Determination of Aflatoxins in Medicinal Plants by High-Performance Liquid Chromatography–Tandem Mass Spectrometry

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ABSTRACT – **Purpose.** The intention of the proposed work is to study the presence of the aflatoxins B_1, B_2, G_1 and G_2 in medicinal plants, namely *Mucuna pruriens*, *Delphinium denudatum* and *Portulaca oleraceae*. **Methodology.** The aflatoxins were extracted, purified by immunoaffinity column chromatography and analysed by high-performance liquid chromatography–tandem quadrupole mass spectrometry with electrospray ionisation (HPLC–MS/MS). Fungal count was carried out in PDA media. **Results.** A good linear relationship was found for AFB_1, AFB_2, AFG_1 and AFG_2 at 1–10 ppb (r>0.9995). The analyte accuracy under three different spiking levels was 86.7–108.1 %, with low per cent relative standard deviations in each case. The aflatoxins can be separated within 5 to7 min using an Agilent XDB C18-column. We found that AFB_1 and AFB_2 were in trace amounts below the detection limit in *M. pruriens* whilst they were not detected in *D. denudatum*. *P. oleraceae* was found to be contaminated with AFB_1 and AFB_2. AFG_1 and AFG_2 were not detected in *M. pruriens, P. oleraceae* and were below the detection limit in *D. denudatum*. This was consistent with very low numbers of fungal colonies observed after 6 hr of incubation. **Conclusion.** The analytical method developed is simple, precise, accurate, economical and can be effectively used to determine the aflatoxins in medicinal plants and therefore to control the quality of products. The aflatoxin levels in the plant extracts examined were related to the minimal fungal load in the medicinal plants examined.

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INTRODUCTION

The plants *Mucuna pruriens*, *Delphinium denudatum* and *Portulaca oleraceae* have the potential for wide therapeutic applications. *Mucuna pruriens* L (Velvet beans; Fabaceae), a herbaceous forage and food legume, has found widespread usage in the management of several diseases, including diabetes, rheumatoid arthritis, atherosclerosis, male infertility and nervous disorders, and is a good source of 3,4- dihydroxyphenylalanine (DOPA) (1, 2). In addition to health benefits the seeds possess potent aphrodisiac, geriatric tonic and vermifuge activities (3). The major constituent of *Mucuna* seeds was found to be L-dopa (5%), along with minor amounts of methylated and nonmethylated tetrahydroisoquinolines (0.25 %) (4). *Delphinium denudatum* Wall (Jadwar; Ranunculaceae) is one of the important medicinal drugs used as indigenous medicine in India, and the entire plant is reported to be useful in a variety of ailments (5). The root is used in various medical formulations in Unani and Ayurveda to reduce the withdrawal symptoms in people on de-addiction therapy (6). It is a natural analgesic and is prescribed for relief from toothache (7). Herbalists recommend the roots in the treatment of fungal infections, asthma, cough, jaundice and nervous problems (8). It is considered as a natural blood purifier and prescribed for people who have digestive problems and provides relief from painful wounds and inflammation (9). Chemically *D. denudatum* contains many active compounds, some of which are alkaloids including delphocurarine, staphisagrine, delphine, condelphine, denudatin, talatizidine, acetylhetero-phylistine and a

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diterpenoid alkaloid identical to condelphine (10). *Portulaca oleracea* L. (Purslane; Portulaceae) grows widely in different areas of the world including India. The seeds are regarded as valuable in the treatment of urinary problems, digestive problems and cardiovascular diseases (11). Studies demonstrated a variety of pharmacological activities, including hypoglycemic (12, 13), hypocholesterolemic, antioxidant, analgesic, anti-inflammatory, skeletal muscle relaxant, smooth muscle relaxant, neuroprotective (14), anxiolytic, sedative, anticonvulsant, antiulcerogenic, bronchodilatory, wound healing, and antifungal effects (15). It contains a variety of bioconstituents, including catecholamines, l-noradrenaline, dopamine, α-amyrin, β-amyrin and portuloside A (16, 17, 18).

