Pharmacogn. Res., 2025; 17(3):740-754.
https://www.phcogres.com

Review Article

Aflatoxin: A Prevalent Toxin in the Demesne of Existence

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ABSTRACT

Aflatoxin, a mycotoxin origin from food-borne microbes, poses a significant and remarkable risk to the safety and integrity of food. Mycotoxin-producing fungi can infect goods intended for human and animal consumption, impacting both populations. Among mycotoxins, aflatoxin B_1 harms many living organisms, including microorganisms, plants, laboratories, pet animals, and cultured cells. Aflatoxins are incredibly liposoluble and easily absorbed from the exposure site, typically entering the bloodstream through the respiratory and gastrointestinal systems. In humans and animals, the reactive form of aflatoxin, AFB_1, is metabolized through cytochrome P450 microsomal enzymes into aflatoxins-8,9-epoxide, which then binds with DNA and albumins present in blood serum, forming an active-adducts that damage DNA. Aflatoxins are the well-researched class of mycotoxins, with clear correlations to carcinogenic, hepatotoxic, mutagenic, and teratogenic effects. Therefore, aflatoxin detection is crucial for maintaining food safety and public health, as mycotoxins can withstand high temperatures during storage and cooking. Several analytical and chemical methods can detect, identify, and control aflatoxin contamination with precision, contributing to food safety and public health. Further worldwide research is crucial to deepen our understanding of toxic pollutants, toxicity patterns, regulatory mechanisms, and decontamination strategies, as preventing aflatoxin contamination needs adequate funding and collaboration.

Keywords: Food-borne microbes, Mycotoxins, Aflatoxins, Carcinogenic, Hepatotoxic, Mutagenic and Teratogenic.

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Received: 27-02-2025;
Revised: 09-04-2025;
Accepted: 14-06-2025.

INTRODUCTION

The agricultural and food sectors should prioritize food for human and animal use. However, mycotoxins, produced from a food-borne microbe, pose the greatest risk to the safety and integrity of food.[1,2] Sporadic metabolites of fungus are called mycotoxins that thrive on various cultivated products, such as cereals, almonds, soybeans, and other crops. These mycotoxin-producing fungi can contaminate goods intended for human and both pet and wild animal consumption, impacting both populations. Food safety experts must also consider the possibility of mycotoxins entering animal products like meat, milk, and eggs when animals resistant to particular mycotoxins are fed contaminated feed.[3-6] Unlike bacterial toxins and macromolecular proteins that cause symptoms within hours, mycotoxins are low-weighted chemicals that cause delayed symptoms. This delay occurs as the body identifies them as antigens and mounts an antibody-mediated response. Mycotoxins can impair human and animal performance, cause illness, or even be fatal when consumed, inhaled, or absorbed through the skin.[1,7] The signs of mycotoxin exposure vary on the types and variants of mycotoxin, the duration and intensity of exposure, the exposed person's age, their health condition, and gender, as well as several inadequately comprehended synergistic effects, including heredity, dietary condition, and interactions with additional toxic effects. Conditions like vitamin deficiencies, calorie restriction, alcohol misuse, and the presence of infectious diseases can all exacerbate the severity of mycotoxin poisoning. The exact number of people affected by mycotoxins is unknown. While the overall number is thought to be lower than that of bacterial, protozoan, and viral infections, fungal illnesses still pose a severe threat to global health.[8,9] A closely related group of heterocyclic chemicals known as aflatoxins is primarily produced by two conidial fungi, *Aspergillus flavus* and *Aspergillus parasiticus*.[10,11] These fungi belong to the *Aspergillaceae* family, class Hyphomycetes, and subdivision Deuteromycotina. *Aspergillus flavus* is the most significant and economically significant member of the *Aspergillus* genus due to its production of aflatoxins. An overview of the major aflatoxins produced by the different *Aspergillus* species is tabulated in Table 1. *A. flavus* is a saprobe that can thrive on various organic nutrients, including debris of plants, animal dung, cotton, animal carcasses, compost piles, dead insects, grains that are stored and

