

Full Length Research Paper

## Inhibition of growth and aflatoxin production of *Aspergillus parasiticus* by guaraná (*Paullinia cupana Kunth*) and jucá (*Libidibia ferrea Mart*) extracts

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In the present study, fruits extracts (*Paullinia cupana* K. & *Libidibia ferrea* M. - guaraná & jucá, respectively) of the Amazon Region were tested for antifungal and antimycotoxigenic activities against *Aspergillus parasiticus* using the agar dilution method. The treatments utilized were at three different concentrations (1.08, 1.62 and 3.24%). The effect on both extracts on growth diameter of fungal colony was time and concentration dependent. No treatment completely inhibited fungi growth, however *A. parasiticus* was significantly reduced by the treatments when compared to the control group. The production of AFB<sub>1</sub>, AFB<sub>2</sub>, AFG<sub>1</sub> and AFG<sub>2</sub> by *A. parasiticus* grown on guaraná and jucá extracts treatment was significantly smaller when compared to controls. *A. parasiticus* strain produced aflatoxins in all concentrations when grown on guaraná extracts medium treated and different of that observed when on jucá's treated extracts.

**Key words:** *Aspergillus parasiticus*, *Paullinia cupana*, *Libidibia ferrea*, aflatoxin.

### INTRODUCTION

Aflatoxins, the secondary metabolites produced by strains of *Aspergillus flavus* (*A. flavus*) and *Aspergillus parasiticus* (*A. parasiticus*) are known to be potent carcinogens and hepatotoxic agents and pose a severe hazard to animal and human health (Stoloff, 1977). Aflatoxin B<sub>1</sub> (AFB<sub>1</sub>) is the most potent of the four naturally occurring aflatoxins namely (AFB<sub>1</sub>, AFB<sub>2</sub>, AFG<sub>1</sub> and AFG<sub>2</sub> (Figure 1). Thus, foods contaminated with these toxigenic fungi and presence of aflatoxin is a major concern, which has received world wide attention due to

their deleterious effects on human and animal health as well as their importance in international food trade (Mishra and Das, 2003). A major challenge for several foods production is the control of contamination by aflatoxigenic fungi and aflatoxins production. Although, there are many synthetic fungicides in use, their safety in foods are yet to be fully established and also the chances of obtaining newer fungicides that meet stringent environmental and food safety requirements through empirical synthesis do not appear to be high

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(Gopalkrishnan et al., 1997). The use of numerous plant extracts and their constituents may provide an alternative way to prevent fungal growth and aflatoxin formation (Fan and Chen, 1999; Mahmoud, 1999). Medicinal plants constitute the basis of health care systems in many Amazonian communities in Brazil. Ethnopharmacological studies in this region indicate jucá (*Libidibia ferrea* Mart) as an antifungal, antimicrobial and anti-inflammatory healing plant of the Amazonian forest. Locals use it in the form of tea (leaves, fruits or peel), syrup (peel) and as oral mouth wash steeping the fruits in alcohol for days (Vieira, 1992; Di Stasi and Hiruma-Lima, 2002; Borrás, 2003; Sampaio et al., 2009).

In spite of its antimicrobial properties, most of the studies with the crude extract of jucá had focused on its high content of polyphenols and the analgesic, anti-inflammatory, antiulcer and cancer chemopreventive properties (Port et al., 2013, Bacchi et al., 1995; Queiroz et al., 2001; Nakamura et al., 2002). The crude extract of jucá contains anthraquinones, alkaloids, depsides, depsidones, flavonoids, lactones, saponins, sugars, tannins, sesquiterpenes and triterpenes. Tannins are regarded as the major component (Souza et al., 2006).