Aflatoxins are a group of structurally related toxic metabolites of mycotoxins produced by *Aspergillus flavus* and *Aspergillus parasticus* (19). In the aflatoxin group, about 16 compounds are known, but only aflatoxin B_1, B_2, G_1, G_2, M_1 and M_2 are routinely monitored (20, 21). Aflatoxin B_1, B_2, G_1 and G_2 are chemical derivatives of difurancoumarin (Fig. 1). Aflatoxin B_1 is the prevalent compound in plant samples and it is acutely toxic and the most carcinogenic amongst the aflatoxins (22). Aflatoxins may increase stress susceptibility and compromise growth efficiency. The clinical signs of aflatoxicosis in humans are extremely varied. Signs of acute aflatoxicosis include depression, nervousness, abdominal pain, diarrhea and death (23). The aflatoxins occur widely in food, especially in corn, nuts, peanuts, coconut, fruits, dried fruits and beer (24, 25). High moisture and temperature are two main factors that cause the occurrence of mycotoxins at pre-harvest and post harvest stages (26). Fungi are widely distributed as environmental contaminants (27), in fact, under favorable conditions of temperature and humidity, they grow on many commodities including cereals, oil seeds, nuts, herb-teas and spices (28). They have also been found in medicinal herbs that are not fully dried or stored improperly (e.g. at high temperature and humidity) (29, 30). Despite many studies on mycotoxins in agricultural products, only a few are concerned with spices and herbal medicines. The determination of aflatoxins in herbs is becoming important for medicinal safety, as more and more people are using medicinal herbs for complementary and alternative therapy and they play an important role in the economy, especially in developing countries. Previously aflatoxins were analyzed in peanut, cocoa, pistachio, chili, ginger (31, 32), chocolate, cotton-seed, job's tears, pepper, paprika, feed and wheat samples (33).
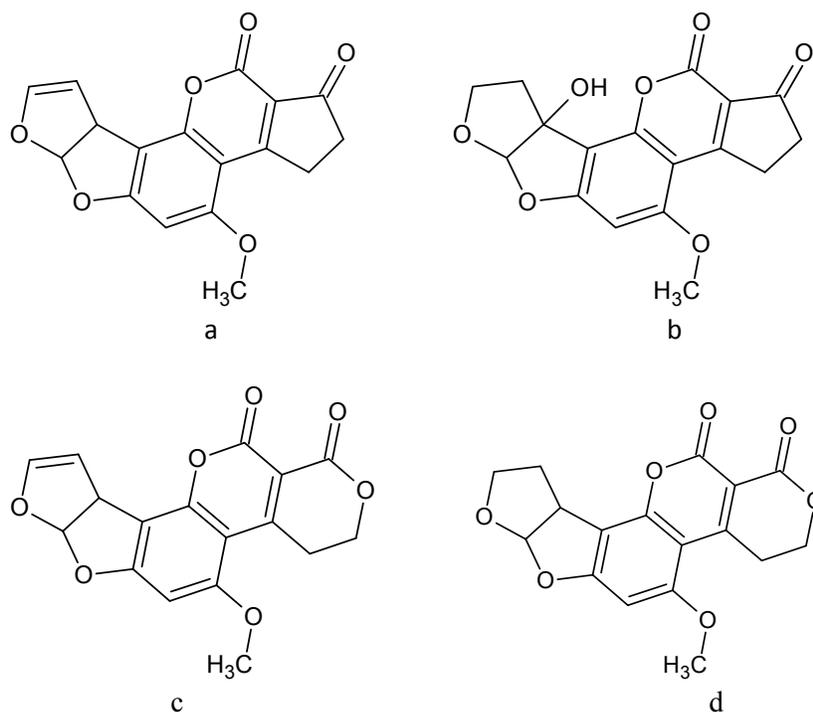
Figure 1. Structure of (a) AFB_1 (b) AFB_2 (c) AFG_1 (d) AFG_2

