

OCCURRENCE OF AFLATOXIN B₁ IN NATURAL PRODUCTS

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ABSTRACT

The media claims for the consumption of natural resource-based food have gradually increased in both developing and developed countries. The interest in the safety of these products is partially due to the possible presence of toxigenic fungi acting as mycotoxin producers, such as aflatoxins produced during the secondary metabolism of *Aspergillus flavus*, *A. parasiticus* and *A. nomius*. Aflatoxins, mainly aflatoxin B₁, are directly associated with liver cancer in human beings. This paper is aimed at evaluating the presence of aflatoxin B₁ in a few vegetable drugs, dried plant extracts and industrialized products traded in 2010 in the city of Belo Horizonte, State of Minas Gerais, Brazil. The method used for the quantification of aflatoxin B₁ was based on extraction through acetone:water (85:15), immunoaffinity column purification followed by separation and detection in high efficiency liquid chromatography. Under the conditions of analysis, the Limits of Detection and Quantification were 0.6 µg kg⁻¹ and 1.0 µg kg⁻¹ respectively. The complete sets of analyses were carried out in duplicate. Aflatoxin B₁ was noticed in a single sample (< 1.0 µg kg⁻¹). The results revealed low aflatoxin B₁ contamination in the products under analysis. However, it is required to establish a broad monitoring program in order to obtain additional data and check up on the actual extension of contamination.

Key words: medicinal plants, fungi, mycotoxin, high-performance liquid chromatography (HPLC)

INTRODUCTION

The use of plants for medicinal purposes for the treatment, cure and prevention of diseases is one of the earliest known

medical practices in History. At present, a significant amount of medicinal plant commercialization is carried out in drugstores and natural product stores, where vegetable preparations are marketed under industrialized labeling (16).

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The intake of certain plants deemed as medicinal ones in the form of teas has always been meaningful in Brazil, mainly in the lower economic level populations, owing to low costs and the popular belief ascribed to their effects (30). In industrialized countries, it is believed that 30% to 50% of the populations make regular use of medicinal plants and/or vitamin and mineral supplements (29).

The increase in the consumption of natural products has become a public health issue. Practices of cultivation, harvest, storage and distribution make natural products subject to a great variety of contamination. Within such a context, the microbiological risk of medicinal plants may vary according to the different stages presented by the production line. The practices of field cultivation and harvest in association with the absence of an effective sanitary control stand for a potential risk for this type of product (18, 21, 31).

The interest in the safety of these products is greatly due to the possible presence of pathogenic bacteria and toxigenic fungi that produce mycotoxins such as aflatoxins B₁, B₂, G₁ and G₂. Aflatoxin B₁ (AFB₁) is the most common and most toxic one produced mainly by filamentous fungi as *Aspergillus flavus*, *A. parasiticus* and *A. nomius*. The toxic effects of the aflatoxins include immunosuppressive, mutagenic, teratogenic, and hepatocarcinogenic activity. The most potent hepatocarcinogen agent described in mammals is AFB₁, which is classified by the International Agency for Research on Cancer as Group 1 (probable carcinogen). (2, 7, 14, 19, 22).

The occurrence of toxigenic fungi in medicinal plants in Brazil has already been verified by several authors. Bugno, Almodovar, Pereira, Pinto, Sabino (2006) evaluated 91 samples of medicinal plants, composed by 65 different species marketed in São Paulo. *Aspergillus flavus* was the dominant and often isolated species (58 isolates/23.39%). Among these, 16/27.6% were able to produce aflatoxin B₁ or B₁ and B₂. Aquino, Gonçalves, Rossi, Campos Nogueira, Reis, Corrêa (2010) analyzed 80 samples, including 20 samples of each one of the four tested plants: Boldo (*Peumus boldus*), green tea (*Camellia sinensis*), Espinheira-Santa (*Maytenus ilicifolia*), and