The therapeutic properties of jucá fruits have been described and studied over the years and they include the treatment of wounds and bruises and chronic cough and asthma relief (Hashimoto, 1996). Jucá fruits feature antiulcerogenic (Bacchi and Sertie, 1994; Bacchi et al., 1995), anti-inflammatory and analgesic properties (Carvalho et al., 1996). Souza et al. (2006) in their study to assess genetic toxicity, from three different concentrations of the crude extract obtained from the fruits of jucá investigated their potential cytotoxic and clastogenic and described that extract in rats showed no toxic effect. Guaraná (*Paullinia cupana* Kunth) is one of the best-known native examples of Brazilian Amazonian biodiversity, in addition to having great economic value (Kuskoski et al., 2005).

Guaraná, a low-growing bush-type plant, is the richest vegetable source of caffeine (Mehr et al., 1996; Weckerle et al., 2003). The seeds of this plant also contain theophylline, theobromine, xanthine derivatives and tannins and also catechin, epicatechin and proanthocyanidins (Majhenic et al., 2007).

Due to its high caffeine content, its seed has been suggested to have natural protection against mycotoxins from toxigenic fungi. The activity of guaraná extracts against bacteria and fungi was tested demonstrating significant inhibition for several species (Basile et al., 2005; Majhenic et al., 2007).

Several substrates with antifungal activity of plant origin have been studied as a way to prevent the effects of toxigenic fungi, for example *Satureja hortensis* L. essential oil (Razzaghi-Abyaneh et al., 2008), traditional medicinal plants from Tamil Nadu, India (Duraipandiyan and Ignacimuthu, 2011), citrus species (Salas et al., 2011),

combined plant extracts (Sindhu et al., 2011), eugenol (Komala et al., 2012), *Caesulia axillaris* Roxb. essential oil (Mishra et al., 2012), *Callistemon lanceolatus* (Sm.) sweet essential oil (Shukla et al., 2012), essential oil from *Cicuta virosa* (Tian et al., 2012).

To the best of our knowledge, there has not been a relevant study on the effectiveness of any species of *Paullinia cupana* K and *Libidibia ferrea* M. against aflatoxin production by *A. parasiticus*. The objectives of the present investigation were to evaluate the efficiency of guaraná and jucá extracts against the growth and aflatoxin production by *A. parasiticus*.

## MATERIALS AND METHODS

### Plant material

#### Obtaining the vegetable raw material

The samples of guaraná (only the seeds) were acquired in a market in the city of Manaus. The jucá fruit (except its seeds) were collected in the city of Manaus-AM, Brazil, in September 2011. The identification was carried out in the Herbarium National Institute for Amazonian Research (INPA), which was incorporated under the voucher specimen number 228022. The seeds and fruits of both Amazonian vegetables respectively were ground in a blender and grinder. Conventional sieves with a mesh of approximately 1 mm<sup>2</sup> were used to obtain homogeneous flour which was wrapped in plastic pots for later use in the tests.

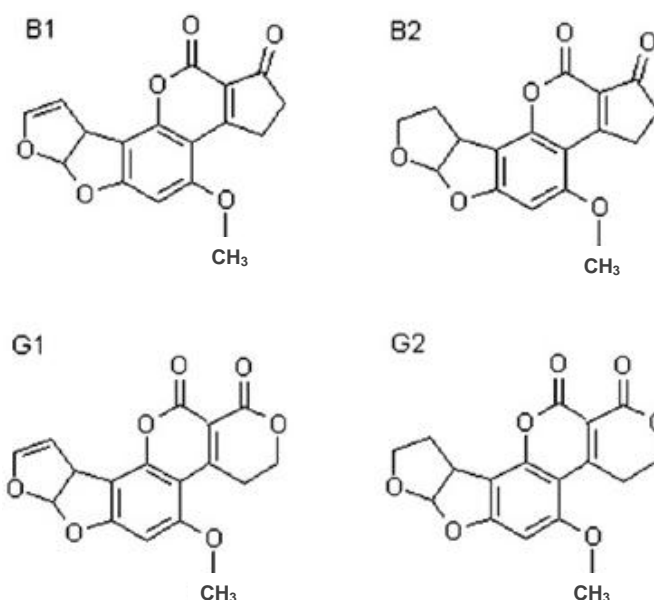
### Extract preparation

The guaraná and jucá extracts were obtained according to the method described by Majhenic et al. (2007), with some modifications. Samples were weighed into 6, 9 and 18 g quantities and added to 100 ml of ethanol (Synth, Diadema, Sao Paulo) /water (60:40 v/v). The extraction was carried out with magnetic stirring for 2 h (room temperature), followed by Buchner funnel filtration through filter paper (Nalgon, porosity 3 and diameter 12.5 µ) with the aid of a vacuum pump. The final volume was adjusted to 100 ml with the solvent (ethanol/water 60:40 v/v).

