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REVIEW

A big threat: Aflatoxin

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Abstract: The past decade has witnessed a tremendous surge of interest in herbal medicines throughout the world. Aflatoxins are naturally occurring mycotoxins that are mainly produced by *Aspargillus flavus* and *Aspargillus parasiticus* and primarily contaminate food crops such as corn, groundnuts, and tree nuts as well as herbal medicinal plants in tropical and subtropical regions of the world. It is a lethal substance that intensely or at slower ingestion influences the strength of humans and animals. Aflatoxin study is vital for a safety perspective as they are extremely lethal and cancer-causing to overcome the health effect of aflatoxins and for better assessment and standardization of herbal plant drugs. The investigation includes worldwide regulations on aflatoxins with their acceptable ranges in commodities. With more controls for adequate dimensions of aflatoxins set up, present-day analytical techniques have turned out to be very modern, capable of accomplishing results with high accuracy and precision, appropriate for administrative research centers and post-reap sample testing in developed countries.

Keywords: aflatoxin, herbal plants, herbal medicines, pharmacopeias

1 Introduction

Currently according to WHO, Herbal plants are widely used as primary therapeutic care by more than 60% of the world's population and 80% of the population in developing countries. Today patients seeking alternate and herbal therapy is growing exponentially. Herbal medicines are now in great demand for their better acceptability, less expensiveness and better compatibility with human body with minimum side effects.

India is a country that has a very safe, continuous, and long usage of many herbal drugs in the officially recognized alternative systems of health like Ayurveda, Yoga, Unani, Siddha, Homeopathy, and Naturopathy. Millions of Indians use herbal drugs regularly, such as spices, home remedies, health foods as well as OTC as self-medication or also as drugs prescribed in non-allopathic systems. As there is increasing demand for herbal plant medicines, there are increasing concerns about the safety, efficacy, standardization, quality, availability, and preservation of herbal products by health professionals as well as the general public. As aflatoxins are very prone to their growth in plant origins under their favorable growth condition, it becomes very important to identify the presence of aflatoxins to overcome the health hazards [1, 2]. (Figure 1)

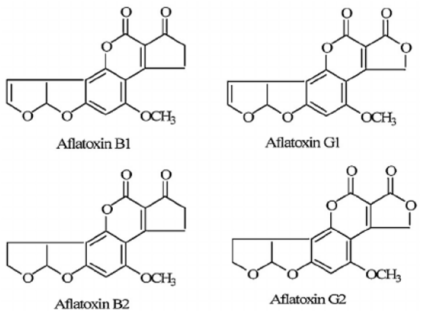

Figure 1 Chemical structure of aflatoxins

Aflatoxins occur as natural, genotoxic, and unavoidable contaminants in a variety of food commodities posing potential health hazards. Aflatoxins are very potent and carcinogenic naturally occurring mycotoxins that are produced by *Aspargillus* species, mainly by *Aspargillus flavus* and

Aspargillus parasiticus. These fungi tend to promote aflatoxin in storage, transportation, and food processing [3]. Favorable conditions for aflatoxins are a temperature of 12-40°C and 3-18% moisture [4]. They may be found present in corn, peanuts, oil seeds, cereals, tree nuts, spices, plants grown in high moisture content environment and also may be found in milk and milk products. There are major 4 types of aflatoxins: aflatoxinB1 (AFB1), aflatoxinB2 (AFB2), aflatoxinG1 (AFG1), and aflatoxinG2 (AFG2); AFM1 and AFM2 are two additional metabolic products which are significant contaminants produced from milk and milk products. IARC classified AFB1 as the most carcinogenic and potent type [5]. The climate of the region, the genotype of the crop planted, soil type, crop maturity, mechanical injury, insect/bird damage, fungus, minimum and maximum daily temperatures, and daily net evaporation are the factors that affects aflatoxin contamination [6].

2 Health impact of aflatoxins

The potential hazard of mycotoxins to humans and animals was forcefully revealed during a serious outbreak of "Turkey X Disease" in England in 1960 when more than 100,000 young turkeys died in England over the course of a few months. The causative agent was traced to a mixture of complex lactones, the aflatoxins, produced by some strains of *Aspargillus flavus* growing on peanut meal. Since then *Aspargillus* have been found that produce aflatoxins on other foodstuffs and grains.

