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*Research Article*

# Detection of Total Aflatoxins in Groundnut and Soybean Samples in Yemen Using Enzyme-Linked Immunosorbent Assay

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Aflatoxins are fungal toxins that have mutagenic and carcinogenic effects, especially hepatocellular carcinoma effect. This work aimed to investigate the presence of aflatoxins in groundnuts and soybeans that are consumed in Yemen. The samples were collected from three different regions in Yemen (Sana'a, al-Hodeida, and Aden), and they were divided into two groups. The concentration of total aflatoxins was analyzed by enzyme-linked immunosorbent assay (ELISA). Aflatoxins were determined in 89 groundnut and 65 soybean samples. The results showed that 85.39% (76/89) of groundnut and 72.3% (45/65) of soybean samples were contaminated with aflatoxins. In addition, in 49.44% and 27.6% of the groundnut and soybean samples, total aflatoxins exceed the acceptable level of European Commission (4 $\mu$g/kg), while in only 6.2% of soybean samples and 22.47% of groundnut samples, total aflatoxins were beyond the maximum limit of FDA/Yemen standards (20 $\mu$g/kg). The results showed that the aflatoxin contamination in the groundnut and soybean samples may be considered a significant risk for public health. The present study is the first to report the data on the presence of aflatoxins in groundnut and soybean samples in Yemen.

## 1. Introduction

Mycotoxins are a group of secondary toxic metabolites of some strains of filamentous microfungi. Generally, these are found in animal feed and plant products such as rice, maize, copra, soya, peanuts, and wheat [1]. These toxins are produced during growth, harvesting, and storage phases of feed and grains [2]. Until now, 450 various types of mycotoxins are identified, but only a few of them which are concerned with human beings are important. Most of the mycotoxins are produced by five major fungal genera, i.e., *Penicillium*, *Aspergillus*, *Fusarium*, *Claviceps*, and *Alternaria* [3]. The diseases caused by mycotoxins in animals and humans called mycotoxicosis. The severity of fungal diseases ranges from acute toxic to carcinogenic or immunosuppressive. Mycotoxins are not only harmful to humans but also a source of antibiotics (e.g., penicillin) which are used as medicine for the treatment of various diseases, immunosuppressants (e.g., cyclosporine), and compounds used for control of migraine headaches and postpartum hemorrhage (e.g., ergot alkaloids) [4]. Aflatoxins are the most studied groups of mycotoxins on account of their natural carcinogenic properties and their pathogenetic effects to animals and humans. These secondary metabolites are produced by five species of fungi, i.e., *Aspergillus flavus*, *A. parasiticus*, *A. nomius*, *A. tamari,* and *A. pseudotamarii*. Only *A. flavus* and *A. parasiticus* produce high enough concentration to be considered economically important. Aflatoxins B1, B2, G1, and G2 are produced by special strains of *A. flavus* and *A. parasiticus*. The species *A. flavus* merely produces aflatoxin B, while other species produce both aflatoxin B and aflatoxin G [5–7]. Aflatoxin B1 (AFB1) is metabolized to aflatoxin M1 (AFM1), which is excreted in milk and urine [8, 9]. According to the International Agency of Research on Cancer (IRCA), AFB1 and AFM1 have been considered as a human carcinogen [10]. AFs are acute toxic compounds and have shown to be

immunosuppressive, mutagen, teratogen, and carcinogen [11]. The occurrence of acute aflatoxicosis is uncommon in humans because contaminated foods are usually avoided. However, exposure to aflatoxins may cause mutations (mutagenesis) and cancers (carcinogenesis), especially hepatocellular carcinoma. The impacts of toxins such as immunosuppression and nutritional interference take place as a result of exposure to low doses of aflatoxins for long periods [12].

Groundnuts are a good source of protein and vitamin E and are consumed by people of all age groups because of their good flavor and are considered as a very popular snack worldwide [13]. Also, Yemenis use groundnuts as flavor with sweets and desserts and also as raw, cooked, or mixed with other food substances and considered as an everyday meal by many rich and poor families. On the other hand, soybeans are an Asiatic legume planted in various parts of the world for their oils and proteins, which are extensively used in the manufacture of foodstuffs and feedstuffs [14]. Different soybean-based foods, such as soy protein and soy milk, are used as a nutritional source. Also, soybean is used in fermented foods such as soybean paste and soy sauce. Several previous research studies have reported that aflatoxins were detected in soybeans (raw and fermented products) [15].

