000 rpm for 30 min. The supernatant obtained was concentrated on a rotary evaporator and was freeze-dried. The protein quantification was performed using bovine serum albumin to obtain a curve-standard concentration. The assay was performed in triplicate and the absorbance reading at 595 nm.

Inhibitory activity against trypsin

The inhibitory activity against trypsin was determined by measuring the remaining hydrolytic activity of trypsin towards the substrate N-Benzoyl-DL-arginine-*p*-nitroanilide (BApNA) at pH 8.0 after pre-incubation with SCE (0.1; 0.2; 0.4; 0.8 and 1 µg protein). Trypsin inhibition was determined by the residual activity of the enzyme and expressed in relation to the hydrolysis promoted in the absence of the SCE which corresponds to 100%. The reduction of residual activity corresponds to the inhibition of enzymatic activity.

Polyacrylamide gel electrophoresis

Sodium dodecyl sulfate-polyacrylamide gel (12%) electrophoresis (SDS–PAGE) was performed. The High-Range Rainbow Molecular Weights Marker (12-225 kDa) was used as the molecular weight standard. The proteins were detected by staining with 0.05% Coomassie Brilliant Blue R-250.

Microorganisms

The American Type Culture Collection (ATCC) strains were used to assess the antimicrobial activity: Gram-negative pathogenic bacteria *Acinetobacter baumani* (ATCC 19906), *Escherichia coli* (ATCC 35218), *Klebisiella pneumoniae* (ATCC 13883), *Proteus mirabilis* (ATCC 12453) and *Pseudomonas aeruginosa* (ATCC 9027); Gram-positive pathogenic bacteria *Staphylococcus aureus* (ATCC 29213), methicillin-resistant *Staphylococcus aureus* (MRSA) (ATCC 43330) and *Staphylococcus epidermidis* (ATCC 12228); pathogenic fungal strains *Candida albicans* (ATCC 5314), *Candida glabrata* (ATCC 90030), *Candida guillermondii* (ATCC 6260), *Candida krusei* (ATCC 6258), *Candida parapsilosis* (ATCC 90018), *Candida tropicalis* (ATCC 750) and *Cryptococcus gattii* (AFLP4).

Determination of minimum inhibitory concentration (MIC), minimum bactericidal concentration (MBC), and the minimum fungicidal concentration (MFC)

MIC and MBC/MFC were determined by broth microdilution method (CLSI 2008), protocols M07-A9 and M27-A2. The MIC assay was performed in a 96-well microplate with a serial concentration of 4 to 0.0078125 mg/mL of SCE. MIC was defined as the lowest extract concentration capable of inhibiting visible microbial growth in the microplate. Each assay was performed in triplicate using three independent experiments.

CBM and MFC were determined using 10 µL aliquots of the subculture from the wells corresponding to the MIC, which were inoculated onto Mueller Hinton Agar (MHA) or Sabouraud Dextrose Agar (SDA), respectively. MBC and MFC were defined as the lowest concentration of extract that did not allow visible microbial growth in the solid medium after the incubation period (CLSI 2008).

Potential antifungal mechanism of action D-sorbitol and ergosterol assay

MIC values of SCE with *C. gattii* and *C. albicans* were determined by microdilution methodology, in the absence and presence of 0.8 M of D-sorbitol to investigate whether SCE acts on the fungal cell wall (CLSI 2002). To investigate whether SCE acts on the fungal cell membrane, the ergosterol assay was performed (CLSI 2002). All the assays were carried out with *C. gattii* and *C. albicans* in triplicate.

Inhibition of Candida albicans biofilm formation

The biofilm formation inhibition activity of *C. albicans* was performed in a sterile 96-well microplate. Percentage survival was determined based on the comparative survival of treated and untreated biofilms (Sardi et al. 2017).

Preparation of Carbopol gel base

Purified water (3 mL) and methylparaben (0.2 g) were heated (75 - 80 °C) until complete solubilization. To this solution were added 3 ml of glycerin and, under constant agitation, Carbopol-940 gel (1 g) and triethanolamine (1 ml dissolved in 3.0 ml of water). The formulation remained under agitation until it became homogeneous. Purified water was added until obtaining 100 g (final mass). Five concentrations of Carbopol gel + SCE were tested: 1, 5, 10, 15 and 20% (w/w).

Evaluation of stability study of gel preparation containing I. laurina protein extract

In the accelerated stability test, Carbopol gel + SCE was evaluated after storage (40 ± 2 °C) for 30 days. The following were evaluated: appearance, color, odor, system homogeneity, pH and microbiological activity at day zero (freshly prepared) and after 7, 15, 21 and 30 days. The negative control (Carbopol gel without SCE) was subjected to the same conditions and tests.