The current acceptance level of total aflatoxin in maize set by the United States- Food and Drug Administration (USFDA) is 20 µg/kg (34). Health Canada has set acceptance criteria of < 20 µg/kg (ppb) for aflatoxins ($B_1+B_2+G_1+G_2$), and < 5 µg/kg (ppb) for aflatoxin B_1 for evening primrose oil, sugar cane, sugar beets, cottonseed, peanuts, corn, ginseng or any substance derived from these sources (35). Recently, the Codex Alimentarius Commission, the Joint FAO/WHO Food Standards Program, adopted a limit of 15µg/kg for total aflatoxins (36). In 2008, the Codex Alimentarius set a maximum level of 10 µg/kg total aflatoxins in ready-to-eat almonds, hazelnuts, and pistachios (37). In the European Union, the total aflatoxins and AFB_1 levels in human commodities are regulated with maximum residue levels (MRLs) that cannot be greater than 2 and 4 µg/kg, respectively (38). In the year 2002 the European Food Safety Authority (EFSA) set maximum limits in spices (*Capsicum* spp., *Piper* spp., *Myristicafragrans*, *Zingiber officinale*, *Curcuma longa*) (AFB_1 5 µg/kg; total AFs 10 µg/kg) (02/472/EC) (39).

Environmentally sound and most widespread methods applicable for quantitative determination of aflatoxin content in different samples are thin-layer chromatography (40, 41) and high performance liquid chromatographic (HPLC) (42, 43). Aflatoxins can be separated and detected using either normal- or reversed-phase HPLC methods mainly with fluorescence spectrometric detection. The use of two-dimensional thin-layer chromatography (2D-TLC) (44, 45) and high performance thin-layer chromatography (HPTLC) for the determination of aflatoxins has been reported (46). An alternative complex media based method is reported to detect the natural fluorescence of aflatoxins released by the growing mycelium (47) or relies on multiplex polymerase chain reaction (PCR) and real time polymerase chain reaction (RT-PCR) detection of genes or their transcripts involved in the aflatoxin biosynthetic pathway (48, 49). An enzyme-linked immunosorbent assay (ELISA) for AFB_1, and levels of total aflatoxins were quantified and confirmed by liquid chromatography combined with tandem mass spectrometry (LC-MS/MS) (50). However, a lot of time and a tedious pre-or post-column derivatization is required with conventional HPLC in order to provide better detection limits of AFB_1, AFB_2, AFG_1 and AFG_2 (51). Furthermore, many other structurally similar components to the AFs in the plants can also be retained by the aflatoxin immunoaffinity column, and as a result produce false positive results in many chromatographic techniques.

Despite the numerous uses and the various biologically active chemical constituents reported in *M. pruriens*, *D. denudatum* and *P. oleracea*, no data on aflatoxin detection of these plants are available in the literature. In order to ensure safety and quality we have collectively developed a cost efficient, flexible and rapid method for the quantification of aflatoxins in *M. pruriens*, *D. denudatum* and *P. oleraceae* as these plants are commonly consumed by people without any awareness of their safety. There exist no well-accepted scientific methods for proper determination and quality control of aflatoxins in selected plant materials. The aim of this study was to screen the content of aflatoxins as well as providing a sensitive, accurate and reproducible analytical method for the detection of aflatoxins. Further, the purpose of this study is to provide useful information on aflatoxin levels in *M. pruriens*, *D. denudatum* and *P. oleraceae* and to achieve a confirmatory method for quick determination of AFB_1, AFB_2, AFG_1 and AFG_2 by high performance liquid chromatography–electrospray ionization tandem mass spectrometry (HPLC–MS/MS). The proposed method overcame the drawbacks of HPLC by using tandem MS/MS detection, which can determine the presence of a specific substance more accurately than HPLC and avoids the tedious derivatization process. The method is validated for linearity, recovery, sensitivity, precision and repeatability, and was shown to successfully determine the levels of AFs in plants samples.

METHODS

Equipment and chemicals

The analysis was performed on an Agilent 1200 system using an Agilent XDB-C18 4.6-50 mm column containing 1.8 mm particles. MS/MS detection was achieved on an Agilent 6410B tandem quadrupole mass spectrometer using an electrospray ion (ESI) source in positive mode. Data were acquired and processed using the Masshunter software (Agilent). Acetonitrile, methanol, ammonium acetate and HPLC grade water were supplied by Merck (Darmstadt, Germany). AFB_1, AFB_2, AFG_1 and AFG_2 were purchased from Sigma (St. Louis, MO). All other inorganic chemicals and organic solvents were of reagent grade.