### Antifungal activity test

#### Fungal strains and inocula

Strains of *A. parasiticus* were obtained from the culture collection of the Laboratory of Mycotoxicology and Contaminants in Foods - LABMICO Federal University of Santa Catarina - UFSC. The identification of fungi genera and species: for the (a) morphological characterization of the isolated strains was carried out for the *Aspergillus* genera on MEA, GN25 and CYA media. On the other hand, the (b) species identification was performed through microculture Czapek-dox for *Aspergillus* as described by Weber and Pitt (2000) and followed the Samson et al. (2006) keys. For the (c) species micromorphological observation, the isolates were examined under the light microscope (100x and 400x magnification) with species identification carried out according to the taxonomic keys and guides available as follows (Pitt and Hocking, 1997; Raper and Fennel, 1965). The culture was maintained at 25°C on slants of Potato Dextrose Agar (PDA). These fungus were previously cultivated in strains containing malt extract agar (MEA)



**Figure 1.** Chemical structure of aflatoxins B1, B2, G1 and G2.

over the course of 7 days to assure fungus purity. Then, 10 ml of Tween 80 (Biokar) (0.02%, dissolved in distilled water) was added and the tubes were shaken for 1 min in a vortex to separate the conidia from the rest of the medium.

The conidia concentration in suspension was determined, using a Neubauer counting chamber. The value obtained was as follow: *A. parasiticus*,  $1.0 \times 10^7$  conidia /ml.

#### Agar dilution method

Fungi were tested using the agar dilution method according to APHA (1992) described by Salas et al. (2011). In brief, the extracts (in the following concentrations: 1.08, 1.62 and 3.24%) were added to culture medium at a ratio of 18/2 v/v (18 ml of medium and 2 ml of extract). The extracts were added to the medium and then 20 ml of the mixture was poured into a disposable petri dish (90 × 15 mm). The medium in question was MEA, which contained 20 g of malt extract, 20 g of agar, 1 g of glucose and 20 g of peptone per litre of distilled water. After the addition of medium, the plates were allowed to dry for two days. MEA plates without guaraná and jucá extracts (control), and MEA plates with the guaraná and jucá extracts were inoculated with the same inoculum in triplicate for each mould. For each treatment, a volume of 10 µL of the conidia concentration in suspension was inoculated ( $10 \pm 0.2$  µL). The plates were incubated at  $28 \pm 1^\circ\text{C}$  in the darkness for 8-12 days (for toxin production). In order to evaluate the fungi growth, two colony diameter measurements were taken to assess the treatment efficacy twice daily for 86 h. The colony radius was plotted against time, and linear regression was used to obtain the growth rate constant (k).

#### Antimycotoxin activity test

To evaluate the mycotoxin production, *A. parasiticus* was grown on MEA medium containing the different concentration extracts and the control (MEA without extracts-treatment), at temperature of

$30^\circ\text{C}$  and moisture content of 90% for 12 days. The fungi grown on each MEA medium was transferred into separating funnel and stirred with chloroform for aflatoxins extraction, followed by filtration through anhydrous sodium sulphate ( $\text{Na}_2\text{SO}_4$ ), which was performed three times. The aflatoxin extract was evaporated with nitrogen stream at  $60^\circ\text{C}$  and quantified by thin layer chromatography (TLC) in plates of silica gel 60 G. The detection of AFB1, AFB2, AFG1 and AFG2 (produced by the *A. parasiticus* strains) was performed according to the methods of Moss and Badii (1982). The extracts were resuspended and the chromatogram developed in the following solvent system chloroform/acetone (90:10). Standard solutions and the fungi toxin extracts (10 µL) were applied onto silica plates and the chromatographic run performed within 90 min.