Aflatoxins cause around 90,000 instances of liver malignant growth every year and are firmly connected with hindering and invulnerable concealment in kids [7]. Diseases resulting from consumption of grains contaminated with aflatoxins range from acute liver disease, including necrosis and hemorrhage, Immunological disorders (Table 1) to more chronic forms with extensive fibrosis and bile duct proliferation [8]. Jaundice, hemorrhage, and diarrhea, along with decreased performance, may be evident in affected animals with acute disease [9, 10]. Hemorrhagic anemia syndrome is characterized by massive hemorrhagic lesions in the major organs and musculature. Possible hemolytic anemia with bone marrow hyperplasia and an increase in bone marrow nucleic acid occurred in broiler chicks consuming aflatoxin.

Table 1 Effect of Aflatoxins on the Immune System [11]

Effects of Cellular Responses	Effects on Humoral Factors
Phagocytosis by macrophages reduced	Immunoglobulins (lgG and lgA) concentrations in serum may be reduced
Delayed cutaneous hypersensitivity reduced	Complement activity reduced
Lymphoblastogenesis reduced (response to mitogens)	Bactericidal activity of serum reduced
Graft versus host response reduced	

Typical but nonspecific changes in patients with acute aflatoxicosis include jaundice, low-grade fever, depression, anorexia, and diarrhea, with fatty degenerative changes in the liver evident, upon histopathology examination, as Centro lobular necrosis and fatty infiltration. Tenderness near the liver was evident in patients with acute, aflatoxin-caused hepatitis in Kenya; ascites may develop. Mortality reached 25% in outbreaks in India. Samples of liver obtained from patients who died contained detectable levels of aflatoxin B1 [11]. Outbreaks of acute aflatoxin poisoning are a recurrent public health problem. Outbreaks that cause serious health hazards to humans and animals result in hepatocellular carcinoma, liver cirrhosis, impaired growth, immunosuppression, reproductive problems, and anemia followed by death [12].

These effects could be mitigated or prevented through the effective and integrated use of current agricultural knowledge, public health practice, and prevention techniques.

3 Global regulations on aflatoxins

To ensure food and feed safety, more than 100 countries have established regulations for mycotoxins in food and feed. On a worldwide basis, at least 99 countries had mycotoxin regulations for food and/or feed in late 2003, an increase of 30 percent compared to 1995. Many national and international public health and governmental authorities such as the USFDA, WHO, FAO, and the EFSA are paying serious attention to mycotoxin contamination in food and feed and addressed this global problem by adopting strict regulatory guidelines for major mycotoxin classes in food and feed [13].

Generally, Regulatory guidelines like FDA mycotoxin regulatory guidance, Commission Regulation (EC) No 401/2006, European Communities (Sampling Methods and Methods of Analysis for the Official Control of the Levels of Certain Contaminants in Foodstuffs) (No. 2) Regulations 2006,

Guidance document for competent authorities for the control of compliance with EU legislation on aflatoxins. Guidance for Industry: Action Levels for Poisonous or Deleterious Substances in Human Food and Animal Feed by USFDA are currently followed by various organizations for aflatoxins.

The USA also has regulations like GIPSA, FGIS provides mycotoxin testing services as official criteria for corn, sorghum, wheat, barley, oats, rye, and soybeans under the USGSA, as amended. Testing is also provided for rice, popcorn, distillers dried grains, corn grits, corn meal, corn gluten meal, corn/soy blend, wheat middlings, and other processed commodity products under the authority of the AMA of 1946, as amended.

The maximum levels of aflatoxins established in Commission Regulation (EC) 1881/2006 apply to all groundnuts, other oilseeds, derived products, and cereals placed on the market except for those groundnuts, other oilseeds, derived products, and cereals that are intended for uses other than human consumption either directly or indirectly and also for groundnuts and other oilseeds intended for crushing for refined vegetable oil production.