HPLC–MS/MS analysis

Chromatographic analyses were carried out using a binary gradient elution with 70:30 v/v methanol: acetonitrile as eluent A and 5 mM aqueous ammonium acetate as eluent B. The total run time was 5 to 8 min. The injection volume was 5 mL, the flow rate was set at 5 µL/min and the column temperature was maintained at 30°C. The ionisation source of the MS/MS detector had 4.0 kV capillary voltage, 350 °C source temperature and 9 L/min desolvation gas flow (both gases were nitrogen).

Sample collection

All sample species were collected from a local market of New Delhi, India and were authenticated by Dr. H.B. Singh (Chief Scientist & Head, Raw materials Herbarium & Museum, NISCAIR, New Delhi). A voucher specimen (RHMD/1704/04) has been deposited in the Department of Pharmacognosy and Phytochemistry, Faculty of Pharmacy, Jamia Hamdard, New Delhi. All samples were ground to powders and kept in moisture proof paper bags to keep their water content constant before analysis. The herbs and their parts used are summarized in Table 1.

Sample preparation

The samples (5 g) were sonicated in 100 mL 70:30 v/v methanol:water for 45 min and centrifuged (Remi Compufuge-CR30) for 10 min, then 5 mL of the supernatant was drawn, diluted with 30 mL milli-Q water, and passed through the immunoaffinity column at no more than 5 mL/min (the column was previously conditioned with 30 mL distilled water). The column was rinsed with 15 mL double distilled water to remove the matrix components and then dried by passing air through to remove any adherent water. The eluate was diluted to 2 mL with water and passed through a 0.22 mm filter, and the filtrate was injected into the HPLC–MS/MS.

Peak recognition

The AFs in samples were recognized by comparing their retention times and MS spectra (MRM mode) with the standards. The standards used are summarized in Table 1.

Fungal count in the plant materials

All the plant materials were diluted with sterile water and 500 µL of the samples were spread over potato dextrose agar (PDA)-Ox bile media. They were incubated for 5 days at 28 °C and 75 % relative humidity, and the colony forming units (CFU) of the fungal strains were observed.

RESULTS

Selection of mobile phase

The HPLC mobile phase was optimized by considering the retention time, peak response, sensitivity, peak sharpness as well broadening and separation efficiencies. Methanol–water in the combination of 60:40 v/v completely separated the analytes but was found not suitable for the column, while acetonitrile–water 60:30 v/v failed to separate the AFs. Methanol:acetonitrile in a combination of 70:30 v/v with 5.0 mM aqueous ammonium acetate was found the best mobile phase for AF standards separation (Fig. 2).

Optimisation of the mass spectra

Mass spectrometry detection was carried out on positive ionisation mode because this mode gave sharp and sensitive signals. Mass spectrometry detection was optimised by injecting the AF standards (100 ng/mL) under positive ionisation mode. The ion fragments were evaluated by their run time to the most abundant m/z and the ion with the uppermost intensity was selected as the basic ion for quantitation (Fig. 3).