Color

The color analysis of each formulation was performed by visual colorimetry, comparing the color of the Carbopol gel + SCE and the control.

Odor

The odor analysis was performed directly through the sense of smell, by comparing the Carbopol gel + SCE and the control, which were stored under the same conditions.

Homogeneity

In a minicentrifuge with a rotation of 3000 rpm, 1 g of Carbopol gel + SCE was centrifuged for 30 minutes at room temperature.

Weight loss

The formulations were weighed and the respective masses (g) were recorded at the end of each experiment and before starting the next experiment, at day zero and after 7, 15, 21 and 30 days, to verify if during the time under 40 °C ± 2 °C, there was a significant loss of water mass.

pH determination

The pH of the Carbopol gel and Carbopol gel + SCE 5% was determined using a digital pHmeter at 18 °C, dissolving the formulation in purified water (1:10).

Evaluation of antimicrobial activity

In vitro antimicrobial activity was determined by the agar diffusion technique (Valgas et al. 2007) against *C. albicans* (1 x 10^6 CFU/mL). The inoculum was seeded onto Sa-

bouraud Dextrose Agar, and four equidistant holes (4 mm deep and 6 mm in diameter) were aseptically drilled into the agar surface. Nystatin cream (100,000 IU/4 g, 0.43% w/w, positive control) and Carbopol gel + SCE (150 mg) were introduced into the wells. Plates were incubated at 37 °C for 24 h and antimicrobial activity was determined by measuring the diameter (mm) of inhibition zones.

Evaluation of acute toxicity in vivo in an invertebrate model of Galleria mellonella

Random *G. mellonella* larvae weighing 0.2-0.3 g were maintained in sterile Petri dishes and treatments consisted of SCE (250 and 1250 µg/mL in sterile 0.9% saline) and 0.0% saline 9% sterile (negative control). For each treatment and control, ten larvae were used in triplicate. Larvae were considered dead when they showed immobility to the touch and a high degree of melanization (De Barros et al. 2020).

Statistical analysis

Data from biofilm assays and stability studies were analyzed by one-way analysis of variance (ANOVA), with a significance level of 5%, followed by Tukey's multiple comparison post-test. For *G. mellonella* model, the log-rank test was performed in the Mantel-Cox survival curves, with $p<0.05$.

Results and Discussion

Extraction of soluble proteins and obtaining the saline crude extract

From 74 g of *Inga laurina* seed flour, 4.26 g of SCE was obtained, (5.43% yield). Analysis of the SCE protein profile by SDS-PAGE revealed two protein bands with molecular weights between 20 and 52 kDa. Additionally, the trypsin inhibition assay confirms the occurrence of ILTI in the crude saline extract (Macedo et al. 2007).

The ILTI (isolated from the *I. laurina* seed extract in our research group - Macedo et al., 2007) has an approximate size of 20 kDa, consistent with what was observed in this study. It also shows similarity with other protease inhibitors of the Kunitz family (18-26 kDa): EATI (*Entada acaciifolia* trypsin inhibitor) (De Oliveira et al. 2014), ECTI (*Enterolobium contortisiliquum* trypsin inhibitor) (Zhou et al. 2013), (EVTI (*Erythrina velutina* trypsin inhibitor) (Machado et al. 2013) and EPTI (*Erythrina poepgiana* trypsin inhibitor) (De Barros et al. 2021) In this study, the crude extract also demonstrated high ILTI content, confirming the efficacy of protein extraction from *I. laurina* seeds (Macedo et al. 2007).

Antimicrobial activity and determination of MIC, MBC/MFC

In the SCE antifungal activity assay, different strains of *Candida* and *Cryptococcus gattii* were tested. The SCE was able to inhibit the fungal growth of all strains tested (MIC between 31.25 and 1000 µg/mL), showing the best MIC and MFC results for *C. gattii* (MIC and MFC = 31.25 µg/mL) (**Table 1**). Macedo et al. (2016) demonstrated total inhibition of *C. tropicalis* by ILTI at 125 and 250 µg/mL concentrations. At a concentration of 250 µg/mL, the authors found 9 and 12% inhibition for *C. albicans* and *C. parapsilosis*, respectively.

In this study, we tested other bacteria to verify the selectivity of the SCE. The results indicate that SCE did not inhibit the growth of the Gram-positive and Gram-negative bacterial strains tested at a concentration of 4 mg/mL (data not shown). These results corroborate what was found in the literature and indicate the selectivity of SCE against fungi.