Regulations have become more diverse and detailed with newer requirements regarding official procedures for sampling and analytical methodology. At the same time, several regulations have been harmonized between countries belonging to economic communities, or they are in some stage of harmonization. Nevertheless, the regulatory requirements remain substantially different across many countries [14]. In the European and United States Union strict rules and legislative limits defined by the European Commission and USA respectively have been set for all of the aflatoxins in certain food- and feedstuff to protect animals and humans. In Asia, differences regarding legislation exist in different regions. Globally, there are many accepted legislative limits to be followed for aflatoxins (Table 2).

Table 2 Globally accepted legislative limits for aflatoxins

Name of Country	Maximum Level			
	Aflatoxin in Food		Aflatoxin in Feed	
	AFB1	Total Aflatoxins	AFB1	Total Aflatoxin
USA	5 ppb	20 ppb	-	20-300 ppb
Europe	0.1-12 ppb	4-15 ppb	5-20 ppb	-
India	-	30 ppb	-	-
China	0.5-20 ppb	10 ppb	$<$ 50 ppb	-
Australia	-	5 ppb	-	-
Malaysia	0.1 ppb (food for infants and children)	5-15 ppb	-	-
Japan	-	10 ppb	10-20 ppb	-
Indonesia	15 ppb	20 ppb	-	20-50 ppb
Korea	10 ppb	15 ppb	-	-
Taiwan	-	50 ppb	-	-

The choice of limits for aflatoxins depends on the availability of toxicological data, the occurrence of aflatoxins in various commodities, the availability of methods, the implication of methods, and the existence of sufficient food supply. The need for regulations for limiting the concentration of mycotoxins in food and feed is generally recognized and has already been mentioned in some developing countries. According to that aflatoxins in food/feed should not be more than 20ppb.

4 Case studies

A comprehensive update on worldwide regulations was published as FAO Food and Nutrition Paper 64 recently. According to that at least 77 countries now have specific regulations for mycotoxins, 13 countries are known to have no specific regulations, whereas no data are available for about 50 countries [15]. However, with estimated 25 % to 50 % of the world's food crops affected by mycotoxins, the economic costs are likely to be considerable. 9 Numerous reports focusing on different countries/regions, commodities, toxins, and cost categories (e.g., costs of regulations, testing, production loss, trade losses) offer some indication of these losses. Generally, there are a number of contextual investigations were done for aflatoxins on various commodities, livestock, animals as well as humans (Table 3).

These case studies proved that aflatoxins, when ingested, breathed in, or absorbed through the skin, have cancer-causing, hepatotoxic, teratogenic, and mutagenic impacts in human and creatures (rodents, ferrets, ducks, trout, hounds, turkeys, dairy cattle and pigs) even at little fixations, because of this sort of risks it turns out to be imperative to do aflatoxin studies.