Finally, the plates were placed into a 356 nm UV light cabinet to detect the toxins fluorescence, limit of detection (LOD): 2 µg/kg. AFB1 and AFB2 exhibited a bluish and AFG1 and AFG2 a yellow fluorescence, verified at the following Rfs: 0.55, 0.50, 0.45 and 0.40, respectively.

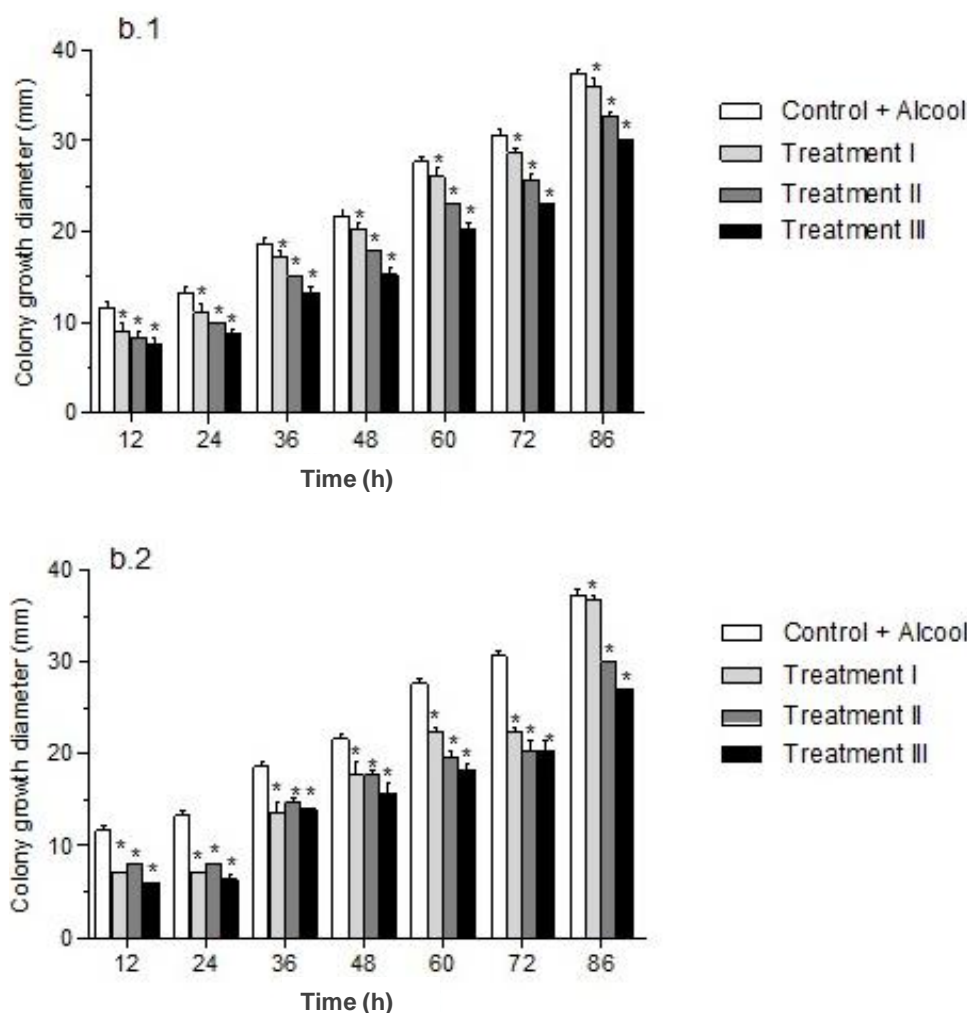
#### Statistical analysis

Colony fungal growth and antimycotoxin activity data were evaluated by analysis of variance (ANOVA) followed by the Bonferroni post-test. All analyses were expressed as the mean  $\pm$  S.D. and *p* values < 0.001 were considered statistically significant. The growth rate constant (k) of the two fungal species was estimated by linear regression. The analysis was conducted using the software programme R (statistical software).