Potential antifungal mechanism of action D-sorbitol and ergosterol

The potential mechanisms involved with the observed antifungal activity were investigated: action on the fungal cell wall and action on the fungal cell membrane. Drugs that act on the cell wall cause cell lysis without D-sorbitol, which acts as an osmotic stabilizer (Levitz 2010). Ergosterol is the principal sterol in the

Table 1 Minimum inhibitory concentration and minimum fungicidal concentration of saline crude extract from *Inga laurina* (Fabaceae), and Amphotericin B (positive control) against fungal strains.

Yeast	MIC (µg/mL)	MFC (µg/mL)	Amphotericin B (µg/mL)
Cryptococcusgattii(AFLP4)	31.25	31.25	0.5
Candidanivariensis(ATCC 9983)	125	125	-
Candidaalbicans(ATCC 5314)	250	250	0.5
Candidaguillermondii(ATCC 6260)	250	250	0.25
Candidakrusei(ATCC 6258)	250	250	0.5
Candidabracarensis(ATCC 10154)	250	250	-
Candidaparapsilosis(ATCC 2209)	250	250	0.25
Candidaglabrata(ATCC 90030)	500	500	0.5
Candidatropicalis(ATCC 750)	1000	1000	0.5

ATCC: American type culture collection; **MIC:** Minimum inhibitory concentration; **MFC:** Minimum fungicidal concentration

fungal cell membrane and an essential component associated with the membrane's fluidity, permeability and integrity (Rodrigues 2018). Our results demonstrate a significant difference in the MIC values of the SCE in the absence and presence of D-sorbitol (**Table 2**), suggesting that the SCE acts by inhibiting fungal cell wall synthesis in *Candida albicans* and *Cryptococcus gattii*. Similar to what was observed in the D-sorbitol assay, it was possible to verify that the presence of exogenous ergosterol also affected the MIC value, increasing it both for *C. albicans* and *C. gattii* (**Table 2**).

Effects of SCE on biofilm formation

Biofilm formation represents a significant problem in its treatment. The dose required to eradicate a biofilm can exceed the highest concentrations allowed in clinical treatments with antifungals, up to 1000 times more resistant to antimicrobial agents than the planktonic (free) form (Stewart and Costernon 2001).

In the results of this work, the MIC found for *C. albicans* in its planktonic form was 250 µg/mL. However, not even a concentration of 10 x MIC (1250 µg/mL) was efficient in inhibiting *C. albicans* biofilm formation. Considering the complexity of the biofilm structure, an antimicrobial agent can inhibit microbial growth in its planktonic form but not necessarily in its biofilm

Table 2 Minimum inhibitory concentration of saline crude extract from *Inga laurina* (Fabaceae) against *Candida albicans* and *Cryptococcus gattii* in the absence and presence of D-sorbitol (0.8 M), and ergosterol (400 µg/mL). Values are expressed in µg/mL.

Treatment	Candidaalbicans		Cryptococcusgattii	
	D-sorbitol		D-sorbitol	
	Absence	Presence	Absence	Presence
SCE	250	>4000	31.25	>4000
Caspofungin (Control)	0.12	15.6	0.06	4.0
	Ergosterol		Ergosterol	
	Absence	Presence	Absence	Presence
SCE	250	>4000	31.25	>4000
Amphotericin B (Control)	0.5	16.0	0.5	8.0

SCE: Saline crude extract from *Inga laurina*

form (Pletzer and Hancock 2016). Amphotericin B (0.5 µg/mL) prevented biofilm formation but is also associated with several adverse patient effects due to its high toxicity (Cavassin 2021).

Acute toxicity of extract in Galleria mellonella model

An acute toxicity test was performed on *G. mellonella* at 250 and 1250 µg/mL (10 mg/Kg/larva and 50 mg/Kg/larva, respectively). The control curve was obtained by systemic administration of sterile 0.9% saline solution into the larval hemocoel. The results of the toxicity analysis showed that the tested doses did not kill the larvae, indicating that the SCE at these concentrations was not toxic (**Figure 1**).

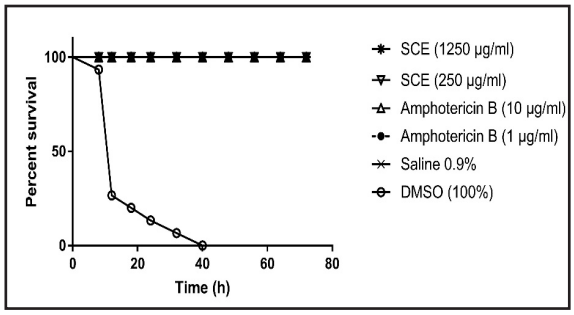


Figure1: Survival (%) of Galleria mellonella larvae injected with SCE (1x and 5x the MIC value, corresponding to 10 mg/kg/larva and 50 mg/kg/larva, respectively) and controls ($p<0.05$, log test -rank).