Table 1. Summary of aflatoxins and herbs used in this study			
Standard aflatoxin	Tested herb		
	Part used	Species	Family
B$_1$			
B$_2$	Seed	*Mucuna pruriens*	Fabaceae
G$_1$	Root	*Delphinium denudatum*	Ranunculaceae
G$_2$	Seed	*Portulaca oleraceae*	Portulacaceae
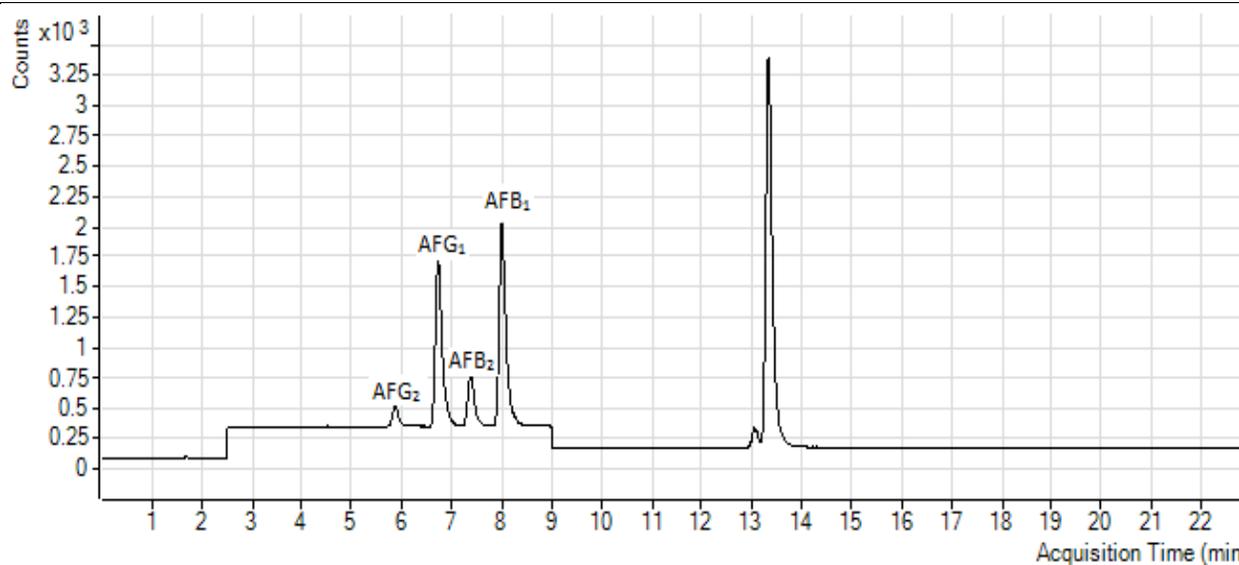
Figure 2. HPLC-MS/MS Chromatograms of standard mycotoxins comprising a mixture of aflatoxins (B_1, B_2, G_1 and G_2). Mobile phase; methanol: acetonitrile (70:30 v/v) eluent A and aqueous ammonium acetate (5 mM) eluent B. Total run time (5 to 8 min), injection volume (5 mL) and flow rate (5 µL/min).
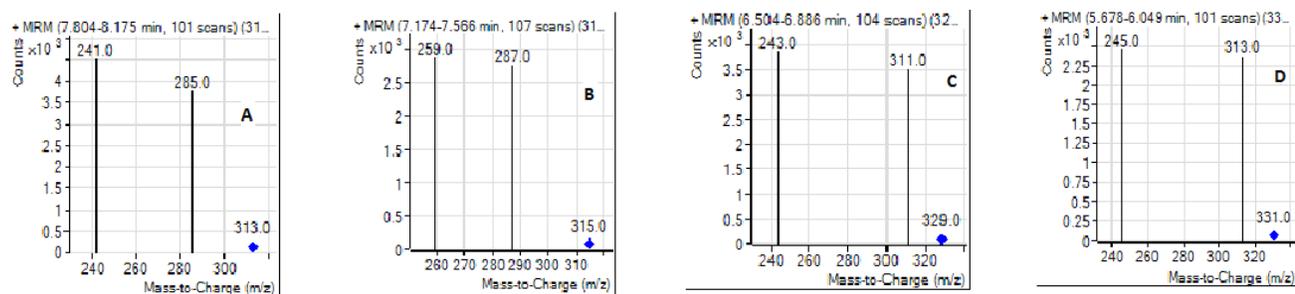
Figure 3. Mass spectral analysis of standard aflatoxins. (A) Std. AFB_1 run time (7.8-8.1) and m/z (313.0) $[M+H]^+$ (B) AFB_2 run time (7.1-7.5) and m/z (315.0) $[M+H]^+$ (C) AFG_1 run time (6.5-6.8) and m/z (329.0) $[M+H]^+$ (D) AFG_2 run time (5.6-6.0) and m/z (331.0).

METHOD VALIDATION

Limits of detection and limits of quantification

The AF analyses showed good results in the MRM mode, the LOD reaching 0.28–1.10 µg/kg and LOQ 0.79-3.34 µg/kg, with a signal-to-noise ratio (S:N) of 3. Linear calibration curve for various concentrations of AFs ranging from 1 ppb–10 ppb (three points) were analysed. The calibration curves were constructed by plotting the response against the concentration. A linear relationship was obtained for each compound, and the correlation coefficients ranged from 0.9992 to 0.9995 (Table 2). The relative standard deviations (RSDs) of precision of AF mixtures were 7.04–2.17 % (n=6). Excellent retention times for AFB_1 (7.973±0.018), AFB_2 (7.361±0.017), AFG_1 (6.689±0.015) and AFG_2 (5.830±0.026) were observed for corresponding mixtures of AFs (Table 2).