## RESULTS AND DISCUSSION

### Extract antifungal activity

Guaraná and jucá extracts showed inhibitory effects on



**Figure 2.** Antifungal activity of guaraná (a) and jucá (b) extracts on *A. parasiticus* on MEA at different concentrations (data are given as mean values and standard deviations of the diameter of fungal colonies - each point represents the mean of measurements in triplicate). Symbols indicate statistically significant compared with the control group \*  $p < 0.001$ .

growth and different degrees of antifungal activity was observed in the concentrations applied. Figure 2 and Table 1 shows the colonies growth and the growth constant ( $k$ ) for different extracts types and concentrations, respectively. Our results show that the effect of guaraná and jucá extracts on growth diameter of fungi colony was time and concentration dependent (Figure 2). For the tests we used three different concentrations of extracts, 1.08% (treatment I), 1.62% (treatment II) and 3.24% (treatment III) and not treated control group. No treatment completely inhibited fungi growth. *A. parasiticus* was significantly reduced by all treatments when compared to the control. All treatments following 86 h of incubation showed better inhibition compared to the control (mainly the treatment 1). The  $k$  was calculated for all fungi tested. Table 1 shows the values obtained for  $k$  as calculated by

linear regression. Amongst previously the toxigenic strains of *Aspergillus* evaluated, *A. parasiticus* was found to be highly toxigenic, producing AFB1, AFB2, AFG1 and AFG2, and selected to verify the ability to produce aflatoxins in medium containing different extracts of plants of Amazon region in the present investigation. Although, there are earlier reports on variation of antifungal activity of plant products against different species of a particular genus of a fungus (Prakash et al., 2010; Shukla et al., 2009) literature is mostly silent on variation of their efficacy at strain level of a particular fungal species.

The efficacy of guaraná and jucá extracts has so far been not well-explored against storage fungi and mycotoxin contamination. However, there are earlier reports on antimicrobial activity of guaraná extracts against three

**Table 1.** Growth constant (k) of colonies of *A. parasiticus* subjected to different types and concentrations of extract.

Species	Group	Concentration (%)		
		1.08	1.62	3.24
<i>A. parasiticus</i>	Guaraná	0.3660 ± 0.0112	0.3291 ± 0.0094	0.3010 ± 0.0119
	Jucá	0.3774 ± 0.0263	0.2809 ± 0.0183	0.2793 ± 0.0143
	Control		0.3933 ± 0.0102	

species of fungi: *Aspergillus niger*, *Trichoderma viride* and *Penicillium cyclopium*, and two species of Gram negative bacteria: *Escherichia coli* and *Pseudomonas fluorescens* and one species of Gram positive bacteria: *Bacillus cereus* (Majenic et al., 2007), and antimicrobial activity of jucá extracts against the following oral pathogens: *Candida albicans*, *Streptococcus mutans*, *Streptococcus salivarius*, *Streptococcus oralis* and *Lactobacillus* (Sampaio et al., 2009). To the best of our knowledge, there has not been a relevant study on the effectiveness of any species of *Paullinia cupana* K and *Libidibia ferrea* M against aflatoxin production by *A. parasiticus*. Recent studies have demonstrated the antifungal activity of natural compounds extracted from different sources, for example, traditional medicinal plants from Tamil Nadu, India (Duraipandiyan and Ignacimuthu, 2011), citrus species (Salas et al., 2011), *C. axillaris* Roxb. essential oil (Mishra et al., 2012), sweet essential oil (Shukla et al., 2012), essential oil from *C. virosa* (Tian et al., 2012).

In spite of its antimicrobial properties, most of the previous studies with crude jucá extract were focused on its high polyphenols content and its analgesic, anti-inflammatory, antiulcer and cancer chemopreventive properties (Bacchi et al., 1995; Queiroz et al., 2001; Nakamura et al., 2002).