Senna (*Cassia angustifolia*). Except for three samples of *P. boldus* e two samples of *C. sinensis*, all the samples presented fungal contamination, with 75% above the limit established by the World Health Organization for the Total Fungal Count: 10³ UFC/g (32). Prado, Andrade, Oliveira, Leal, Oliveira, Batista (2009) identified 8 *A. flavus* isolates in chamomile (*Matricaria recutita*) sold in Belo Horizonte, two of which were aflatoxin producers (B₁ and B₂). The same research reported the presence of *Aspergillus ostianus*, ochratoxin A-producer in artichoke (*Cynara scolymus*). The total fungal count reached values over 10⁵ UFC/g in these plants. In Argentina, Rizzo, Vedoya, Maurutto, Haidukowski, Varsavsky (2004) detected 52% of the genus *Aspergillus* in 152 medicinal plant samples, corresponding to 56 species. *A. flavus* and *A. parasiticus* were the prevalent species, 50% among the 40 aflatoxin-producer isolates. In Croatia, Malaysia and Nigeria, the widely prevailing fungi in researched medicinal plants belonged to the genera *Penicillium* and *Aspergillus*, which are potential producers of mycotoxins (10, 12, 23). In South Africa, 15 out of 16 samples of traditional medicinal plants were contaminated by several fungi species. *A. niger* was the most common isolated contaminant (50% of the samples), followed by *Fusarium* (6/16) and *Penicillium* (5/16). Approximately 60% of the samples were co-contaminated by *Alternaria* and *Rhizopus* spp (17). Roy, Sinha, Chourasia (1988) isolated 15 different vegetable drugs in India, in addition to 158 *A. flavus* isolates, 49 of which being aflatoxin B₁-producers.

In Brazil, the National Health Surveillance Agency (ANVISA) issued Regulation no. 10 (March 9, 2010), which brings in norms about microbiological contaminants for vegetable drugs, medicinal plants and their parts, which will undergo a heat extraction process (infusion and decoction), and plants that will not be submitted to an extraction process (ground sample). The norm sets forth the absence of aflatoxins (6).

Due to the absence of data about the occurrence of aflatoxins in natural products in Brazil, the aim of this paper was to evaluate the presence of aflatoxins B₁ in some vegetable

drugs, dried plant extracts used in the preparation of phytotherapy drugs and industrialized products commercialized in 2010 in Belo Horizonte.

MATERIALS AND METHODS

Samples

A number of 37 samples were purchased in natural-product stores in the city of Belo Horizonte in 2010, including: (1) 8 dried extracts of each of the species quoted below, which are used for the preparation of phytotherapeutic medications 1 sample belonging to each species; (2) Green Tea (*Camellia sinensis*): 5 samples presented as leaves and 4 industrialized products; (3) Espinheira-Santa leaves (*Maytenus ilicifolia* Martius – 2 samples); (4) Valerian Root (*Valeriana officinalis* L. – 3 samples); (5) Horse Chestnut seed (*Aesculus hippocastanum* L. – 2 samples); (6) Cascara Sagrada bark (*Rhamnus purshiana* D. C. – 2 samples); (7) Senna leaflets (*Cassia angustifolia* Vahl – 2 samples); (8) Passion Fruit leaves – *Passiflora* sp Sims – 2 samples) and (9) 7 samples of Guarana powder (*Paulinia cupana* H. B. K).

Chemicals

Aflatoxin B₁ was purchased from Sigma Chemicals Co. St. Louis, MO. It was diluted in benzene:acetonitrile, chromatographic grade. Benzene came from Tedia (Fairfield, OH, USA) and acetonitrile from Merck (Darmstadt, Germany). Acetone, HPLC grade, employed for the extraction of aflatoxin B₁ came from Merck (Darmstadt, Germany). The methanol, HPLC grade, used for the preparation of the mobile phase and elution of aflatoxin B₁ in the immunoaffinity column came from Carlo Erba (Rodano, Milan, Italy). The water used in the analytical process was obtained through a Milli-Q purification and filtration system with an 18 MΩ cm⁻¹ resistivity (Millipore, Bedford, MA, USA). The present study used EASI-EXTRACT Aflatoxin immunoaffinity columns, Product code RP71/70N, R-Biopharm Rhône, Glasgow, Scotland. Column storage took place at a temperature ranging from 2 and 8° C and they were

used at room temperature. The entire glassware used for aflatoxin determination was decontaminated by Alkaline Extran MA 01, 7555 (Merck, Darmstadt, Germany) at 20%, (pH > 12), remaining in contact for 24 hours and further washing with distilled water.

