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Evaluation of ochratoxin A and fungi in powdered guarana (*Paullinia cupana* Kunth), a caffeine rich product from Amazon forest

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Guarana (*Paullinia cupana* Kunth) is a Brazilian commodity well known to stimulate human metabolism due to its alkaloid components (mainly caffeine rich) and is used as an ingredient in the beverage industries. Moreover, the processed form (powdered guarana), may have similarities to other products (coffee, tea and cocoa) regarding its effects on ochratoxin A. This study was to evaluate the effect of the alkaloids of powdered guarana on the OTA production and the toxigenic fungal strains presence, with the specific aim of preventing risks to the consumers and to provide scientific data for those involved in the guarana productions chain. The ochratoxin A levels production of guarana were below the method of LOQ (0.50 µg/kg) and the presence of toxigenic strains was observed only in 2% of the samples. The average level of caffeine was 2.6% (minimum 1.8%; maximum 2.9%) and tannins of 14.7% (minimum 10.0%; maximum 23.1%). The effects of alkaloids from guarana on OTA levels and fungal strains was studied and despite the toxigenic strains were observed in guarana, the OTA levels were below the LOQ (2.0 µg/kg), probably due to the high caffeine content fungi inhibition properties.

Key words: Tannin, caffeine, water activity (Aw), *Paullinia*, *Aspergillus.*

INTRODUCTION

The guarana (*Paullinia cupana* Kunth var. sorbilis), is one of the best-known *Sapindaceae* family from the Amazon biome, and it has an important economic value. The guarana tree provides a fruit with singularly shaped seed (Figure 1a) and is described by Amazonian Indians tribes in their legendary stories. Commercially traded, guarana products are mostly in the form of dried seeds (Figure 1b), which are de-shelled and then used for caffeine extraction by the food industry. Powdered guarana has application in the beverage production and in medicine, as stimulatory and energetic of human metabolism (Kuskoski et al., 2005). These effects are derived from chemical compounds present in the guarana seeds, particularly the xanthines, such as caffeine, theophylline and theobromine (Belliardo et al., 1985). The largest fraction of caffeine is located in different parts of the seed: the seed kernel (embryo with bulky cotyledons) and the seed coat (testa), that are high in caffeine content (4.28 and 1.64%, respectively) (Weckerle et al., 2003).

The different forms of guarana and its seed parts are described in Figure 1a to d. Powdered guarana (Figure 1d) has high levels of antioxidant compounds, such as

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Figure 1. Illustrations of different forms of guarana (*Paullinia cupana* Kunth). (a) fruit, (b) seeds, (c) powder and (d) shells.

polyphenols (tannic/caffeic/gallic acids) and catechins, which have anti-bacterial and anti-fungal properties (Majhenic et al., 2007; Martins et al., 2014).

However, the presence of tannins is notable because tannins are recognized as having anti-nutrient and carcinogenic effects, although they possess anti-microbial activity (Okuda, 2005). In addition, powder guarana is useful in medicinal formulations and is a major source of caffeine in the Brazilian diet, particularly for consumers of certain caffeinated beverages and athletes interested in its stimulatory effect.

Tfouni et al. (2007) presented guarana as an important source of caffeine in the Brazilian diet and related it to other sources of caffeine whose ingestion should be controlled. Caffeine is described as a central nervous system stimulant that is distributed throughout the entire body after ingestion. The caffeine in guarana has pharmacological activity through transdermic diffusion (Heard et al., 2006), anti-plaque (Espinola et al., 1997), psychostimulatory (Otobone et al., 2007) and anti-hepatocarcinogenesis activities (Fukumasu et al., 2006). Powdered guarana (Figure 1c) is the most common type of guarana product and is obtained from artisanal process, particularly in the Amazon region; which is described in Figure 2.

The seeds are collected manually from the tree and transported in baskets to the artisanal facilities. They are fermented for several days under environmental conditions of relative humidity (RH) of >70% at approximately 25°C. Afterwards, the husk is removed from the seeds, then oven roasted open and finely ground. The ground product is packaged in polyethylene bags under environmental conditions. This guarana processing involves several indian (Amazon natives) communities. Brazil also produces guarana by large-scale systematic cultivation. In addition to powdered guarana, there are other forms of guarana products, such as sticks and husks.