SCE concentration in the gel formulation

The inhibitory activity of the Carbopol + SCE gel was evaluated at concentrations of 1, 5, 10, 15 and 20% (w/w) in the agar diffusion assay (**Figure 2**). Carbopol gel + SCE (1%) showed no statistical difference with the positive control Nystatin, forming an inhibition halo of 10.15 ± 0.58 and 11.68 ± 1.17, respectively. Carbopol gel + SCE (5%) showed an inhibition halo of 18.09 ± 1.63, significantly higher than Nystatin. Carbopol gel + SCE 10, 15 and 20% showed no significant difference with Carbopol gel + SCE (5%). The results suggested that the inhibition of *C. albicans* is due to the action of ILTI, leading to a rupture in the architecture of the microorganism's cell wall and cell membrane.

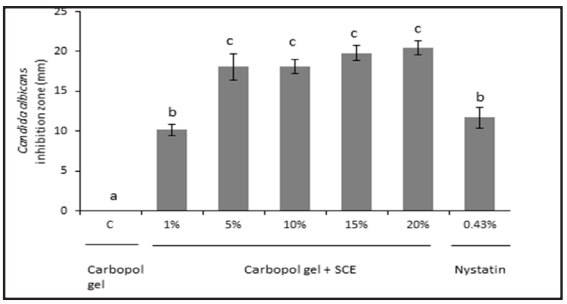


Figure2: Inhibition zones (mm) of the Carbopol gel + SCE at different concentrations: 1, 5, 10, 15, and 20%, and Nystatin 0.43% (positive con-trol) on Candida albicans. Different letters indi-cate a statistically significant difference (one-way ANOVA at $p<0.05$ followed by Tuckey's test).

Stability study of Carbopol gel + SCE

Carbopol gel + SCE was stable and kept organoleptic characteristics for 30 days. The gel containing 5% extract (w/w) was stored and evaluated at intervals of 7, 15, 21 and 30 days after day zero. Activity parameters were evaluated for color, odor, weight loss, pH, and biological activity against *C. albicans*.

After 30 days, both Carbopol gel + SCE (5%) and Carbopol gel base (control) did not show phase separation after centrifugation, indicating that the systems remained stable after the test period. At day zero, the Carbopol gel stain was clear and translucent and remained so after 30 days. However, Carbopol gel + SCE (5%) showed a dark brown color (**Table 3**) from its preparation until the end of the test period. After 30 days, the color did not change in the preparations or controls. However, the glossy aspect observed in the Carbopol gel + SCE (5%) changed to matte after 30 days and the weight loss reported was 1.29 ± 0.71 g when storage at 40 ºC.

As for the microscopic analysis, it was observed that the Carbopol gel + SCE, about the control, presented small particles corre-

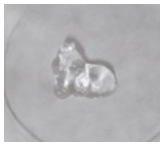
sponding to the SCE. In **Table 3**, it is possible to observe the granules dispersed in the preparations, presenting different sizes and without uniformity on the slide. Regarding the initial mass and the final mass, it was observed that the samples showed no significant reduction in mass.

pH

Topical preparations with pH close to physiological (4.0 to 7.0) are appropriate for preventing and treating skin diseases (Proksch 2018; Lukic et al. 2021).

The pH of the Carbopol gel showed pH values between 6.98 and 7.65 and the Carbopol + SCE gel showed pH values between 4.88 and 7.27 during the 30 days. Thus, all formulations in this study were within the pH range of the skin and did not present a significant difference from the average pH of the control (**Table 3**).

Table 3: Characteristics of the Carbopol gel + SCE (5% w/w) after the end of the stability study (30 days).

Properties	Formulations	
	Carbopol gel	
+ SCE 5% (w/w)	Carbopol gel	
(Control)		
Appearance		
Color	Marrom escuro	Incolor
Odor	-	-
Formação de fases	No odor	No odor
pH	6,57±0,96	7,56±0,1
Weightloss (g)	1.29 ± 0.71	1.63 ± 0.86
Light microscopyanalysis (100x)		
SCE: Saline crude extract from Inga laurina.		