Standard Aflatoxin B₁ (AFB₁) Solution

The stock standard solution of AFB₁ (8,2105 µg mL⁻¹) was prepared by dissolving the solid standard in benzene:acetonitrile (98:2, v/v). The precise concentration was measured in Shimadzu UV-1601 PC spectrophotometer, Shimadzu Scientific Instruments, Japan, as described by AOAC (4). An intermediate standard solution from the stock solution was prepared in benzene:acetonitrile (98:2, v/v) in a concentration of 9.855 ng mL⁻¹. This solution was utilized for the elaboration of a calibration curve in the range 0.1-9.8 ng/mL. All the solutions were packed in amber vials at -18° C.

Extraction and clean-up procedures for high-performance liquid chromatography (HPLC) analysis

Samples were analyzed using a validated method by reversed-phase HPLC separation and fluorescence detection after post-column derivatization (3). A ground sample (10 g) was blended with 100 mL extraction solvent: acetone:water (85:15, v/v) for 30 min. Then the mixture was filtered through Whatman no. 1 filter paper. After filtration, the extract (5 mL) was diluted with water (75 mL). The immunoaffinity column was connected to the vacuum manifold, and the reservoir was attached to the immunoaffinity column. A number of 40 mL of diluted sample extract were added to the reservoir and passed through the immunoaffinity column at a flow rate of ca. 3 mL/min (ca. 1 drop/s; gravity). Do not exceed a flow rate of 5 mL/min. The column was washed twice with 10 mL water at a flow rate of maximum 5 mL/min and dried by applying little vacuum for 5-10 s. Finally, aflatoxin B₁ was eluted with 0.5 mL methanol and passed through by gravity. The eluate was collected in a vial. After 1 minute, a second portion of 0.5 mL methanol was applied. After 1 minute, a second portion of 0.5

mL methanol was applied. Most of the applied elution solvent was collected by pressing air or vacuum through. The extract was evaporated to dryness under a nitrogen stream at ca. 50 °C and reconstituted with 250 µL with methanol:water (2:3), v/v. Aflatoxins are subject to light degradation, thus it was necessary to protect the work from light by using amber vials. As a result, the method was found to be fit-for-purpose for the determination of AFB₁ in medical herbs at levels of 1.0 µg kg⁻¹ and above.

Determination of AFB₁ by HPLC method

The presence of AFB₁ was detected by HPLC after post-column derivatization with the electrochemical generation of bromine (KOBRA cell – Rhone diagnostic technologies, UK) with a current of 100 µA and a fluorescence detector (Shimadzu LC-10 AD Model; 360 nm excitation wavelength; 435 nm emission wavelength; with Shim-Pack CLC – ODS column, 5 µm, 4.6 x 250 mm, preceded by a guard column Shim – Pack G – ODS, 5 µm, 4 x 10 mm). The mobile phase was deionized water-acetonitrile-methanol (60:20:20, v/v/v) with the addition of 350 µL of 4M HNO₃ and 120 mg of KBr at a flow rate of 1 mL/min. The injection volume was 50 µL.

The quantification of AFB₁ was performed by measuring their peak areas at AFB₁ retention time (23.4 min.) and comparing it with the calibration curve. (25).

The performance of the method, aflatoxin B₁ recovery and effectiveness of the cleanup procedure, was evaluated by the samples of medical herbs spiked with AFB₁, in duplicate, at level of 2.96 ng/g.