Aflatoxin contaminations in groundnuts and soybeans could cause many severe problems. Most countries have set legislation to regulate the levels of aflatoxins and protect their population. The European Union (EU) has set tolerance limit of total aflatoxins in the groundnut and soybean as 4 $\mu$g/kg, whereas Food and Drug Administration of the United States has set the acceptable limit for total aflatoxins not exceeding 20 $\mu$g/kg [16]. Aflatoxins in oilseeds such as the groundnut and soybean are very common contamination in many countries, and in Yemen there is limited information about aflatoxin contamination in these products.

The current study aims to investigate the level of aflatoxins in groundnuts and soybeans consumed in Yemen. The aflatoxin content was measured in samples which were collected from different areas of Yemen. Aflatoxins were evaluated by enzyme-linked immunosorbent assay (ELISA).

## 2. Materials and Methods

*2.1. Sample Collection and Materials.* The samples were collected in triplicate from different domestic districts in Yemen, i.e., Sana'a, Al-Hodeida, and Aden. The samples were divided into two groups according to the district of collection, i.e., group 1 (Sana'a and Al-Hodeida) and group 2 (Aden district). The samples were brought to the laboratory and preserved for further analysis. In this work, a total of 89 groundnut and 65 soybean samples were collected for the study of total aflatoxins and analyzed by enzyme-linked immunosorbent assay (ELISA). AgraQuant®, a total aflatoxins kit, purchased from ROMER was used for the analysis.

*2.2. Sample Preparation/Extraction.* The samples were prepared and extracted according to the instruction given by the manufacturer of the enclosed kit. The extraction is based on the solubility of aflatoxins in organic solvents. A representative sample was obtained and ground by using a Romer Series II® Mill (75% passed through a 20-mesh screen), and then the subsample portion was completely mixed. Twenty grams of the ground sample is weighed into a clean conical flask (250 mL) with a glass lid that can be tightly sealed. Solvent solution (methanol: distilled water; 70 : 30) was prepared by v/v, and 100 mL of the solvent solution was added to samples in the conical flasks, and the flask was sealed tightly. The samples were extracted in a ratio of 1 : 5 ($w : v$) of sample to extraction solution, respectively, and then stirred thoroughly for 30 minutes at room temperature by using a shaker. Finally, the sample was allowed to settle and then filtered using the Whatman # 1 filter paper, and the filtrate was collected for the analysis. For each sample to be tested, the preparation was according to the protocol of the ELISA kit as described below.

*2.3. Wash Solution Preparation.* The content of the wash solution concentrate bottle was transferred to a 500 ml plastic squeeze bottle (i.e., 1 ml wash solution to 19 ml DW). 475 ml of distilled/deionized water was added and swirled to mix.

*2.4. Detection of Total Aflatoxins by ELISA.* All reagents and kit elements were brought to room temperature (18–30°C (64–86°F)) before use. A sufficient number of green-bordered dilution strips were inserted into a microwell holder. One dilution well was used for each sample or standard (0, 1.0, 2.0, 10.0, and 20.0 ppb). The same number of antibody-coated microwell strips was inserted into a microwell holder. By using a single channel pipettor, 100 $\mu$l of each standard or sample was added into the micro-titer plate followed by addition of 200 $\mu$l enzyme conjugate and mixed carefully by pipetting it up and down three times. Then, 100 $\mu$l of the contents from the dilution well was transferred into the antibody-coated well to initiate the reaction. This was then incubated for 15 min at room temperature (20–25°C) for reaction to take place. After incubation for 15 min at room temperature (20–25°C), the contents of the wells were discarded and the wells were washed four times to remove any unbound toxin. One-hundred microliters of substrate (Chromogen) was added to each well and mixed gently by shaking the plate manually. Following 5 min incubation at room temperature in the dark, the reaction was stopped by adding 100 $\mu$l of stop solution into each well, and the colour changes to yellow. Finally, absorbance was measured photometrically at 450 nm by the ELISA reader (reference wavelength 630 nm against an air blank) within 30 min after the addition of stop solution.

*2.5. Calculations.* To calculate the sample concentration, the curve had been obtained according to the formula that was used to calculate the %absorbance:

$$\frac{\text{absorbance of standard or sample}}{\text{absorbance of zero standard}} \times 100 = \%\text{absorbance}. \quad (1)$$

The zero standard is thus made equal to 100%, and the absorbance value is obtained in percentages. A calibration curve was got by charting %absorbance values for the standards against the AF concentration ($\mu$g/kg). The concentration of AFs in the samples was calculated from the calibration curve. Also, the concentration of AFs in samples was obtained by the software of the ELISA machine directly.