Accuracy

Accuracy analysis was performed by testing samples at three different concentrations 1 ppb, 5 ppb and 10 ppb. All plants were used for the accuracy test to ensure that the method has wide adaptability. The plant sample (5 g) was spiked with high, medium and low levels of AF standards. The accuracy ranged from 86.7% to 108.1% (Table 3), indicating good accuracy of the method.

Determination of aflatoxins in plant samples

HPLC-MS/MS Chromatograms (Counts *vs* Acquisition time (min)) for AFB_1, AFB_2, AFG_1 and AFG_2 were determined for *M. pururiens* (Fig. 4), *D. denudatum* (Fig. 5) and *P. oleraceae* (Fig. 6). The study revealed mass peaks of AFB_1 and AFB_2 in *M. pruriens* below the detection limit whilst a resultant mass peak of AFB_1 and AFB_2 was observed in *P. oleraceae,* whereas no mass peak was found in *D. denudatum* (Figs. 4, 5 and 6). The aflatoxin levels in the plants investigated is summarized in Table 4.

Fungal count

Total fungal colonies observed per gram of *M. pruriens*, *D. denudatum* and *P. oleraceae* were 18×10^2, 4×10^2 and 20×10^2 respectively in PDA-ox bile media. The total fungal CFU also show that there was very low growth of fungus in these tested plant materials, which is consistent with the low levels of mycotoxins.

DISCUSSION

An LC-MS method was developed which was found to be suitable for the rapid and accurate assessment of aflatoxin levels in selected plant samples. The method was subsequently applied to determine the natural occurrence of AFs in selected widely used commercial medicinal plant samples (Table 1). We found that *P. oleraceae* was contaminated with AFB_1 (1.675 µg/kg) and AFB_2 (1.335 µg/kg), whilst levels in *M. pruriens* were below the detection limit for AFB_1 (0.04 µg/kg) and AFB_2 (0.05 µg/kg) and were not detected in *D. denudatum*. AFG_1 and AFG_2 were absent in *M. pruriens* and *P. oleraceae,* whilst a relatively low value (0.07 µg/kg) of AFG_2 was found in *D. denudatum* (Table 4).

Table 2. Linearity, limit of detection, limit of quantification and precision for the analysis of AFB_1, AFB_2, AFG_1 and AFG_2 by HPLC–MS/MS. Data expressed as mean±SD. [a]n=6; [b, b1]S/N=3 (Signal-to-noise ratio); [c]n=6. Abbreviations: RT: Retention Time; LOD: Limit of detection; LOQ: Limit of quantification; RSD: Relative standard deviations.

Compound	RT±SD	Regression Slope	Correlation Intercept	(LOD)[b] Coefficient[a] R^2	(LOQ)[b1] µg/kg	Precision[c] µg/kg	(RSD %)
B_1	7.973±0.018	-44.8238	927.2519	0.9992	0.2849	0.7996	0.704988
B_2	7.361±0.017	-11.4898	742.1691	0.9995	0.6903	2.0918	0.155408
G_1	6.689±0.015	-180.806	787.3258	0.9859	0.0839	0.2545	0.048938
G_2	5.830±0.026	-12.9685	277.9480	0.9991	1.1044	3.3468	0.217735

Table 3. Recovery of aflatoxins AFB_1, AFB_2, AFG_1 and AFG_2 in low level, medium level and high level, Data expressed as mean±SD. Abbreviations: L: Low level (1 ppb); M: Medium level (5 ppb); H: High level: (10 ppb), [a]n=6.

