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Effect of γ-irradiation on mycoflora of guarana (*Paullinia cupana*)

S. Aquino[a,*], E. Gonçalez[c], T.A. Reis[b], I.T. Sabundjian[a], R.A. Trindade[a], M.H. Rossi[c], B. Corrêa[b], A.L.C.H. Villavicencio[a]

[a]*Instituto de Pesquisas Energéticas e Nucleares – IPEN-CNEN/SP. Centro de Tecnologia das Radiações. Travessa R. No. 400, Cidade Universitária, CEP: 05508-910, São Paulo, Brazil*
[b]*Instituto de Ciências Biomédicas (USP). Departamento de Microbiologia. Av. Prof. Lineu Prestes, 1374. CEP: 05508-900, São Paulo, Brazil*
[c]*Instituto Biológico de São Paulo. Av. Cons. Rodrigues Alves, 1252, CEP 04014-002, São Paulo, SP, Brazil*

Abstract

Guarana (*Paullinia cupana*), originally from Amazon, Brazil, is currently used to cure headaches and as a stimulant, besides being used on depressive patients. This study was developed to evaluate the presence of toxigenic moulds, to detect mycotoxins, to determine the water activity (Aw) and to verify the effects of γ-radiation in the fungal mycoflora in 30 samples of guarana (*P. cupana*) in powder and grain forms purchased from industry, pharmacies and street market in five cities of São Paulo.

Keywords: Guarana; *Paullinia cupana*; *Aspergillus*; γ-radiation; Fungi; Mycotoxins

1. Introduction

Microbiological contamination of medicinal plants is a common public health problem in Brazil. It is a concern of the pharmaceutical industry to obey the requirements of the Brazilian legislation. In 1998, the World Health Organization published a document about quality control methods for medicinal plant materials and some suggestions regarding general limits for contaminants were included. The general limits to untreated plant materials harvested under acceptable hygienic conditions: mould propagules, maximum $10^5\,g^{-1}$. For plant materials that have been or that have been used as topical dosage forms, the maximum limit to yeasts and moulds are $10^4\,g^{-1}$. Finally, to plant materials for internal use: yeasts and moulds, maximum of $10^3\,g^{-1}$.

Brazilian consumers use powdered guarana by mixing it with water and drinking it, without a previous heating treatment. There may be a risk of occurring mycotoxicosis in patients after oral administration since many medicines are made of plant material. Under hot and humid conditions, the grains of Brazilian guarana are vulnerable to contamination by mould such as *Aspergillus flavus* that produces proven carcinogens known as aflatoxins, which are the strongest natural carcinogens that affect the liver and are probably the most common mycotoxins to which humans are exposed to through ingestion (IARC, 1993), and several environmental factors are known to influence aflatoxin production, but temperature and relative humidity are considered to be the most critical (25–30 °C and 97–99%, respectively). Additional factors such as water activity, moisture, substrate composition, storage time, insect damage and presence of a shell also influence fungal growth and aflatoxin production.

The use of γ-radiation is an important way of prevention against fungal contamination. However, according to Brazilian legislation (Brasil, 2004) the use of methods to eliminate contaminants should be studied regarding that matter possibility and eventually changed in raw material. The present study was performed to evaluate the fungal burden, to isolate toxigenic moulds, to detect mycotoxins, to analyze chemical changes on irradiated guarana (grains and powdered) collected in five cities of São Paulo State, from industry, pharmacies and open-air markets.

*Corresponding author. Fax: +55 11 3816 9292.
E-mail address: siaq@dglnet.com.br (S. Aquino).

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2. Experimental

2.1. Evaluation of fungal contamination

Twenty samples of powdered guarana (control group) were portioned in 10 g and were mechanically homogenized for 30 min in separated bottles containing 90 ml of sterile distilled water. For fungal counts and identification, 0.1 ml of the dilutions in a serial dilution from 10^{-1} to 10^{-5} of the samples were seeded in duplicates and plated while applying the method in dichloran 18% glycerol agar (DG 18). A total fungal concentration was counted after 5 days at 25 °C and was visually and microscopically examined for the fungal growth. The isolated moulds were sub-cultured on potato dextrose agar for identification and evaluation of the toxigenic potential of *Aspergilli*. The isolation of 10 samples of guarana grains was performed by direct plating using DG 18 agar for each dose. Thirty-three grains in two replicated plates were prepared (11 grains for each plate). The counting was made by frequency of fungi in grains, after 5 days at 25 °C.