DOI: 10.5530/pres.20252183

even immunodeficient humans and animals. It is one of the most common soil-borne molds.[12,13] Although *A. flavus* can tolerate temperatures between 12°C and 48°C where the ideal range for the growth is 28°C to 37°C. It has the ability to expand at high temperatures, which leads to its pathogenicity toward human beings and other existed warm-blooded creatures. For most of its life cycle, the fungus is mycelium or conidia, which are asexual spores.[14,15] The fungal mycelium may adapt to difficult environmental circumstances by transforming into resistant structures known as sclerotia, which can endure a lack of water or nutrients. The fungus are overwinter as spores, mycelium, or sclerotia in the leftovers.[13,16] The sclerotia usually germinate to create new colonies renowned as conidiophores that contain conidia when the right circumstances are met. Aflatoxins are naturally occurring carcinogens that contaminate various dietary items for humans and animals. Agronomic and agricultural techniques and geographic location influence the prevalence of aflatoxins in foods and food products. Food products are vulnerable to fungal attacks during pre-harvest, transit, storage and processing.[17,18] Aflatoxin-contaminated food products are a widespread issue in tropical or subtropical locations, particularly in developing nations with inadequate sanitation systems and warm, humid climates that encourage the growth of fungi, such as in sub-Saharan Africa. Around 100,000 turkeys died from "Turkey X disease" in England and the USA in 1960 as a result of liver necrosis, and aflatoxins were initially linked to the illness. The usage of peanut meal tainted with mold was the cause of the deaths.[19,20]

Chemistry of Aflatoxins

In chemical nature, aflatoxins are derivatives of di-furanocoumarins with either a lactone ring (in the case of AFGs) or a pentanone ring (in the case of AFBs) linked to the coumarin nucleus. They are derived via a polyketide pathway. Aspergillus species of mold create four primary naturally occurring aflatoxins: AFB_1, AFB_2, AFG_1, and AFG_2.[3] The letters “B” stand for blue fluorescent and “G” stand for green fluorescent colors as aflatoxins produce these colors on thin-layer chromatography plates when exposed to UV light, although the major and minor compounds are indicated by the subscript numerals 1 and 2, respectively. Since the metabolites of aflatoxins, such as M_1 and M_2, were first discovered in milk from moldy grains infected with aflatoxins, the M designation was given. These toxins belong to a special class of highly oxygenated heterocyclic chemicals that exist naturally and have similar structures.[10] Aflatoxins B_2 and G_2 are the compounds known to be the di-hydroxy derivatives of aflatoxin B_1 and aflatoxin G_1 respectively. However, 4-hydroxy aflatoxin B_1 is known as aflatoxin M_1, while 4-dihydroxy aflatoxin B_2 is known as aflatoxin M_2. Among the four main aflatoxins (B_1, B_2, G_1, and G_2) the most dangerous one is AFB_1, while G_2 is present in higher concentrations but is less hazardous.[12,21] Structures of different aflatoxins are depicted in Figure 1.

Biosynthesis of Aflatoxin

Understanding the biosynthetic pathway of aflatoxins is crucial for developing strategies to control their production and reduce their impact on food safety. The biosynthesis of aflatoxins involves several steps, as illustrated in Figure 2 and described below.[9,10]

Norsolorinic Acid production from Acetate

Acetate is first transformed into acetyl-CoA, a key player in various metabolic processes and a precursor for numerous pathways in the body, including the synthesis of aflatoxin. Acetyl-CoA combines with malonyl-CoA through the action of a Polyketide Synthase (PKS) enzyme to produce a polyketide chain. Versicolorin B is the product of this chain's several alterations, including cyclization and reduction. Versicolorin B is then converted into Norsolorinic Acid (NOR) through enzyme-driven reactions involving reduction, dehydration, and epoxidation processes. Specific enzymes catalyze these transformations to convert the polyketide chain into NOR.

Averantin production from Norsolorinic Acid

The initial step in converting NOR to AVN involves lowering the ketone group in NOR to create a hydroxyl group, a process typically aided by a reductase enzyme. Next, the hydroxyl group in the altered NOR is hydroxylated at a designated location with the help of a hydroxylase enzyme, which introduces a hydroxyl group to a specific carbon within the molecule. Following hydroxylation, the molecule goes through further changes like methylation and oxidation to produce the end product, Averantin (AVN).

5'-Hydroxyaverantin production from Averantin

This step involves transforming AVN into HAVN and hydroxylation of AVN at its 5' position. A hydroxylase enzyme catalyzes this reaction by attaching a hydroxyl group to the AVN's 5' carbon. Following hydroxylation, the molecule goes through oxidation to produce a carbonyl group at the 5' position, which an oxidase enzyme facilitates. The oxidized AVN then changes methylation and reduction to yield 5'-Hydroxyaverantin (HAVN) product.