*2.6. Statistical Analysis.* All the values were expressed as mean ± standard error. The data was obtained from the analysis by statistical analysis of variance (ANOVA) using the SPSS statistical package from IBM.

## 3. Result and Discussion

*3.1. Recovery Evaluation.* In order to validate our method, the samples (10 gm each) were spiked with AF standard at levels 20 $\mu$g/L and mixed for 10 min to ensure toxin dispersion. The total aflatoxin concentration was determined using the previously indicated procedure. Under these conditions, the mean recovery score in spiked samples was 97.20% with a coefficient of variation (CV) of 2.6. The results of recovery for three spiked samples were 94.31%, 97.87%, and 99.40% (Table 1). Figure 1 illustrates the curve of ELISA and equations of the regression curves with their coefficients of determination.

*3.2. Soybeans.* In the current study, a total of sixty-five soybean samples were collected from Sana'a, Al-Hodeida, and Aden districts to detect total aflatoxins by ELISA. It can be seen from Tables 2 and 3 that 47 of 65 soybeans samples were contaminated with aflatoxins, ranging from 1 to 30.20 $\mu$g/kg, and the mean content was 4.13 ± 0.87 $\mu$g/kg. According to the Yemen and FDA permissible limit of total aflatoxins (20 $\mu$g/Kg), in only two samples from each group, total aflatoxins exceeded the acceptable limit with contamination level ranging from 22 to 30.20 $\mu$g/Kg. On the other hand, in 34.3% (12/35) of group 1 samples, total aflatoxins were beyond the admissible level of the EC regulation which was 4 $\mu$g/kg, whereas in 20% (6/30) of group 2 samples, they were above the EC regulation and the level of aflatoxins ranged from 4.10 to 30.20 $\mu$g/kg. Figure 2 shows the total aflatoxins (B1, B2, G1, and G2) in soybean samples in a general way and according to the region of sample collection.

In the previous studies on soybeans, the U.S. Department of Agriculture (USDA) conducted analysis for 1046 soybean samples collected from various regions of the US to detect aflatoxin contamination. In the same study, AFs were present in only two of the tested samples with low levels (7–14 $\mu$g/kg) [17]. Another study conducted in Germany on 55 samples of soybeans to determine mycotoxin reported that only aflatoxin B1 was detected in 32 of the 51 samples with a maximum concentration level of 0.41 ppb [18]. Also, a study conducted in Asia and the Pacific region reported that for all soybean samples, the occurrence of aflatoxins was in low levels (0.02–13 $\mu$g/kg) [19].

Generally, soybean is considered resistant to *Aspergilli* formation and contamination with aflatoxins. So, contamination with total aflatoxins in soybeans is not considered a significant problem as compared to cereals such as corn, rice, wheat, etc. [20].

Table 1: Spiked samples with aflatoxin standard at 20 $\mu$g/kg.

| Spiked sample | Average OD | Conc. in $\mu$g/kg | Theoretical conc. | Recovery (%) |
|---|---|---|---|---|
| 1 | 0.111 | 18.86 | 20 | 94.31 |
| 2 | 0.118 | 19.57 | 20 | 97.87 |
| 3 | 0.121 | 19.88 | 20 | 99.40 |

OD = optical density.

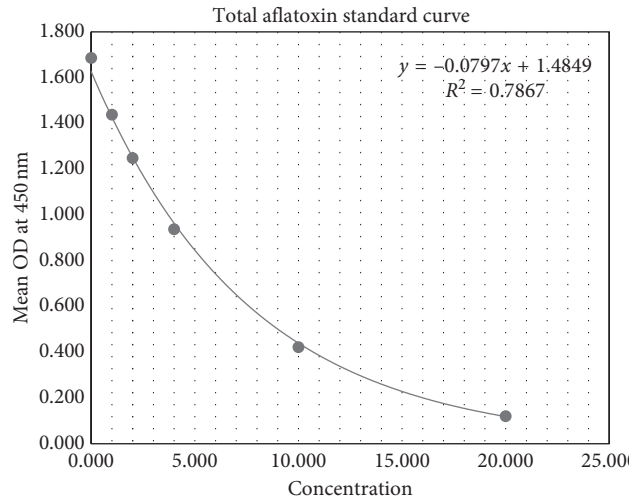


Figure 1: Linear regression curve and equation for the aflatoxin standard in ELISA (given in $\mu$g/kg).