RESULTS AND DISCUSSION

The linearity was evaluated within the range under study and calculated from the linear regression equation and determined by the least squares method. The linear correlation coefficient (r^2) was used as the indicator of the straight line as a mathematical model. The values were always over 0.99 as recommended by Green (11). Figures 1, 2 and 3 show an aflatoxin B₁ calibration curve, the chromatogram of a mixture of aflatoxins standards, and the extract of a horse chestnut seed sample contaminated by 2.96 ng/g of aflatoxin B₁, after post-column derivatization with the electrochemical generation of bromine.

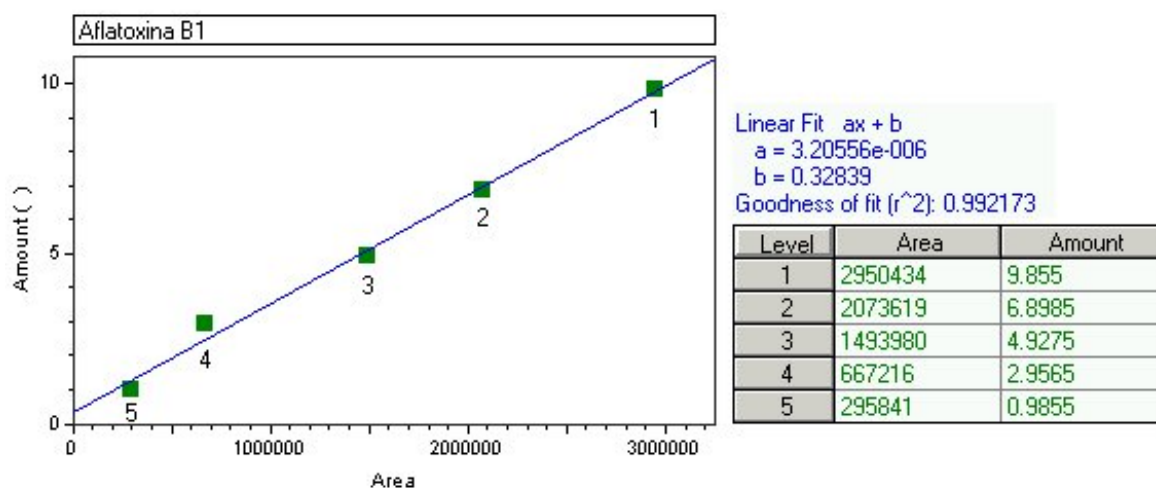


Figure 1. Standard curve used for the quantification of aflatoxin B₁ with the area obtained in the readings, the concentration of aflatoxin B₁ in ng/mL, the linear fit equation and the r^2 value.

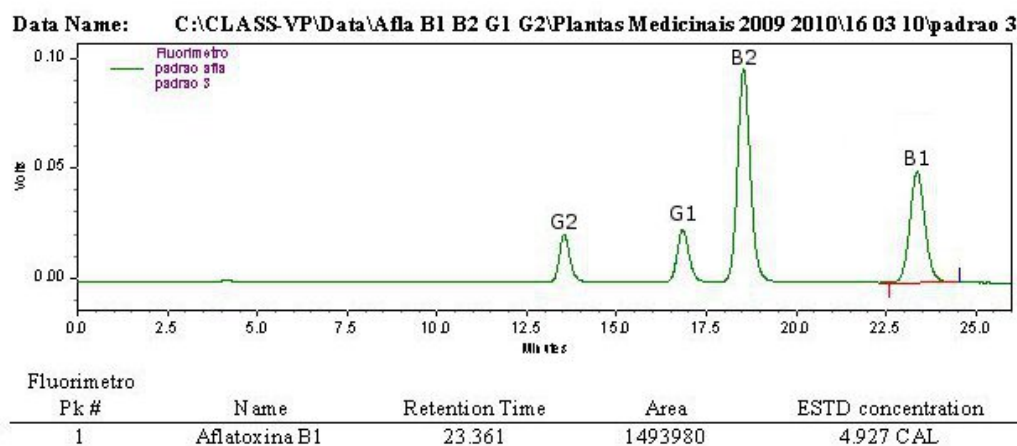


Figure 2. HPLC chromatogram with fluorescence detection post-column derivatization (electrochemically) – Kobra cell of a mixture of aflatoxin standards; Shim-pack CLC-ODS column and water:metahanol:acetonitrile, 60:20:20,v/v/v as mobile phase. Aflatoxin B₁ (4.93 ng/mL).