Oxoaverantin and Averufin production from 5'-Hydroxyaverantin

The enzyme oxidase catalyzes the oxidation of HAVN at the 7' position to produce Oxoaverantin (OAVN). OAVN is then oxidized at the 5' position by another oxidase enzyme, forming Averufin (AVF). AVF can be reduced at the 5' position by a reductase enzyme to create Averufinhydroxy (AVFH).

Versiconal Hemiacetal Acetate production Averufin

The process of converting AVF to VHA involves the oxidation of AVF at the 3' position. An oxidase enzyme is typically responsible

for catalyzing this oxidation reaction, which adds an oxygen atom to the 3' carbon of the AVF molecule. Following oxidation, the molecule undergoes a ring closure reaction to form a hemiacetal. A cyclase enzyme mediates this reaction by facilitating the formation of a new bond between the 3' carbon and a hydroxyl group on the molecule. The hemiacetal group is then acetylated, usually at the 5' position, to create the final product, Versiconal Hemiacetal Acetate (VHA). An acetyltransferase enzyme catalyzes this acetylation reaction by transferring an acetyl group from acetyl-CoA to the hydroxyl group on the hemiacetal.

Versiconal production from Versiconal Hemiacetal Acetate

The process usually involves a reductase enzyme that changes the hemiacetal group in VHA to a hydroxyl group, creating Versiconal (VAL). Versiconal can then be modified by enzymes through oxidation and cyclization to produce various intermediates in the aflatoxin biosynthesis pathway.

Versicolorin B production from Versiconal

The process of converting versiconal to versicolorin B begins with the oxidation of the hydroxyl group in versiconal to a ketone group. An oxidase enzyme typically facilitates this oxidation. Following oxidation, the molecule undergoes a ring closure reaction to create a dihydrofuran ring, catalyzed by a cyclase enzyme. This enzyme helps form a new bond between two carbon atoms in the molecule. Subsequent enzymatic reactions, such as reduction and cyclization, further modify the dihydrofuran ring to produce the end product, versicolorin B.

Versicolorin A production from Versicolorin B

The initial step in transforming versicolorin B into versicolorin A involves reducing the ketone group at the C-1 position of the dihydrofuran ring in versicolorin B. A reductase enzyme typically carries out this reduction, creating a hydroxyl group at the C-1 position. Following the reduction, the molecule undergoes a rearrangement reaction called a Baeyer-Villiger oxidation, which entails the migration of an oxygen atom from the C-1 position to the C-10 position of the molecule. A Baeyer-Villiger monooxygenase enzyme facilitates this rearrangement. The reconfigured molecule then undergoes a ring closure reaction to form a dihydrofuranone ring. This reaction is initiated by a cyclase enzyme, which aids in the formation.

DMST production from VER A

The first step in the process is the oxidation of VER A, which typically occurs at position C-11 and produces a ketone group. An oxidase enzyme aids in this oxidation process. To create DMST, the ketone group in the oxidised VER A is then methylated at the C-10 position. A methyltransferase enzyme transfers a methyl group from S-Adenosyl Methionine (SAM) to the C-10 position in order to complete the methylation process.

DMDHST production from VER B

VER B is oxidized at the C-11 position to create a ketone group, just like VER A turning into DMST. The ketone group in the oxidized VER B is later transformed into a hydroxyl group at the C-10 position resulting in DMDHST. This reduction process is facilitated by a reductase enzyme.

ST production from DMST

Sterigmatocystin (ST) is formed through a series of reactions starting with the oxidation of DMST at the C-12 position. This oxidation is carried out by an oxidase enzyme. The oxidized DMST then undergoes a rearrangement known as a Baeyer-Villiger oxidation, facilitated by a Baeyer-Villiger monooxygenase enzyme. During this rearrangement, an oxygen atom moves from the C-12 position to the C-11 position. The molecule is further processed through reduction and dehydration to produce Sterigmatocystin (ST).

DHST production from DHDMST

DHDMST undergoes oxidation, usually at the C-12 position, to form a ketone group, similar to the conversion of DMST into ST. The ketone group in oxidized DHDMST is then converted into a hydroxyl group, typically at the C-11 position, resulting in the formation of DHST. This reduction process is facilitated by a reductase enzyme.