Table 2: Total aflatoxins in soybean samples.

| Soybean samples | Total samples | Total aflatoxin-contaminated samples | | | |
|---|---|---|---|---|---|
| | | No (%) | Range | Mean ± SE | SD |
| Group 1 | 35 | 21 (60) | 1.0–30.2 | 4.01 ± 1.16 | 6.88 |
| Group 2 | 30 | 26 (86.67) | 1.0–30 | 4.26 ± 1.33 | 7.26 |
| Total | 65 | 47 (72.31) | 1–30.2 | 4.13 ± 0.87 | 7.00 |

SE = standard error; SD = standard deviation.

Table 3: Total aflatoxins in soybean samples exceeding the Yemen/FDA regulation (20 $\mu$g/kg) and EC regulation (4 $\mu$g/kg).

| Soybean samples | Total samples | Exceeding Yemen/FDA regulation (20 $\mu$g/kg) | | Exceeding EC regulation (4 $\mu$g/kg) | |
|---|---|---|---|---|---|
| | | No (%) | Range | No (%) | Range |
| Group 1 | 35 | 2 (5.71) | 22.9–30.2 | 12 (34.29) | 4.12–30.2 |
| Group 2 | 30 | 2 (6.67) | 22–30 | 6 (20) | 4.1–30 |
| Total | 65 | 4 (6.2) | 22–30.2 | 18 (27.69) | 4.1–30.2 |

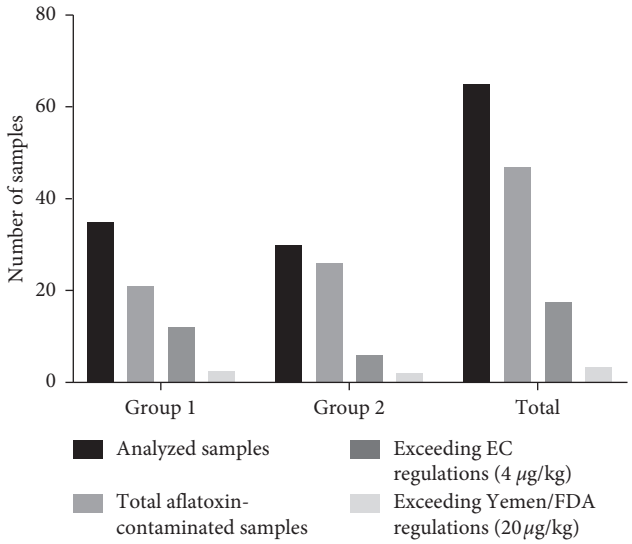


Figure 2: Total aflatoxin contamination in soybean samples (total samples, group 1, and group 2).

*3.3. Groundnuts.* In this study, analysis of eighty-nine groundnut samples for total aflatoxins revealed that 76 samples were contaminated with aflatoxins. The percentage of total aflatoxins was 85.93% in the contaminated groundnut samples and ranged from 1 to 44.20 $\mu$g/kg (mean $9.72 \pm 1.22$) (Table 4). The level of total aflatoxins in groundnuts was higher than the maximum acceptable level of the FAD and Yemen standards (20 $\mu$g/kg) and than that set by the EU commission (4 $\mu$g/kg) (Table 5). Sixty-three groundnut samples were analyzed to detect total aflatoxin concentration in group 1 (Sana'a and Al-Hodeida). The results of group 1 showed that 55 (87.3%) of 63 samples were contaminated with aflatoxin, ranging between 1.0 and 44.20 $\mu$g/kg with a mean value of 8.88 $\mu$g/kg, while 8 (12.7%) groundnut samples were noncontaminated. From group 2 (Aden district), a total of twenty-six groundnuts samples were analyzed to determine total aflatoxin concentration. 21 (80.77%) of 26 groundnut samples were contaminated with aflatoxin with a mean value of 11.70 $\mu$g/kg. The range of contaminations for samples of group 2 was between 2.25 and 44 $\mu$g/kg. There is no such information about the incidence of aflatoxin in groundnuts in Yemen, so this work is the first to investigate the presence of aflatoxin in groundnuts.