Table 3 Case studies on aflatoxins worldwide

Place/Country	Case Studies Done on	Case Study	Summary	Reference
	Rats, trout, ducks, ferrets, mice, monkeys, sheep	Induction of neoplasms (abnormal growth of tissue) by aflatoxins has been extensively studied	By induction of neoplasms, binding of aflatoxin B1 to DNA causes structural alterations in the DNA results to genomic mutations in in vitro and in vivo systems	John L. Richard et al, 2003
USA	Young male Fischer 344 rats, 6 month old mosquitofish (Gambusia affinis), immortalized human hepatoma cells (HepG2) and human bronchial epithelial cells (BEAS-2B).	Acute and combinative toxicity of AFB1	Acute toxic symptoms were observed immediately after treatment in F344 rats; reduction of activity and loss of righting reflex was observed in mosquitofish; cytotoxicity was observed in HepG2 and BEAS-2B.	C. McKean et al, 2005
Kenya	40 case-patients with aflatoxicosis and 80 randomly selected controls	administered questionnaires regarding maize storage and consumption and obtained maize and blood samples from participants	Homegrown (not commercial) maize kernels from case households had higher concentrations of aflatoxins than did kernels from control households. Serum adduct concentrations were associated with time from jaundice to death; Case patients had positive hepatitis B titers	Eduardo AB et al, 2005
USA (California)	Trout fish	Aflatoxin-contaminated cottonseed meal was implicated as the causative agent in this study	Liver cancer was observed after 9 months	John L. Richard et al, 2003
USA (California)	Swine and guinea pigs	General clinical studies were done	Clinical and pathological signs include decreased rate of weight gain, decreased feed conversion efficiency, toxic hepatitis, nephritis, and systemic hemorrhages due to presence of great dose of aflatoxins	John L. Richard et al, 2003
Nigeria	2 pigs 4 weeks old for 4 weeks and 5 others got a meal with 20% Brazilian groundnut meal having aflatoxin	Study on a toxic factor in Brazilian groundnut causing liver damage in pigs	High mortality is reported in young pigs in a herd given Brazilian groundnut meal, 17.5% in the starter pellets and 8.75% in the sow and weaner meal; All developed signs of poisoning, with typical acute or sub-acute liver damage	Loosmore, R. M, 1961
Sudan (Africa)	252 Sudanese children (comprised 44 with kwashiorkor, 32 with marasmic kwashiorkor, 70 with marasmus, and 106 age-matched)	Blood and urine samples were investigated for their aflatoxin content by high-performance liquid chromatography	Aflatoxicol, a metabolite of aflatoxins B_1 and B_2, was detected in the sera of children with kwashiorkor and marasmic kwashiorkor but not in the controls and only once in a marasmic child	Hendrickse, R.G et al, 1982

5 Approaches for detecting aflatoxins

The greater part of the current analytical techniques utilized for the detection and quantification of aflatoxins involve sampling, and sample preparation including extraction and clean-up, trailed by a detection method depending on the precision of the desired result.

5.1 Sampling and sample preparation

A sampling protocol, or plan, is specific to an analytical method and consists of a sampling phase, and an analytical phase, divided into sample preparation and instrumental analysis [16]. Efforts for improving sampling and sample preparation for the detection of aflatoxins, particularly in food and feed, continue to be a priority for regulating agencies worldwide [17].

Expulsion of aflatoxins from a target commodity is accomplished by extracting the mycotoxins with an aqueous polar solvent, most commonly, methanol or acetonitrile [18, 19]. The choice of solvent depends on the chemical composition of the mycotoxin, the extraction matrix, safety concerns including the volume of waste generated, and the chosen analytical method [17, 19]. The extraction method involves blending or shaking ground samples in the preferred solvent, followed by filtration or centrifugation [18, 19]. The filtered liquid is further purified before the determination step or applied directly in methods that do not require clean-up including immune-based analytical methods such as ELISA [20, 21] or LC-MS/MS procedures utilizing 'dilute and shoot' protocols [22].

The most frequently employed clean-up procedures for aflatoxins-determination are SPE methods, recently reviewed by several authors, including multifunctional columns and IAC [18–21]. The SPE column contains a bonding phase such as porous silica, modified to allow selective absorption of impurities or the substance of interest (analyte). IACs have been generally utilized in mycotoxin, especially aflatoxins, examination. The eluted IAC extracts might be read straightforwardly in a fluorometer using economically developed techniques. On the other hand, the eluted extracts might be combined with HPLC-based strategies endorsed by the Association of Official Analytical

Chemists (AOAC), for enhanced quantitative investigations.

5.2 Quantitative determination methods

Reference methods have several purposes: one is to confirm samples that have been determined to contain mycotoxins, based on rapid screening tests. The second is to more accurately quantify the amount of toxin present. Reference methods for mycotoxins generally involve a chromatographic technique for quantification of aflatoxins, comparison of various analytical methods in terms of principle, advantages, disadvantages, and application of a particular method in determination is given. Since the collection of methods has been built up gradually over a period of years, sufficient attention has to be paid to selecting an appropriate method for aflatoxins. (Table 4)