2.2. Aflatoxin analysis

Aflatoxin analysis was performed with 25 g of each sample and extracted with methanol: potassium chloride 4% (9:1, v/v). The extracts were clarified with ammonium sulfate solution 30% and then the aflatoxins were extracted by adding chloroform. The chloroform extracts were collected for dryness on a water bath. Identification was conducted via thin-layer chromatography (TLC) using silica gel 60 Merck plates that were developed in chloroform: acetone (9:1, v/v). The intensities of fluorescence of the separated aflatoxin spots were measured through comparison with standards under 365 nm UV light Soares and Rodrigues-Amaya, 1989).

2.3. Evaluation of toxigenic potential

All isolates of *Aspergillus flavus* were screened for the ability to produce aflatoxins by the inoculation in coconut agar medium at pH 7.0 ± 0.1. All plates were incubated at 25 °C for 10 days and the average culture around it was transferred to glass flasks, weighted and macerated in chloroform at a ratio of 3 ml/g. The material was filtered and the filtrate was evaporated to dryness. Mycotoxins were qualitatively detected by thin-layer chromatography (TLC) as described by Soares and Rodrigues-Amaya (1989).

2.4. Treatment by irradiation process

Twenty samples (10 for each dose assay) were irradiated in polyethylene bags, each containing 10 g of powdered guarana and 10 samples of grains (33 grains for each assay), using a cobalt 60 (^{60}Co) γ-ray source (gamma cell) located at Instituto de Pesquisas Energéticas (IPEN) in São Paulo. The samples were exposed to doses of 5 and 10 kGy. The γ-ray source produced a dose rate of 3.5 kGy/h. After irradiation, the samples were plated as described previously in duplicates and the experiment was repeated in three replicates.

2.5. Water activity

The water activity (Aw) of the samples was determined using equipment from Decagon Devices, AQUALAB CX-2.

2.6. Chemical analysis of guarana extracts

Powdered guarana (200 g) acquired at a regular store was extracted with hexane and ethanol for 3 days (three times consecutively). After the evaporation of the solvents, the residues (hexanic and ethanolic extracts) were submitted to TLC over silicagel 60 G (Merck) and fluorescent silicagel 60 $F_{254+366}$ (Merck). The plates were uncovered under iodine steams and ultraviolet light (254 and 366 nm).

3. Results and discussion

3.1. Powdered guarana

In this study, the isolated species of fungi belonged to the genera *Aspergillus, Cladosporium, Penicilium* and *Rhizopus.* Two species were held to the genus *Aspergillus: A. flavus* and *A. niger*. Based on the results, 80% of all control samples were above the limit established by WHO (1998) for total counting of moulds in colonies forming units per gram (10^3 cfu/g), as shown in Table 1 and Fig. 1. The microbiological analysis showed that 90% of the samples, purchased in open-air market, presented a fungal growth of more than 10^3 fungal colonies per gram (averaged 10^4–10^5) in which only 10% had acceptable values (10^2). The predominant mycoflora obtained from open-air market was distributed in three genera: *Aspergillus* (82%), *Penicillium* (15%) and *Rhizopus* (3%) and two species were isolated from the genus *Aspergillus: A. niger* (43%) and *A. flavus* (39%).

In the samples taken from industry or pharmacies, 70% exceeded the limit determined by WHO (1998) (Table 1). The genus *Aspergillus* was the most dominant recovered (75%), followed by *Penicillium, Rhizopus* and *Cladosporium* (18%, 4% and 3%, respectively). The predominant isolated species were *A. flavus* (35%) and *A. niger* (30%). After irradiation, the contamination was reduced in 85% to the acceptable limit, using the dose of 5 kGy, but the genus *Cladosporium* and *Rhizopus* were isolated (20%) from industry/pharmacies samples and *Penicillium*, recovered (10%) from open-air market, irradiated with the dose of 5 kGy. Meanwhile, 10 kGy was the dose required for complete elimination of fungal contamination of guarana (powdered and grains) (Fig. 2).