The presence of total aflatoxins in groundnut samples has been documented in several countries all over the world [21]. In Turkey (2006), Yentür et al. [22] revealed that aflatoxin B1 and total aflatoxins in butter of peanut samples ranged from 2.06 to 63.70 $\mu$g/kg and from 8.16 to 75.70 $\mu$g/kg, respectively [22]. Iqbal et al. [21] from Punjab, Pakistan, analyzed 198 peanut and peanut products and reported that the existence of aflatoxins in peanuts and their products; around 32%, 17%, 29%, and 36% were contaminated, which were beyond the tolerable limit of the EU (4 $\mu$g/kg) in peanuts in the shell, raw peanut without shell, peanuts roasted in shell, and peanuts roasted without shell samples, respectively [21]. Leong et al. conducted an analysis of 196 peanuts and peanut product samples and found a high level of aflatoxin contamination ranging between 16.6 and 711 $\mu$g/kg [23]. In South Korea (2007), Chen et al. reported that 10.6% of peanut samples were contaminated with aflatoxins ranging between 0.20 and 28.2 $\mu$g/kg [24]. Also, in China (2006), a study evaluated the presence of aflatoxins in peanut and reported that the concentration of aflatoxins in peanut samples was from 80.3 to 437 $\mu$g/kg [25].

The occurrence and level of total aflatoxins reported in the current study were relatively high compared to those quoted in the literature. Furthermore, many researchers have conducted an investigation to detect the incidence of aflatoxins in groundnuts [26].

Table 5 presents the exceeded level of total aflatoxins according to the EU tolerance limit (4 $\mu$g/kg) and the FDA/Yemen regulation (20 $\mu$g/kg). In group 1 samples, 30 of 63 samples (47.63%) were contaminated with total aflatoxins that were above the EU acceptable limit, ranging from 4–44.20 $\mu$g/kg, whereas in group 2, 14 of 26 samples (53.85%) had total aflatoxins that exceed the maximum acceptable level of the EU regulation, ranging between 4.30 $\mu$g/kg and 44 $\mu$g/kg. On the other hand, the percentage of contamination that exceeded the acceptable limit of the FDA and Yemen regulation (20 $\mu$g/kg) was 20.63% (13/63), ranging between 20.10 and 44.20 $\mu$g/kg, and 26.9% (7/26), ranging between 23.60–44 $\mu$g/kg, in group 1 and group 2, respectively. Figure 3 shows the total aflatoxins (B1, B2, G1, and G2) in groundnut samples in a general way and according to the region of sample collection.

This present study showed that a high level of aflatoxin contamination in groundnut samples in both groups. Generally, the aflatoxin concentration is shown at much higher percentages depending on the European Union (EU) regulation than the FDA and Yemen regulation. The EU regulation limit of total aflatoxins is 4 $\mu$g/kg, but the tolerance level of total aflatoxins in a few developing countries and the US shall not exceed 20 $\mu$g/kg in foodstuffs intended for human consumption [26]. Considering this high level of aflatoxins, attention should be given to routine inspection and surveillance of these products and control measures to reduce groundnut contamination with aflatoxin on top priority. Aflatoxin is associated with many health hazards and, therefore, such actions contribute to food security.

In our present study, the high level of total aflatoxin contamination in groundnuts can be attributed to many factors such as preharvest and postharvest environmental factors and poor management practices during planting, harvesting, drying, transportation, and storage of the product [27]. Furthermore, damage by pest and unawareness of good storage practices were noticed to be significant in increasing aflatoxin contamination during storage phases. These bad practices favor fungal contamination and growth and aflatoxin production [28, 29]. Additionally, several factors lead to the growth of fungi in groundnuts during the storage stage, such as seed temperature and moisture, which increase aflatoxin contamination [30]. Other important factors that influence the growth of molds that produce aflatoxin are water activity, absorption of moisture, and infestation by insects [31, 32]. So, it is possible to reduce the contamination with mycotoxins to a great extent or to permissible limits by

Table 4: Total aflatoxin contamination in groundnut samples.

| Sample | Analyzed samples | Negative samples | Total aflatoxin-contaminated samples | | | |
|---|---|---|---|---|---|---|
| | No. | No. (%) | No. (%) | Range | Mean ± SE | SD |
| Group 1 | 63 | 8 (12.69) | 55 (87.30) | 1.0–44.2 | 8.88 ± 1.38 | 10.92 |
| Group 2 | 26 | 5 (19.23) | 21 (80.77) | 2.25–44 | 11.77 ± 2.48 | 12.66 |
| Total | 89 | 14 (14.61) | 76 (85.39) | 1–44.2 | 9.72 ± 1.22 | 11.46 |

SE = standard error; SD = standard deviation.