Compound	Concentration (ppb)	Response[a]±SD	Accuracy (%)
AFB_1	L	955.66±5.13	108.1
AFB_2	L	224.33±4.50	105.8
AFG_1	L	665±4.0	107.4
AFG_2	L	77±2.64	108.7
AFB_1	M	4456.66±5.508	97.1
AFB_2	M	1074.66±3.512	97.9
AFG_1	M	3232.33±4.163	86.7
AFG_2	M	391.66±3.055	96.9
AFB_1	H	9286.66±5.132	100.6
AFB_2	H	2224.66±4.509	100.5
AFG_1	H	8158.33±5.033	105.9
AFG_2	H	826.66±4.509	100.7
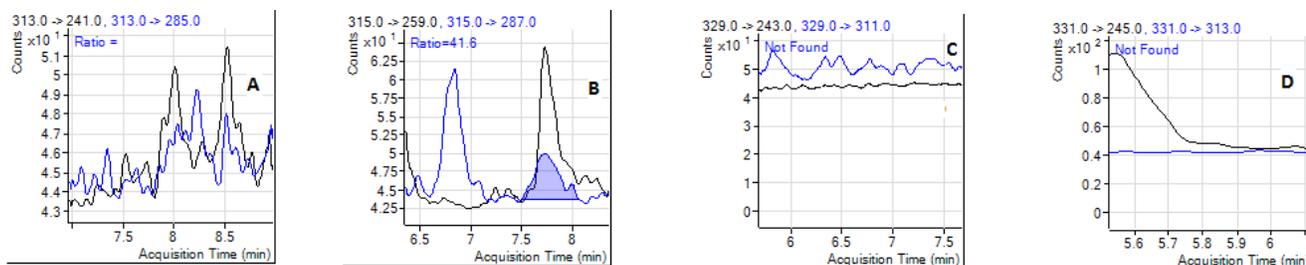
Figure 4. HPLC-MS/MS Chromatogram of *M. pururiens* Counts *vs* Acquisition time (min); (A) Resultant mass peak of AFB_1 observed below the detection limit (0.04 µg/kg),(B) Resultant mass peak of AFB_2 observed below the detection limit (0.05 µg/kg) (C) No resultant mass peak of AFG_1 observed (D) No resultant mass peak of AFG_2 observed. Mobile phase; methanol: acetonitrile (70:30 v/v) eluent A and aqueous ammonium acetate (5 mM) eluent B. Total run time (5 to 8 min), injection volume (5 mL) and flow rate (5 µL/min). The black line in the chromatogram is for quantifier ion and the blue line shows the qualifier ion for the MRM transition for LC-MS/MS of aflatoxins. Ratio in the chromatogram is quantifier to qualifier ion ratio for the compound (which determines confirmation of a compound).
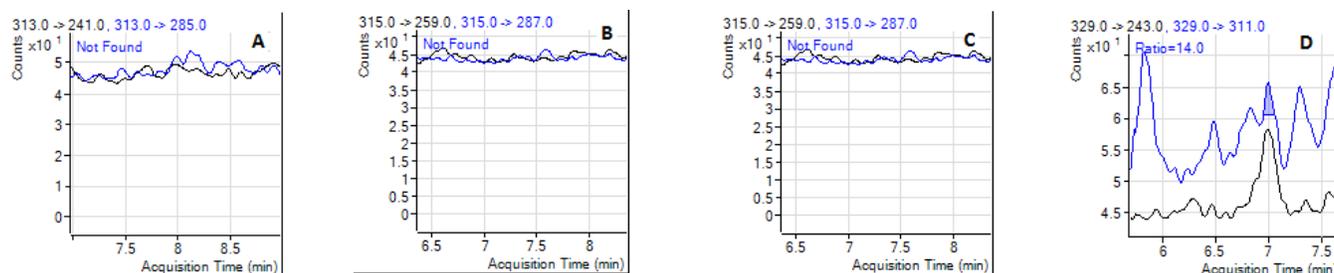
Figure 5. HPLC-MS/MS Chromatogram of *D. denudatum.* Counts *vs* Acquisition time (min); (A) No resultant mass peak of AFB_1 observed (B) No resultant mass peak of AFB_2 observed (C) No Resultant mass peak of AFG_1 observed (D) Resultant mass peak of AFG_2 observed below the detection limit (0.07 µg/kg). Mobile phase: methanol: acetonitrile (70:30 v/v) eluent A and aqueous ammonium acetate (5 mM) eluent B. Total run time (5 to 8 min), injection volume (5 mL) and flow rate (5 µL/min). The black line in the chromatogram is for quantifier ion and blue line shows the qualifier ion for the MRM transition for LC-MS/MS of aflatoxins. Ratio in the chromatogram is quantifier to qualifier ion ratio for the compound, (which determines confirmation of a compound).
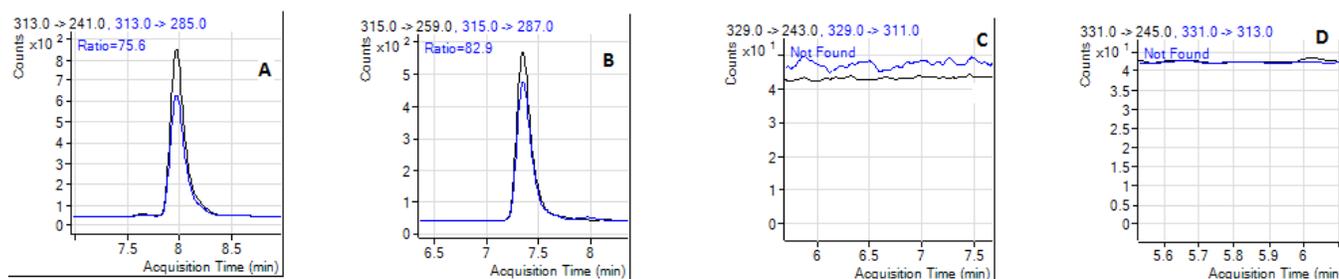
Figure 6. HPLC-MS/MS Chromatogram of *P. oleraceae* Counts *vs* Acquisition time (min) (A) Resultant mass peak of AFB_1 observed (1.675 µg/kg) (B) Resultant mass peak of AFB_2 observed (1.335 µg/kg) (C) No resultant mass peak of AFG_1 observed (D) No resultant mass peak of AFG_2 observed. Mobile phase: methanol: acetonitrile (70:30 v/v) eluent A and aqueous ammonium acetate (5 mM) eluent B. Total run time (5 to 8 min), injection volume (5 mL) and flow rate (5 µL/min). The black line in the chromatogram is for quantifier ion and the blue line shows the qualifier ion for the MRM transition for LC-MS/MS of aflatoxins. Ratio in the chromatogram is quantifier to qualifier ion ratio for the compound, (which determines confirmation of a compound).