OMST production from ST

In order to create OMST, ST is methylated at the C-10 location during the transformation process. A methyltransferase enzyme helps to assist this methylation by moving a methyl group from S-Adenosyl Methionine (SAM) to the C-10 position.

DHOMST production from DHST

DHST gets methylated, usually at the C-10 spot, to create DHOMST. A methyltransferase enzyme facilitates this methylation process, transferring a methyl group from SAM to the C-10 position.

AFB_1 and AFG_1 production from OMST

During the process, OMST gets oxidized at the C-9 position to create a ketone group with the help of an oxidase enzyme. Following this, the oxidized OMST goes through a ring closure reaction to produce a dihydrofuran ring, mediated by a cyclase enzyme that facilitates the bonding of two carbon atoms in the molecule. The dihydrofuran ring is further altered through enzymatic reactions such as reduction and dehydration to yield the ultimate products, AFB_1 and AFG_1.

AFB_2 and AFG_2 production from DHOMST

DHOMST is oxidized primarily at the C-9 position to produce a ketone group, akin to the process by which OMST is transformed

into AFB_1 and AFG_1. The resulting oxidized DHOMST then experiences a ring closure reaction, generating a dihydrofuran ring, mirroring the transformation from OMST to AFB_1 and AFG_1. This dihydrofuran ring is subsequently altered through further enzymatic processes involving reduction and dehydration, ultimately yielding the end products AFB_2 and AFG_2.

Synthetic Approach of Aflatoxin B_1

Exceptional contributions in the overall synthesis of aflatoxins have been made by the Büchi group. This group has successfully identified multiple full synthesis of difficult aflatoxin-family compounds. The Pechmann Condensation and the Cascade Reduction Rearrangement are features of these synthesis. Büchi's group has identified the first total synthesis of aflatoxin B_1 as early as 1966, as Figure 3 illustrates. Acetyl benzene 1-(2,4,6-trihydroxyphenyl)ethan-1-one(A) was converted into the aldehyde 5-(benzyloxy-7-methoxy-2-oxo-2H-c hromene-4-carbaldehyde(B) by five processes: non-selective acylation followed by methylation and deacylation then selective benzylation, veticilienylation, and allyl oxidation completes the synthesis procedure. In the presence of zinc/acetic anhydride, the tricyclic intermediate 4-hydroxy-6-methoxy-3a,8a-dihydrofuro[2,3-b]benzofuran-2(3H)-one(C) is produced by the tricyclic skeleton esterification reaction and the subsequent removal of the benzyl protecting group. Subsequently, the β-keto ester and hydrochloric acid were used to form the D-ring using a Pechmann condensation process in methanol. Notably, methanol's C-ring opened when hydrochloric acid was present. The C-ring was then re-cycled as a result of the two ester groups undergoing acetal methyl hydrolysis in the presence of hydrochloric and acetic acids. Following the carboxyl group's activation, the 4-methoxy-2,3,9,9a-tetrahydroc yclopenta[c] furo[3',2':4,5] furo[2,3-h] chromene-1, 8, 11(6aH)-trione(D) was created using the Friedel-Crafts reaction, which $AlCl_3$ catalyzed. By selectively reducing the C-ring, acylating the hemiacetal hydroxyl, and pyrolysing at 240°C, aflatoxin B1 was created. This leads to the synthesis of aflatoxin.[22]