Table 5: Total aflatoxin contamination in groundnuts exceeding the EU tolerance limit (4 $\mu$g/kg) and FDA/Yemen regulation (20 $\mu$g/kg).

| Sample | Total | Exceeding EU regulation (4 $\mu$g/kg) | | Exceeding FDA/Yemen regulation (20 $\mu$g/kg) | |
|---|---|---|---|---|---|
| | | No. (%) | Range | No. (%) | Range |
| Group 1 | 63 | 30 (47.63) | 4–44.2 | 13 (20.63) | 20.1–44.2 |
| Group 2 | 26 | 14 (53.85) | 4.3–44 | 7 (26.92) | 23.6–44 |
| Total | 89 | 44 (49.44) | 4–44.2 | 20 (22.47) | 20.1–44.2 |

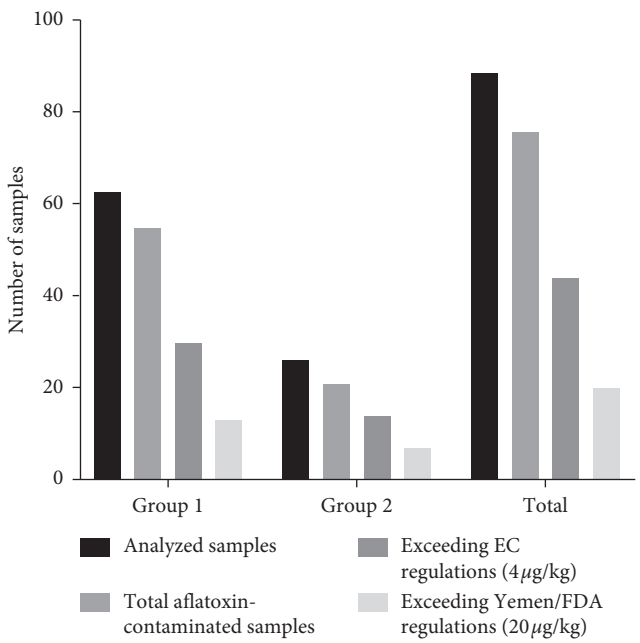


Figure 3: Total aflatoxin contamination in groundnut samples (total samples, group 1, and group 2).

applying appropriate technologies for production, harvests, processing, and storage [27]. Contamination with aflatoxin is the most surveyed and regulated of all the toxins all over the world, especially in more economically developed countries [33], while in the developing world, such protection is limited or absent due to high poverty [29]. Many authors have confirmed that the problem of aflatoxin is more severe in the developing world where the climatic condition (temperature, water activity, or moisture), marketing, transportation, and practices of storage are improper and considered conducive to the growth of fungi and mycotoxin formations [34–36].

The results of this study revealed that the incidence of aflatoxin contamination in groundnuts and soybeans reached up to 85.4% and 72.3% of total samples, respectively. In groundnut samples, approximately 49% of the total sample and 22.4% of the contaminated samples were exceeded the European Union tolerance limit and the FDA/Yemen regulation, respectively. However, in soybeans, only 6.2% and 27.7% of the total samples were above tolerance levels of the European Union tolerance limit and FDA/Yemen regulation, respectively. The present results demonstrated that a high level of contamination with aflatoxin across the study areas. So, precautions must be taken during all times of groundnut and soybean storage (low temperature, low moisture content, and low humidity) because these depress the fungus growth and thus eliminate the incidence of aflatoxin contaminations. The results of this work suggest that safety limits for aflatoxin are needed for controlling, regulating, and ensuring the quality of groundnuts and soybeans. Aflatoxins are considered a real hazard to human health and cause serious risks to consumers. So, groundnuts, soybeans, and other foodstuffs must be monitored by public health authorities through a set of standard regulations and by tireless inspection and surveillance on all foodstuffs and feedstuffs with regard to aflatoxins. On the basis of the results, we suggest that investigation on groundnut contaminations by toxic fungi and correlated mycotoxin must take place across the country to create a complete picture of grain contaminations and to work on adequate control measures.

## Data Availability

Data used to support the findings of this study are available from the corresponding author upon request.

## Conflicts of Interest

All authors declare that the research was conducted in the absence of any commercial or financial relationships that could pose potential conflicts of interest.

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