Table 4. Summary of contamination levels of aflatoxins in herbs (µg/kg) obtained from LC-Mass spectroscopy
Abbreviations: BDL: Below detection limit; ND: Not detected.

Name of herb	AFB_1	AFB_2	AFG_1	AFG_2	Total AF
Mucuna pruriens	BDL (0.04)	BDL (0.05)	ND	ND	0.09
Delphinium denudatum	ND	ND	ND	BDL (0.07)	0.07
Portulaca oleraceae	1.675	1.335	ND	ND	3.01

Amongst the four AFs, AFB_1 was detected as the most common contaminant. The AFs occurred in the order of $AFB_1 > AFB_2 > AFG_1 > AFG_2$. *Aspergillus flavus* is a very common fungus which infects plants and produces AFB_1 and AFB_2. However levels of AFG_1 and AFG_2 are less often detected in plants because the fungus *Aspergillus parasiticus* does not infect plants very often (52). This may account for the high incidence and concentrations of AFB_1 and AFB_2 compared with AFG_1 and AFG_2 in the three plants investigated in this study. Rhizomes, roots, seeds and fruits have apparently higher AFs than in flowers, indicating that the herbs rich in starch and oil might be more favorable for fungal growth (53). In particular, contamination levels of some samples i.e. root of *D. denudatum*, seeds of *M. pruriens* and *P. oleraceae* were notable, as all of these have a content of fat or fatty oil, suggesting that plants and their products rich in fat or fatty acids should be carefully monitored. The plant samples should be dried as soon as possible and stored in moisture-proof containers with good aeration to avert the growth of AFs, as inappropriate storage and moisture content in samples encourages augmentation of mycotoxins (54). We conclude from this study that the risk of AF contamination in medicinal herb like *M. pruriens, D. denudatum and P. oleraceae* appears low but the presence of AFs in these plants needs to be analyzed before processing to confirm the absence of mycotoxins. The consumer must be cautious while consuming these plants for medicinal purposes. The monitoring scheme described could be applied to determine the aflatoxin contamination in a large number of samples in a cost and time effective manner. The data obtained from this monitoring can be used as a basis for risk analysis of aflatoxin contamination, thereby maintaining the aflatoxin contents at the lowest possible levels.

ACKNOWLEDGMENTS

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