Table 1
Total of colonies forming units per gram (cfu/g) of fungal contamination of powdered guarana of control (non irradiated samples), 5 and 10 kGy

Guarana samples[a]	Control		
	0 kGy	5 kGy	10 kGy
1[b]	2.0×10^4	0	0
2[b]	8.0×10^4	0	0
3[b]	2.1×10^4	0.8×10^2	0
4[b]	0	0	0
5[b]	2.9×10^4	0	0
6[b]	0	0	0
7[b]	5.0×10^4	0	0
8[b]	1.7×10^5	0.1×10^2	0
9[b]	2.9×10^3	0	0
10[b]	1.0×10^3	0	0
11[c]	2.2×10^5	0	0
12[c]	4.4×10^4	0	0
13[c]	3.5×10^5	0.5×10^2	0
14[c]	2.1×10^5	0	0
15[c]	4.2×10^5	0	0
16[c]	1.3×10^5	0	0
17[c]	5.0×10^2	0	0
18[c]	6.3×10^4	0	0
19[c]	1.2×10^4	0	0
20[c]	2.0×10^5	0	0

[a]Average of three replicates.
[b]Samples purchased from industry or pharmacies.
[c]Samples purchased from open-air markets.

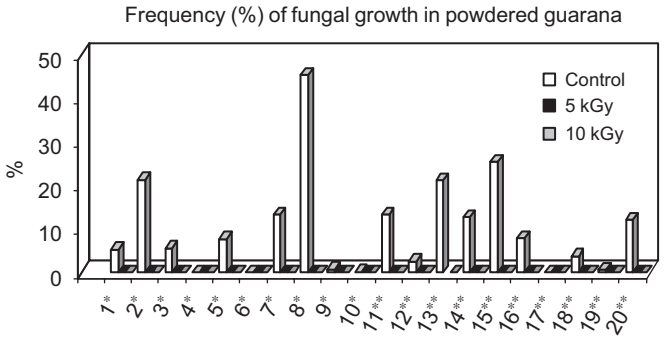

Fig. 1. Total frequency (%) of moulds in control and irradiated samples of powdered guarana purchased from industry/pharmacies (*) and open-air market (**). Average of three replicates.

3.2. Guarana grains

All control samples of guarana grains purchased in industry and in the open-air market were contaminated with *Rhizopus* spp. (Fig. 2). *Rhizopus* spp. is a cosmopolitan filamentous fungus and belongs to a larger group of fungi, the Zygomycetes that are common saprophytes and are ubiquitous in the environment (found in soil, decaying fruit and vegetables, animal feces and old bread). While *Rhizopus* spp. are common contaminants, they are also occasional causes of serious, and often fatal infections in humans and are also associated to allergy process in some immune depressive patients by inhalation. The reduction of fungal contamination with the doses of 5 kGy led up to

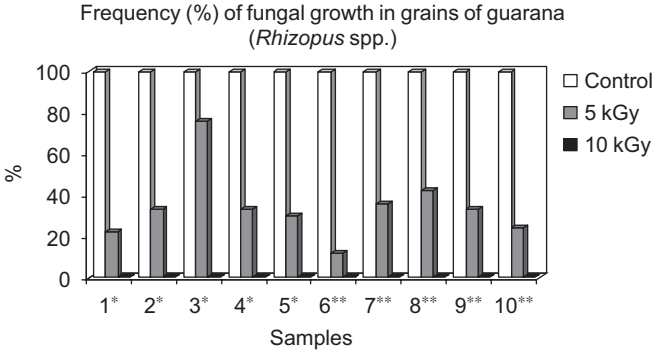

Fig. 2. Frequency of isolated fungi of control and irradiated samples of guarana grains purchased from industry/pharmacy (*) and open-air market (**). Average of three replicates.

acceptable limits. The γ–ray's sensitivity was confirmed with 10 kGy for all isolated fungi from industry, pharmacies and open-air market, where it was observed that the fungal burden was eliminated. El-Zawahry et al. (1991) demonstrated that gamma radiation doses from 5 to 10 kGy were required to completely inhibit fungal and bacterial flora that contaminated different Egyptian and Saudi Arabian food commodities. By the way, the results of the present study agree with these mentioned results.