Antimicrobial activity of Carbopol gel + SCE

The antifungal activity of the preparations was demonstrated using halo inhibition measurements (mm) of *C. albicans* during day zero, after 7, 15, 21 and 30 days. At day zero, the gel incorporated into SCE 5% (w/w) showed an inhibition halo of 14.66 ± 1.22 mm, which was more significant (p<0.05) than the control with nystatin cream (0. 43%), which presented a mean halo equivalent to 11.21 ± 0.53 mm (**Figure 3**).

However, after seven days from day zero, the gel incorporated into the SCE showed significantly decreased antifungal activity. After 15 days, the halo inhibition of the Carbopol + SCE gel again showed a decrease in the halo inhibition (8.98 ± 0.29 mm), and at 21 and 30 days, the gel no longer showed antifungal activity.

Nystatin cream was active against *C. albicans* throughout the analyzed period, with a mean inhibition zone of 10.65 ± 0.50 mm. The carbopol gel (control) showed no inhibition throughout the stability study (**Figure 3**).

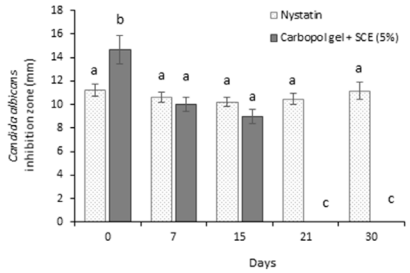


Figure3: Inhibitory effect of the Carbopol gel + SCE 5% and the Nystatin cream 0.43% (positive control) on C. albicans. Different letters indicate a statistically significant difference (one-way ANOVA at p<0.05 followed by Tuckey's test).

Campos et al. developed a chitosan gel with methanolic extract of *Mitracarpus frigidus* for the treatment of vulvovaginal candidiasis. The 10% concentration (w/w) showed more significant and faster antifungal activity than the other concentrations, reducing 50% of the fungal

burden on the third day of treatment. However, none of the formulations managed to eliminate the infection after six days of treatment.

The ability to inhibit the growth of *C. albicans* demonstrated in this study can be understood by the different mechanisms of antifungal action associated with ILTI and other protease inhibitors already isolated from plants. ILTI can damage the cell membrane of *C. tropicalis* and *C. buinensis* (Macedo et al. 2016) by acting on mitochondria and the nucleus of fungal cells, suggesting the generation of reactive oxygen species in *C. tropicalis* and *C. buinensis*. Thus, it is suggested that the mechanisms of action of ILTI that lead to the death of *Candida* species may be associated with targets other than membrane targets (Macedo et al. 2016).

Viscosity can affect the diffusion process and the availability of the active ingredients that make up the extract, as seen in the work by Adeleye et al. (2019), who developed a cream from *Andrographis paniculata* extract and investigated the effect of shea butter in formulations on antimicrobial activity. Different formulations of the cream (5, 10 and 20% (w/w) of *A. paniculata* extract and shea butter) were tested against bacterial and fungal pathogenic strains by agar diffusion. The study revealed that, by incorporating shea butter, the viscosity of the creams increased, as well as their antimicrobial activity. The physical stability of the cream formulations was maintained during the 30 days of analysis, with no change in color, texture or homogeneity, demonstrating that the inclusion of shea butter had a synergistic effect with the antimicrobial activity of the *A. paniculata* cream.

This work opens new perspectives on the antimicrobial potential of ILTI with application in pharmaceutical preparation, encouraging future studies that improve the formulation.

In conclusion, the saline extraction of I. laurina seeds, a native and abundant species in Brazil, presents a high protein content of ILTI. The extract is safe and non-toxic and has demonstrated in vitro antifungal activity against *Candida* species and *Cryptococcus gattii*. The Carbopol + SCE 5% gel could inhibit the microbial growth of *Candida albicans*. However, the proposed formulation showed a loss of activity after 21 days, making it necessary to investigate its reformulation to prolong the durability of the antifungal activity. Thus, additional studies that make its production more efficient and stable can be encouraged to meet the worldwide demand for developing products with antimicrobial action.

Acknowledgements

This work was supported by PROPP/ UFMS, CAPES (Coordenação de Aperfeiçoamento de Pessoal de Nível Superior), FINEP (Financiadora de Estudos e Projetos), FUNDECT (Fundação de Apoio ao Desenvolvimento do Ensino, Ciência e Tecnologia do Estado de Mato Grosso do Sul) and CNPq (Conselho Nacional de Desenvolvimento Científico e Tecnológico).

Ethical approval

Not applicable.

Conflict of Interest

The authors declare no conflict of interests.

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