Table 4 Comparison of various analytical methods for aflatoxin determination

Method	Principle	Advantages	Disadvantages	Reference
Thin layer chromatography	Separation depends on the relative affinity of compounds towards the stationary and mobile phase, compounds under the influence of the mobile phase driven by capillary action over the surface of the stationary phase.	Reliable quantification method when combined with densitometry Accuracy and precision comparable to HPLC methods (HPTLC; OPLC) Official reference methodology for aflatoxins	Outdated equipment Destructive sample preparation Largely replaced with HPLC for quantitative analysis of aflatoxins	Rahmani et al., 2009; Shephard, 2009
High-performance liquid chromatography (HPLC)	A separation technique where a mobile phase passes over a stationary phase causing different components of a mixture to separate. High pressure is required to move the mobile phase over microparticulate particles.	Reliable, sensitive, selective, and repeatable quantification methodology May be automated Official reference method for aflatoxins	Expensive equipment requiring dedicated operator and specialist to interpret results Destructive sample preparation May require derivatization	Cho et al., 2008; Shephard,2009; Turner et al., 2009
Liquid chromatography/ mass spectrometry (LC-MS)	Individual components in a mixture are first separated followed by ionization and separation of the ions on the basis of mass/ charge ratio. The separated ions are then directed to a photo or electron multiplier tube detector, which identifies and quantifies each ions.	Simultaneous analysis of mycotoxins Low limit of detection (LC-MS/MS) Confirmatory method No derivatization required	Very expensive equipment requiring dedicated operator and specialist to interpret results Sensitivity relies on ionisation Matrix assisted calibration for quantitative analysis	Krska et al, 2008; Li et al., 2013; Pascale, 2009; Shephard, 2009
GC-MS	In this method, carrier gas is used as mobile phase and liquid coated onto inert solid particles is acts as stationary phase. Sample to be analyzed into gaseous phase and carried through stationary phase with help of carrier gas.	Simultaneous analysis of mycotoxins Good sensitivity Provides confirmation (MS detector).	Equipment, specialist expertise required Derivatization required Matrix interference problems Non-linear calibration curve drifting response Carry-over effects from previous sample Variation in reproducibility and repeatability.	Alex P. Wacoo et al.; Michelangelo N. Pascale
Enzyme-linked immunosorbent assay (ELISA)	ELISA is a method of target antigen (or antibody) capture in sample using a specific antibody (or antigen), and of target molecule detection/ quantification using an enzyme reaction with its substrate.	Specific, rapid and relatively easy to use Inexpensive equipment Low limit of detection Simultaneous analysis of multiple samples Semi-quantitative (screening) or quantitative analysis possible Limited use of organic solvents	Possible cross reactivity with related mycotoxins Matrix interference Possible false positives/negatives Narrow detection range Confirmatory LC analysis may be required	Pascale et al, 2009; Pittet, 2005; Turner et al., 2009
Immunoaffinity assay	It uses the highly specific binding of antibodies for the retention of a target. This approach can be used in a myriad of techniques for the selective purification, concentration and/or analysis of target compounds.	IAC in combination with liquid fluorometry is comparable to LC for determination of aflatoxins Official method	Sample destruction Limited to analysis of total aflatoxin	Pittet, 2005
Fluorescence polarization immunoassay	It quantifies the change in fluorescence polarization of reaction mixtures of fluorescence labelled tracer, sample antigen and defined antibody.	Rapid, no cleanup required Mycotoxin-specific tracer for analysis Very sensitive Portable	Limited validation with ELISA or HPLC Possible cross reactivity with related mycotoxins Matrix interference Limited to detecting one mycotoxin at a time	Lattanzio et al., 2011; Lippolis and Maragos, 2014; Pascale, 2009
Adsorptive stripping voltammetry	It is based on accumulation and reduction of AFB1 and AFB2 species on the surface of HMDE	Highest yield of aflatoxins Minimum matrix effect Improved sensitivity Quantifies AFB1, AFB2 Extended linear dynamic range Simplicity and speed	Limited use Quite costly as it involves use of hanging mercury drop electrode Reliable compared to HPLC method	Alejandro E et al.
Capillary electrophoresis	Capillary tube is placed between two buffer reservoir and an electric field is applied, separation depends on electrophoretic mobility and electro-osmosis. Electrophoretic separation is measured by detector.	Useful for separating closely related mycotoxins Highly sensitive Capable of multi-constituent analysis when combined with immunoassays	Limited to lab use due to cumbersome instrumentation	Maragos, 2004