Pharmacokinetic Properties and Biological Effect of Aflatoxin

Aspergillus species naturally produce a wide variety of aflatoxin types. Still, four specific aflatoxin kinds, AFB_1, AFB_2, AFG_1, and AFG_2, are specifically harmful to people and various animals because of their linking with contaminated nuts, cereals, and other goods. There is a lot of variety in the biological harm and health risks associated with aflatoxins.[12] Aflatoxins, particularly AFB_1, are extremely harmful to many living organisms, including microbes, plants, lab and pet animals, and cultured cells. The study aids in understanding several notions of the biological negative consequences and health risks associated with aflatoxins, such as their hepatotoxic effect, carcinogenic impact, mutagenic and teratogenic influence. Aflatoxins are highly lipid-soluble substances easily absorbed from exposure, typically entering the bloodstream through the respiratory and gastrointestinal systems.[23] There are two main ways that aflatoxins can enter the system: (a) through direct food consumption or ingestion of aflatoxins that have been transferred into milk products, such as butter, cheese, and milk powder (b) by breathing in Aflatoxins (AFB_1, in particular) dust from contaminated food in factories and industries.[23-25] Aflatoxin absorption occurs across cell membranes after it enters the body and travels to the blood circulation. Blood carries them to many organs, including the liver, the primary organ involved in the metabolism of xenobiotics. Aflatoxin M_1 is a less dangerous form of aflatoxin that is primarily converted by the liver into a very reactive epoxide intermediate or hydroxylated.[26-28] The reactive form of aflatoxins, AFB_1, is metabolized by cytochrome P450 microsomal enzymes in humans and susceptible animal species to aflatoxins-8,9-epoxide, which binds to DNA and albumins present in blood serum to form adducts and damage DNA.[6,14] CYP3A4 and CYP1A2 are the two main human CYP450 isoforms involved in AFB_1 metabolism. Both enzymes catalyze the biotransformation of AFB_1 into the extremely reactive exo-8,9-epoxide of AFB_1. AFM_1 can also be oxidized by CYP1A2 to produce an intermediate endo-epoxide, and it can also be hydroxylated to produce Aflatoxin M_1 (AFM_1), a less potent and poorer substrate for epoxidation than AFB_1.[29,30] AFB_1 is primarily metabolized by CYP3A5 to the exo-epoxide and a small amount of AFQ_1. It has been documented that AFB_1 binding to DNA and DNA adduction by AFB_1 exo-8,9-epoxide results in functional alterations to DNA conformation.[12,31-33] The epoxide is extremely erratic and forms aflatoxin-N7-guanine when it attaches to DNA guanine bases with great affinity. It has been demonstrated that aflatoxin-N7-guanine can cause transversion changes in DNA from guanine (purine) to thymine (pyrimidine), which can impact the p53 suppressor gene, which has an impactful role in the cell cycle. When DNA mutations occur, the p53 gene plays a significant role in stopping the course of the cell cycle and inducing apoptosis.[34-37] An illustration of the toxicity mechanism of aflatoxin is shown in Figure 4.

Carcinogenic Effects

The aflatoxins are now known to be strong hepatocarcinogens because they were the first mycotoxins to be thoroughly investigated for their carcinogenic potential. Much study has been conducted on the acute toxicity and carcinogenicity of aflatoxin to rats since the discovery of the "Turkey X" sickness in agricultural animals.[38,39] In studies it shows that various mice species, some fishes, rat species, marmosets, ducks, tree shrews, and monkeys are all carcinogenic to aflatoxins. Among mycotoxins, aflatoxin B_1 is extremely hazardous and is mainly responsible for liver cancers such as cholangia and hepatocellular carcinoma. In a variety of animals, AFB_1 exhibits the highest carcinogenic activity orally. Also, Liver cancer, Kidney cancer and Colon cancer are primarily caused by aflatoxins. It is found that after seven days, the oral LD_{50}

for the four aflatoxins in ducklings that are one day old are AFB_1 0.36 mg/kg body weight, AFB_2 1.7 mg/kg body weight, AFG_1 0.78 mg/kg body weight, and AFG_2 3.5 mg/kg body weight. Aflatoxin B_1 has an LD_{50} of 7.2 mg/kg body weight orally and 62 mg/kg body weight intraperitoneally in male rats, and 7.9 mg/kg body weight orally and 13.2 mg/kg body weight intraperitoneally in female rats.[40-42] After being administered AFB_1 at a dietary level of 30 µg/kg body weight for 14 months, it was revealed that 8 out of 11 ducks developed liver tumors.[43]

Hepatotoxic Effects

Aflatoxin-induced liver damage is recognized to cause both distinct lesions and non-specific ones, including fatty livers, moderate to severe necrosis, and bleeding. Among the mycotoxin class aflatoxin B_1 is the most well-researched and documented.[44,45] Toxic compounds can be absorbed by the liver's hepatic tissues, eliminating them from the bloodstream. Although an animal's susceptibility to aflatoxin B_1 varies, liver damage is always the main cause of disease and death in these situations. Similar liver lesions to those caused by aflatoxin in ducklings have been found in rhesus monkeys. Aflatoxin feeding at a dose of 0.5 mg/kg body weight per day (60% B_1, 40% G_1) resulted in normal histological alterations in the liver that are usually linked to aflatoxin damage. After receiving a daily dosage of 10-15 mg/kg body weight, every test animal passed away in a span of 28 days.[46,47] In additional research on the effects of aflatoxin experimented on

Table 1: Summary of the major aflatoxins produced by the *Aspergillus* species.