3.3. Chemical analysis of guarana extracts

Concerning chemical changes in the irradiated samples with the doses of 5 and 10 kGy and comparing with non-irradiated samples of powdered guarana intending to determine changes in the chemical structure of the substances contents, the analysis effected for control samples and submitted to gamma radiation showed no significative chemical differences among them, considering that the spots of extracts were observed under iodine steams and ultraviolet light. Koseki et al. (2002) reported that powdered and dehydrated herbs irradiated with doses of 10, 20 and 30 kGy showed the identical therapeutical action of non-irradiated samples of *Rosmarinus officinalis* Linné, *Nasturtium officinale* R. Br, *Cynara scolymus* Linné and *Ocimum basilicum* Linné.

3.4. Aflatoxin analysis and Aw

The results demonstrated that aflatoxins were not found in any of the samples. Hitokoto et al. (1978) studied the fungal contamination and mycotoxin detection of powdered herbal drugs in Japan and they showed no mycotoxins as the natural occurrence in a total of 49 drugs powdered samples analyzed. The Aw of samples (Table 2) were low to produce mycotoxins. Hilmy et al. (1995) evaluated the effects of humidity after gamma irradiation on aflatoxin B_1 production of *A. flavus* in ground nutmeg and peanut and they concluded that there was almost no growth of *A. flavus* under RH of 85% or less.

Table 2
Average of 20 samples of Aw values of powdered and 10 samples of guarana grains

Dose	Samples[a]	
	Powdered	Grains
Control	0.46	0.51
5 kGy	0.44	0.49
10 kGy	0.41	0.47

[a]Average of three replicates.

The Aw and the temperature to fungal germination and growth are of 0.82 (25 °C), 0.81 (30 °C) and 0.80 (37 °C). *Aspergillus* spp. is more common in the tropics; as Brazil is a tropical country, it has high humidity and temperature throughout the year. Warm temperatures and high relative humidity are characteristics of the production areas in Amazon and the highest levels of aflatoxin occurred in Brazilian nuts from Amazon when the relative humidity in the storage environment was close to 97% and was accompanied by temperatures in the range of 25–30 °C (Arrus et al., 2005). Concerning the products that are sold in open-air markets, most of the toxigenic moulds would grow very well in this environment.

3.5. Evaluation of toxigenic potential

Among the *A. flavus* identified in powdered guarana of the control group, screened for the ability to produce aflatoxins, 72% produced aflatoxins B_1 and B_2. Studies on aflatoxin production in strains of *A. flavus* and *A. parasiticus* have shown that 74–100% of strains isolated from groundnuts and cottonseeds produced aflatoxin, while in those isolated from rice aflatoxin was produced by only 20–55% of strains. The presence of toxigenic moulds represents a potential risk of mycotoxin contamination and considering the high worldwide use of herbal products as alternative medicines, it is necessary to set standards for toxigenic moulds in crude herbal drugs in order to reduce the risks for consumers' health (Bugno et al., 2006).

4. Conclusion

Concerning the samples of street markets, it was demonstrated that the powdered guarana presented in high levels, a variety of fungal contamination to consumers. Fungal contamination of this product should be prevented as much as possible during the course of manufacturing. The handling and selling process of such products is an important period to avoid even further contamination. There is a high contamination risk to the manufacturer during the powdered drug production through inhalation. In some open-air markets, it was clearly observed that the powdered guarana was exposed to air, humidity and high temperatures, without a proper package or a seal protection. This study demonstrated that the samples presented high fungal contamination contents, making the product improper for consumption, even when in original packages from industry. It was also observed that guarana sold in bulk, previously wrapped or packaged in pharmacies from industry were contaminated with a high number of moulds (70%). There are no limits of fungal contamination to guarana in the phytotherapic Brazilian legislation (Brasil, 2004). In the same law, there is no mention to the commercialization forms like wrapping or bottling the product to retail. It is also important to report that the results regarding mycoflora, even on the same substrate (guarana) but in different forms of presentation to consumers (grains or powder) were different, and the presence of toxigenic *A. flavus* represented a potential risk to aflatoxin production. The ionization process avoids the risk for consumers and manufacturers, especially if using the dose of 10 kGy as a useful implement of sanitary quality of this product when exposed for long periods in open markets, without protective package, proper temperature and moisture control.

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