Biosensors	Biosensors are operated based on the principle of signal transduction. Bio recognition element is allowed to interact with a specific analyte and signal generated from transduction is then simplified and processed by electronic system.	Rapid, no clean-up High selectivity and low limit of detection Ease of use, low cost and portability Self-contained, simple design	Extraction sample prep for solid samples Extract clean-up needed to improve sensitivity Cross reactivity with related mycotoxins Variation in reproducibility and repeatability (improved with use on novel materials)	Malhotra et al., 2014; Meneely and Elliott, 2014; Pascale, 2009; Rubert et al., 2012a; Tothill, 2011
Near infrared spectroscopy	Interactions of light from different wavelengths with the matter, which represented by electromagnetic spectrum applied. IR instruments usually operated NIR region of electromagnetic spectrum is from 800 to 2500nm.	Rapid, non-destructive No extraction or clean-up User-friendly operation	Calibration model must be validated Knowledge of statistical methods Poor sensitivity (high limit of detection) Costly equipment	Berardo et al., 2005; Dowell et al., 2002; FAO, 2004; Gordon et al., 1999; Hossain and Goto, 2014; Pearson and Wicklow, 2006; Pearson et al., 2001; Tallada et al., 2011
Ion mobility spectrometry	Used for characterization of chemicals on the basis of speed acquired by the gas phase ions in an electrical field. It has been used to detect and quantify the concentration of aflatoxins in food. Sample is evaporated and mixed with carrier gas which the entered to IMS where mixer is ionized and passes through an electric field gradient, where ions of different substances travel at different speeds.	Low detection limit Fast response Simplicity Portability Low cost	Impossible to quantify as low as 0.25ng	Alejandro E et al.
Hyper spectral imaging	It collects and processes information from across the electromagnetic spectrum. The goal of hyper spectral imaging is to obtain the spectrum for each pixel in the image of a scene, with the purpose of finding objects, identifying materials or detecting processes.	Rapid, non-destructive No extraction or clean-up User-friendly operation High spectral and spatial resolution Potential for in-line detecting applications	Calibration model must be validated Knowledge of statistical methods Poor sensitivity (high limit of detection) Low signal level (for fluorescence) Costly equipment	Del Fiore et al., 2010; Hruska et al., 2013; Yao et al., 2008, 2010
Electronic nose	It comprises of: a sample delivery system, a detection system and a computing system; which generates signal pattern that are used for characterizing odors.	Rapid means for controlling and improving the microbiological quality of food	Need to improve selectivity and reduce interferences (e.g. to humidity) Compensate for drift effects Limited feasibility studies and poor validation	De Lucca et al., 2012; Gardner and Bartlett, 1994

6 Conclusion

During the past decade, there has been increasing acceptance and public interest in natural therapies in both developing and non-developing countries. The number of reports of patients experiencing negative health consequences caused by the consumption of affected plant drugs has to be strictly regulated as per globally established regulatory limits. Herbal drugs normally carry several contaminants that should be carefully investigated and/or monitored, since some common species produce toxins, especially aflatoxins which can be dangerous to health even if they are absorbed in minute amounts. This review contains a representative selection of analytical methods for aflatoxins which have been adopted as standard procedures in large commercial laboratories engaged in technical analysis and have been proved to give satisfactory results in the hands of different analysts. In conclusion, a wide spectrum method range is available for the detection and quantification of aflatoxins employed with advantages and limitations. So new methods are still required to achieve high sensitivity and accuracy to overcome existing challenges. This review is a summary of current analytical approaches to aflatoxin detection and the most recent research in quick and non-obtrusive identification techniques.

Even though there are so many studies were already done in the case of aflatoxins, most of the pharmacopeias are working towards this area in fixing/ensuring these particular aflatoxins to enhance quality control and standardization of herbal drugs.

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Conflicts of interest

The authors declare that they have no conflict of interest.

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