Therefore, some of the guarana processing stages are susceptible to fungal contamination. The approximate composition of powdered guarana is as follows: moisture

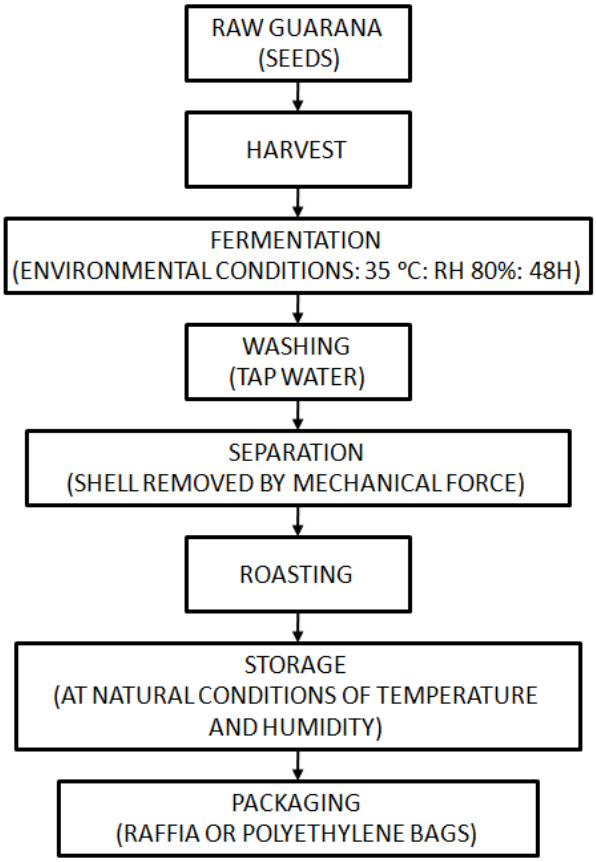

Figure 2. Flowchart of powdered guarana (Paullinia cupana Kunth) processing.

content (*mc*) 8.8 g%; ashes, 1.0%; lipids 2.7%; carbohydrates 70.98% and protein 16.4%. That composition shows its energetic properties and suggests the possibility of contamination which takes place by fungal strains due to high mc and lipid/carbohydrate components. Those components could lead to spoilage and affect the organoleptic characteristics.

Moreover some fungi could affect the guarana safety such as *Aspergillus* sp. because some species are mycotoxin producers. The mycotoxins are fungal metabolites, such as aflatoxins (AFLs) and ochratoxin A (OTA), recognized as being hepato and nephrotoxic, respectively apart from been carcinogenic and teratogenic. Thus, some of those metabolites affect the safety of the food. In addition, some guarana samples evaluated by Bugno et al. (2006) contained toxigenic *Aspergillus* and *Penicillium* strains able to produce mycotoxins.

Although, some fungal strains can spoil the guarana seeds, this subject has been considered controversial as Majhenic et al. (2007) reported that its seeds extract possess anti-bacterial and anti-fungal activities. Commodities with similar properties to those of guarana, such as cocoa and coffee, have been studied and found to allow/-have OTA contamination (Sanchez-Hervas et al., 2008).

The objective of the present study was to evaluate the effect of the alkaloids of powdered guarana on the OTA production and the toxigenic fungal strains presence, with the specific aim of preventing risks to the consumers and to provide scientific data to those involved in the guarana productions chain.

MATERIALS AND METHODS

Samples

Powdered guarana (n = 30) were purchased from grocery stores in Brazil, from 2010 harvest, in the regular labelling form (packs: 500 g each). They were homogenized (particle size <100 µm) and divided into portions of 25 g for mycobiota tests and caffeine / tannin / OTA / mc / Aw analysis.

Mycobiota and toxigenic potential of guarana

25 g aliquot of each sample was diluted with 225 ml of sterile aqueous, 0.1% peptone, then a series dilutions were prepared and aliquots were inoculated on plates containing DG18 medium (Dichloran Glycerol). All analyzes are performed in triplicate. After incubation at 25°C for seven days, plates were examined and all the fungal species were transferred and isolated on plates containing Czapeck yeast extract agar. For identification of fungi genera and species, the isolated strains were sub-cultured on MEA (Malt Extract Agar), and Czapek yeast autolysate (CYA) agar for *Aspergillus* and *Penicillium* genera.