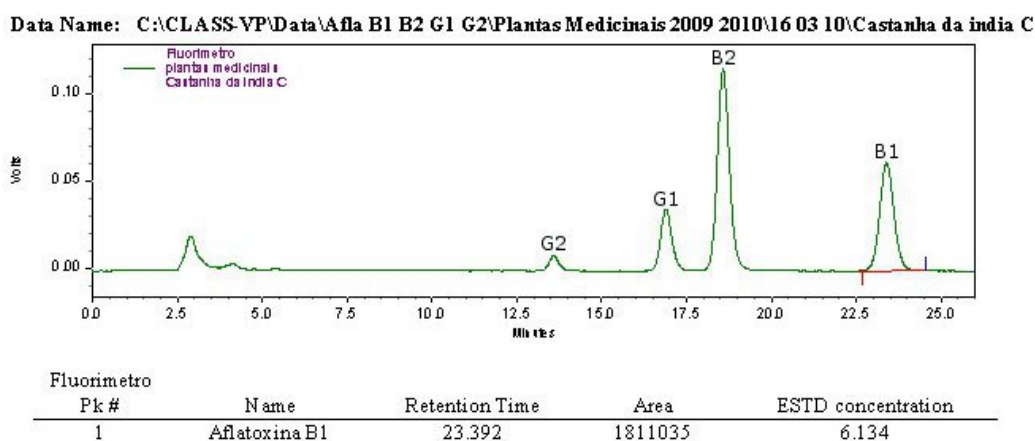


Figure 3. HPLC chromatogram with fluorescence detection of an extract of horse chestnut seed. Aflatoxin B₁-contaminated sample (2.96 ng g⁻¹); immunoaffinity column purification; HPLC using a post-column derivatization (electrochemically) – Kobra cell; Shim-pack CLC-ODS column and water:metahanol:acetonitrile, 60:20:20,v/v/v as mobile phase.

Despite the existence of several methods described towards the determination of aflatoxins in medicinal plants, none of such methods can be applied to all types of samples or all aflatoxins (15). The difficulty stems from the chemical complexity of the compounds of every different medicinal plant. Results of recovery experiments and coefficient of variation for aflatoxin B₁ are showed in Table 1. The recovery values obtained for aflatoxin B₁ revealed that the methodology in use and the analytical conditions developed in the laboratory

are in compliance with the provisions set forth by law no. 401/2006 (02/23/2006) in the European Union (8). All recovery values are within the range of 70 to 110%, which is required when the aflatoxin concentration is in the range of 1-10 µg kg⁻¹. In relation to the variation coefficient values, all stayed below 20% as recommended by Horwitz and Albert (1982), pointing to a good precision of the applied methodology.

Table 1. Recovery in medical herbs spiked with AFB₁ standard (2.96 ng/g)

Sample	Amount recovered (ng/g)	Recovery mean (%)	Coefficient of variation (%)
Senna	2.56		
(<i>Cassia angustifolia</i> Vahl)	2.99	94	10.9
Espinheira-Santa	3.38		
(<i>Maytenus ilicifolia</i> Martius)	2.89	106	11
Passion Fruit	2.53		
(<i>Passiflora sp</i> Sims)	3.13	96	14.9
Guarana Powder	2.40		
(<i>Paullinia cupana</i> H.B.K.)	2.69	86	8.1
Valerian Root	3.04		
(<i>Valeriana officinalis</i> Linné)	3.04	103	0
Cascara Sagrada	2.57		
(<i>Rhamnus purshiana</i> D.C.)	2.04	78	16.2
Horse Chestnut Seed	3.10		
(<i>Aesculus hippocastanum</i> L.)	3.02	104	1.2
Green Tea	2.99		
(<i>Camellia sinensis</i>)	2.36	91	17