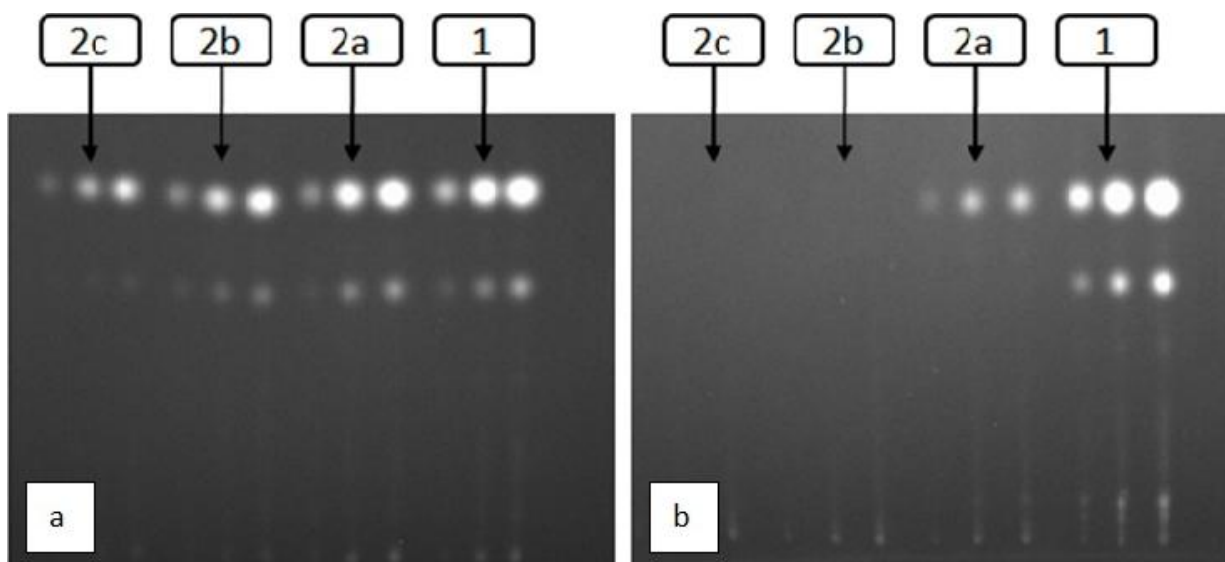
### Antimycotoxin activity test

When different concentrations of extracts were added to the fungal cultures in liquid media, a remarkable reduction in aflatoxin synthesis was observed. The reduction in growth and toxin production was dependent on the concentration of extract. The production of AFB<sub>1</sub>, AFB<sub>2</sub>, AFG<sub>1</sub> and AFG<sub>2</sub> by *A. parasiticus* grown on guaraná and jucá extracts treatment was significantly lower when compared to the controls. *A. parasiticus* strain produced AFLs in all treatments when grown on guaraná extracts medium treated, different of what observed when that fungus grew on extracts jucá (Figure 3). Recent studies have investigated the effects of different compounds [S. *hortensis* L. essential oil by (Razzaghi-abyaneh et al., 2008), combined plant extracts by Sindhu et al. (2011) and eugenol by Komala et al. (2012) on natural strains of

*A. flavus* and *A. parasiticus* and their inhibition of aflatoxin production. Ghorbanian et al. (2008) investigated effect of neem leaf extract on growth of *A. parasiticus* and production of aflatoxin and reported that the inhibition of aflatoxin synthesis by neem leaf extract was found to be time and dose dependent. Bhatnagar and McCormick (1988) also reported that neem leaf extracts added to fungal growth media did not affect fungal growth, but that they essentially blocked aflatoxin biosynthesis at concentrations greater than 10% due to the presence of volatile compounds such as 3-methyl-2-buten-1-ol. Turmeric inhibited spore count and aflatoxin production at the concentration of 0.1 to 1.0% with the highest percent reduction in toxin synthesis (85%) at 1.0% (Gowda et al., 2004). Soni et al. (1992) reported a 90% reduction in aflatoxin production at a 5 to 10 mg/ml concentration of turmeric, an effect attributed to the antioxidant curcumin in turmeric. It has been suggested that the regulation of aflatoxin synthesis and conidiogenesis may be interlinked, since the loss of aflatoxigenic capabilities in the non-aflatoxigenic variant strains of *A. parasiticus* was correlated with alterations in the conidial morphology (Kale et al., 1996).