On the other hand, species identification was performed through microcultive in Czapek-dox as described by Weber and Pitt (2000) and Samson et al. (2006). The isolates were examined under the light microscope and the species identification was carried out according to the taxonomic keys and guides available (Pitt and Hocking, 1997). The isolated *Aspergillus* and *Penicillium* sp. were grown on malt extract agar and were screened for their ability to produce OTA by inoculating coconut agar medium and incubating it at 25°C for 10 days.

Caffeine

Standard solutions were prepared and used to establish a calibration curve. The recovery rate for caffeine was 92% (n=3). A portion (1 g) of the sample was mixed which NH_4OH (1:2) and heated in a boiling water bath. Celite was added to the sample (5 mL), and then this mixture was passed through 150 mL acidic and basic columns to isolate caffeine. The caffeine content was determined at 256 nm, measured by spectrophotometry (Femto®).

Tannins

Standard solutions were prepared and used to establish a calibration curve. The recovery rate was 90% (n = 3). A sample portion (5 mL) was mixed with Folin-Denis mixture sodium carbonate (10 mL) and filtered. Tannin was expressed as mg/100 mL of tannic acid at 760nm by spectrophotometry (Femto®).

OTA

OTA analysis carried out by high performance liquid chromatography

Table 1. Natural elements of powdered guarana (Paullinia cupana Kunth) and OTA distribution.

Variable	Levels (SD[a])	Range
Caffeine (mg%)	2.60 ± 0.27	1.85 - 2.92
Tannin (mg%)	14.75 ± 4.92	10.09 - 23.17
Moisture content (g%)	6.25 ± 1.35	5.00 - 8.78
Water activity	0.49 ± 0.06	0.42 - 0.59
OTA [b] (µg/kg)	n.d.[c]	n.d.[c]

aMean ± standard deviation calculated from the total samples (N=30). bOchratoxin A. cNot detected in the samples evaluated by HPLC method with LOQ for ΣAFL = 2.0 µg/kg.

(HPLC). A calibration curve was prepared by diluting appropriate volumes of an OTA stock solution with toluene-acetic acid (99:1). The Limit of detection (LOD) and quantification (LOQ) for OTA were: 0.35 and 0.50 µg/kg, respectively. To obtain these parameters, powdered guarana, previously tested in LABMICO was certified as free of OTA in a validation test. The OTA free powdered guarana sample was homogenized and spiked with OTA at three concentrations ranging from 1 to 5 µg/kg prior extraction. Five points were used to build an analytical curve to obtain the R values for the LOD and LOQ. The recovery of OTA was 97.1%. A sample portion (15 g) was extracted using methanol- 3% sodium bicarbonate (1:1) with mechanical stirring. For quantification, HPLC (Shimadzu®) was performed using a C_{18} column, with acetonitrile : methanol : acetic acid (35:35:28:) as the mobile phase, a flux of 0.8 ml/min and fluorescence detection with excitation of 332 nm and emission of 476 nm. Confirmation conducted with a methanol solution of BF3 (14%).

Water activity (*aw*)

The *aw* levels of the samples were determined in triplicate at 25 ± 0.1°C in Aqualab Series 3TE instrument (Decagon®).

Moisture content (*mc*)

mc was determined by the gravimetric method.

Statistical analysis

The relationships between the variables of caffeine, tannin, *mc* and *aw* were evaluated by Pearson test. The coefficients of the linear relationships were determined using the method of least squares. Normality of the distribution of the variables was tested using the Shapiro-Wilk test. The statistical analyses were performed using R software.

RESULTS AND DISCUSSION

Fungal strains and OTA production

Some of the fungal strains identified in the samples were previously described in medicinal plants in other researches. Two filamentous strains isolated from 2% of the positive samples were identified as *Penicillium* sp. and *Aspergillus* sp. both of which were OTA producers. The presence of mycotoxigenic strains in powdered guarana was previously reported by Bugno et al. (2006). Our findings emphasize the importance of monitoring the different forms of guarana, because *Aspergillus* and *Penicillium* are widely distributed in the Brazilian ecosystem and are well known producers of mycotoxins. The levels of OTA were below the LOD in all the samples (Table 1). Bugno et al. (2006) identified fungi contaminating medicinal plants, including guarana. They were also found in coffee and cocoa with similar production process (Batista and Chalfoun, 2007).