As far as the remaining aflatoxins are concerned (data shown in Table 1), the recovery values for aflatoxin G₂ were below 60% in all tested plants. Recovery values of 56% were obtained for aflatoxin B₂ in green tea and 53% in guarana powder. The others presented values between 83% and 103%. Recovery values for aflatoxin G₁ were between 95% and 103%, except for horse chestnut seed, Espinheira-Santa and guarana, which presented values over 115%. Low recovery, mainly in relation to aflatoxin G₂ might be due to little mycotoxin affinity towards the antibody, which depends on the extracts used or the insufficient quantity of antibody bound to the gel of the immunoaffinity column (1).

The presence of aflatoxin B₁ was noticed in just a single sample of green tea (*Camellia sinensis*) at a concentration below the Limit of Quantification (1.0 ng/g). A possible explanation for the absence of aflatoxins would be the lack of toxigenic fungi in the samples or the environmental conditions during harvest and storage. It is well reported in literature that temperature and water activity are the main factors that influence fungal invasion and the production of aflatoxins in stored products (9). Kulshrestha, Gupta, Shukla, Kundu, Bhatnagar, Katiyar (2008) evaluated that medicinal plants with

water activity below 0.81 under temperatures of 25 ± 2° C, 30 ± 2° C and 40 ± 2° C and water activity over 0.81 and temperature below 10 ± 2° C did not present aflatoxins, even in the presence of *Aspergillus flavus*, an aflatoxin producer.

Similar results were found by Romagnoli, Menna, Gruppioni, Bergamini (2007) in Italy as 48 infusions and medicinal plants were analyzed. None of the samples presented detectable levels of aflatoxins for an analytical methodology with a limit of detection and quantification of 0.5 and 1.5 ng/g of aflatoxin B₁, respectively. Ali, Hashim, Saad, Safan, Nakajima, Yoshizawa (2005) evaluated 23 traditional medicinal plants from Malaysia and Indonesia. They observed aflatoxin B₁ in 16 samples with an average 0.26 ng/g. In Thailand, Tassaneeyakul, Razzazi-Fazeli, Porasuphatana, Bohm (2004) detected aflatoxins in 5 out of the 28 analyzed samples within a range of 1.7 to 14.3 ng/g. Unlike the quoted authors, Selim, Pependorf, Ibramim, Sharkawy, Kashory (1996) observed high contamination of aflatoxin B₁ in 9 samples in Egypt, after having analyzed 31 medicinal plants within a range of 24 to 105 ng/g and an average 49 ng/g.

In Brazil, a small number of papers evaluated the quantification of aflatoxins in medicinal plants. Aquino,

Gonçalves, Rossi, Campos Nogueira, Reis, Corrêa (2010) evaluated boldo (*Peumus boldus*), green tea (*Camellia sinensis*), Espinheira-Santa (*Maytenus ilicifolia*), and senna (*Cassia angustifolia*), when aflatoxins were not detected through the use of VICAM immunoaffinity columns (AflaTest kit), a monoclonal antibody-based affinity chromatography system and posterior confirmation by thin-layer chromatography. Braga, Medeiros, Oliveira, Macedo (2005) analyzed different samples of *M. ilicifolia* (undisclosed figures) sold in stores and drugstores in the city of João Pessoa, State of Paraíba, when aflatoxins were not detected either. In this case, the method in use involved immunoaffinity column purification and high efficiency liquid chromatography, excluding the derivatization stage.

Owing to the relevance of human exposure to aflatoxin B₁ for public health, the lack of information and the Brazilian climatic conditions, which favor fungal production as well as mycotoxin production, a constant supervision of the quality of medicinal plants and phytotherapeutic products is required to guarantee good health conditions for the users of such products.

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