Ammonia vapors are used to inactivate mycotoxins and one of the characteristics of aflatoxin deactivation processes is that it should destroy the mycelia and spores of the toxic fungi, which may proliferate under favorable conditions (Namazi et al., 2002). Bullerman (1974) studied the effects of cinnamon on growth and aflatoxin production by known toxigenic strains of *A. parasiticus*. It was observed that the cinnamon is an effective inhibitor of aflatoxin production even though mycelium growth may be permitted. The results of the present study are in agreement with these findings. Selvi et al. (2003) in their work on inhibitory effect of *Garcinia indica* extract on growth and aflatoxin production in *A. flavus* found that at lower concentration of 500 and 1000 ppm, the inhibition of aflatoxin production is relatively greater than inhibition of fungus growth. Moreover, the essential oil had a reducing effect on ratio of total aflatoxins per mycelial dry weight, indicating what-ever the concentration of the essential oil increases not only the mycelia growth is prevented but also the ability of aflatoxin production by remaining mycelia is reduced.

Similarly, the concentrations of guaraná and jucá extracts



**Figure 3.** a) Effect of *guaraná* extracts treatment on aflatoxin production by *A. parasiticus* [(1) Control: no treatment, (2) *Guaraná* extracts treated: (2a) 1.08% (2b) 1.62% and (2c) 3.24%]. b) Effect of *jucá* extracts treatment on aflatoxin production by *A. parasiticus* [(1) Control: no treatment, (2) *Jucá* extracts treated: (2a) 1.08% (2b) 1.62% and (2c) 3.24%] by thin layer chromatography (TLC).

that were investigated in this study decreased the fungal growth. This study was conducted for both extracts, providing a confirmation of previous studies that have suggested that these compounds could be natural fungicides. No general rule can be proposed to explain the antifungal activity, and further studies on their activity and mechanisms of action must be carried out. However, the possible use of these compounds as natural antifungal agents reveals an interesting option for the decontamination of natural fungi and toxins in foods.

At the concentrations, applied no extract completely inhibited the growth of the strains under consideration; studies with higher concentrations, or mixtures of different extracts could be tested in order to completely inhibit the growth of fungi. In addition, the extracts should be investigated using an *in vivo* food matrix test. This study also evaluates the potential efficacy of natural Amazonian extracts as compounds that effectively control the aflatoxin production. No significant differences were found between the concentrations of guaraná studied on the inhibition of aflatoxin concentrations and other studies could be performed to reduce the production of these substances in the strains under study.

As shown, it would be possible to work with higher concentrations and mixtures of guaraná extracts to assess total aflatoxin inhibition. Regarding the jucá extracts, was not observed aflatoxins production above the method LOD (2 µg/kg) in two concentrations tested, therefore, further experiments should continue to assess the conditions tested here and should be adapted to an array of foods.

## Conclusions

This work was an approach to study the individual potential efficacy of these extracts as natural compounds that effectively control the growth and aflatoxin production by *A. parasiticus*. Based on the results of this study concluded that jucá extracts were more effective than the guaraná in controlling the growth and production of aflatoxins by *A. parasiticus*; therefore, further experiments should continue to assess the conditions tested here and should be adapted to an array of foods. The important reduction of aflatoxins production by jucá extracts suggests that phytochemical compounds could be used alone or in conjunction with other substances or processes to control the toxic metabolites production. These extracts must be subjected to further study to characterize the active compound define toxicity and evaluate economic feasibility.

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