Caffeine and tannins against OTA

The results for each alkaloid are presented in Table 1 as mean values and range. The mean of caffeine in the samples was 2.6 (from 1.85 to 2.9) g%. Despite this, the OTA levels in the evaluated samples were below the LOQ. The caffeine level of powdered guarana reported in the literature can reach 3.98 mg%, according to Souza et al. (2010). However, other authors have reported higher values of 4.28 mg% (Bauman, 1995) and 1.56-5.58 mg% (Meurer-Grimes et al., 1998). These results demonstrate that the caffeine levels in guarana are above those of other products reported contaminated with OTA, such as cocoa and coffee. The maximum content of caffeine in different varieties of Brazilian coffee reported by Farah and Donangelo (2006) was 1.23 mg%, far below the levels found in our study. Therefore, the OTA levels, in the current study could be below the LOD because of some alkaloids (in higher amount in guarana) ability to suppress the biosynthesis of OTA by *Aspergillus* (Lee et al., 2007). Caffeine, for example, appears to inhibit mycotoxin synthesis by restricting the uptake of carbohydrates and fungi appear to synthesize the toxin. Lenovich (1981) reported that the caffeine level might influence OTA production because it exhibited activity against aflatoxin in cocoa seeds, with caffeine contents ranging from 0.3 to 3.6 mg%. However, Santini et al. (2011) found no correlation between the OTA and caffeine contents of different coffee preparations, with caffeine levels ranging from 0.06 to 0.16 mg/cup. Thus, the method of drying guarana seeds prior to grinding seems to decrease the levels of tannins and caffeine (Ushirobira et al., 2004).

Considering that the standard of the Amazonian preparation is 10 g/cup of powdered guarana, it is possible to consume less than the average amount of caffeine. Another aspect considered is the absence of the skin (husk) of the guarana seeds used in the powdered guarana production. According to Batista et al. (2009), the skin of coffee beans is a significant source of OTA and the cleaning steps and standardization methods effectively reduce the OTA level. During the industrial process of converting coffee beans into roasted coffee and soluble coffee, the OTA levels reduction can reach

up to 90%. In guarana processing, removing the husk and roasting the seeds could positively affect the biosafety of the seeds. Some studies have been performed to understand the effect of UV light on food, as UV exposure is an alternative method to prevent the production of secondary metabolites such as OTA (Schmidt-Heydt et al., 2011). Powdered guarana production occurs under environmental conditions, with drying in sun light, which has a high incidence of UV light. Thus, this method may be a prevention factor. Consequently, it is possible to observe 100% of the guarana samples below the maximum limit allowed of 5 µg/kg. The average for tannins was 14.75 g% (minimum 10.09; maximum 23.16). The tannin levels were higher than the 4.05 g% value reported by SOUZA et al. (2010) for powdered guarana. Friedhelm et al. (1990) reported a level of 12.1 g%. No correlation was observed between the concentrations of caffeine and tannins in the current samples studied (p = 0.0621). Those levels were higher than that reported by Savolainen (1992) for tannins in coffee samples, at 17-18 g%. Therefore, the tannins levels of the samples confirm the antitoxigenic properties of tannins observed in coffee and tea (Hasan, 1999). Although these studies did not examine other phenolic compounds, such as the catechins, it has reported the antioxidant effect and the ability to enhance the antimicrobial effect of guarana (Basile et al., 2005).

Mc and aw levels

The average mc level of 6.25% (minimum 5.0; maximum 8.78%) in the samples was below the Brazilian regulation limit of 12%. Those levels were consistent with the levels of 5.0-10.6% reported by Araujo et al. (2006). It is important to emphasize the influence of environmental conditions, such as relative humidity (RH %) and temperature, on the fungal metabolism, as these parameters provide an adequate caffeine level during the production process. The average of *aw* was 0.49 (minimum 0.42; maximum 0.53), similar to the values of 0.51 (minimum 0.43; maximum 0.58) which was reported by Copetti et al. (2011) in powdered chocolate. Such levels are definitively associated with the production of mycotoxins.

In conclusion, the effects of alkaloids from guarana on OTA levels and fungal strains was studied and despite the observed toxigenic strains in guarana, the OTA levels were below the LOQ (2.0 µg/kg), probably due to the high caffeine content fungi inhibition properties.

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