Class	Type of Aflatoxins	*Aspergillus* specie(s)
Difuranocoumarins	AflatoxinB$_1$ (AFB_1)	*A. flavus, A. arachidicola, A. bombycis, A. minisclerotigenes, A. nomius, A. ochraceoroseus, A. parasiticus, A. pseudotamarii, A. rambellii, Emericella venezuelensis*
	Aflatoxin B_2 (AFB_2)	*A. arachidicola, A. flavus, A. minisclerotigenes, A. nomius, A. parasiticus*
	Aflatoxin B_{2A} (AFB_{2A})	*A. flavus*
	Aflatoxin M_1 (AFM_1)	*A. flavus, A. parasiticus*; metabolite of aflatoxin B_1 in humans and animals and comes from a mother's milk
	Aflatoxin M_2 (AFM_2)	Metabolite of aflatoxin B_2 in milk of cattle fed on contaminated foods
	Aflatoxin M_{2A} (AFM_{2A})	Metabolite of AFM_2
	Aflatoxicol (AFL)	*A. flavus*, metabolite of AFB_1
	Aflatoxicol M	Metabolite of AFM_1
Difurocoumarolactone	Aflatoxin G_1 (AFG_1)	*A. arachidicola, A. flavus, A. minisclerotigenes, A. nomius, A. Parasiticus*
	Aflatoxin G_2 (AFG_2)	*A. arachidicola, A. flavus, A. minisclerotigenes, A. nomius, A. parasiticus*
	Aflatoxin G_{2A} (AFG_{2A})	Metabolite of AFG_2
	Aflatoxin GM_1 ($AFGM_1$)	*A. flavus*
	Aflatoxin GM_2 ($AFGM_2$)	Metabolite of AFG_2
	$AFGM_{2A}$	Metabolite of $AFGM_2$
	Aflatoxin B_3 (AFB_3)	*Aspergillus* species not defined
	Parasiticol (P)	*A. flavus*
	Aflatrem	*A. flavus, A. minisclerotigenes*
	Aspertoxin	*A. flavus*
	Aflatoxin Q_1 (AFQ_1)	Major metabolite of AFB_1 *in vitro* liver preparations of other higher vertebrates

rhesus monkeys, observations were conducted on animals given a daily dose of 1 mg/kg body weight. It was observed that the liver was enlarged and that its fat content was 34% of its net weight.[48] In a study conducted among Kenyan school children, aflatoxin exposure was found to be significantly associated with chronic Hepatomegaly (HM). In 2002, children with HM had higher geometric mean aflatoxin-albumin (AF-alb) levels (176.6 pg/mg) compared to normal children (79.9 pg/mg).[49]

Mutagenic Effects

Among all the aflatoxins, aflatoxin B_1 is found to be most potent mutagen, and significantly there is a clear correlation between the carcinogenic and mutagenic properties of aflatoxins. In plant and animal cells, Chromosomal abnormality and DNA breakage is formed due to microsomal activation.[50] Aflatoxin B_1 has been shown to suppress mitotic cell division in the human embryonic lungs at concentrations of 0.01 mg. Aflatoxin B_1 at 0.05-0.1 mg/mL reduced DNA production in human embryonic cells.[51]

Teratogenic Effects

Aflatoxin's capacity to bind DNA and so prevent protein synthesis makes it a powerful teratogen. Beyond their toxicity or carcinogenicity in people, fungi-produced poisons that may be found on human and domestic animal meals have been shown to have embryocidal and teratogenic effects.[18] It has been observed that injecting female rats with aflatoxin B_1 intraperitoneally caused fetal mortality and bleeding at the uteroplacental junction. There was prenatal growth retardation observed with repeated lower doses. Additionally, they discovered that non-pregnant female rats were more vulnerable to aflatoxin than the pregnant females.[52,53] When aflatoxin B_1 was injected intraperitoneally into pregnant hamsters on the 8th day of their pregnancy, a significant percentage of deformed, dead, or reabsorbed foetuses resulted.

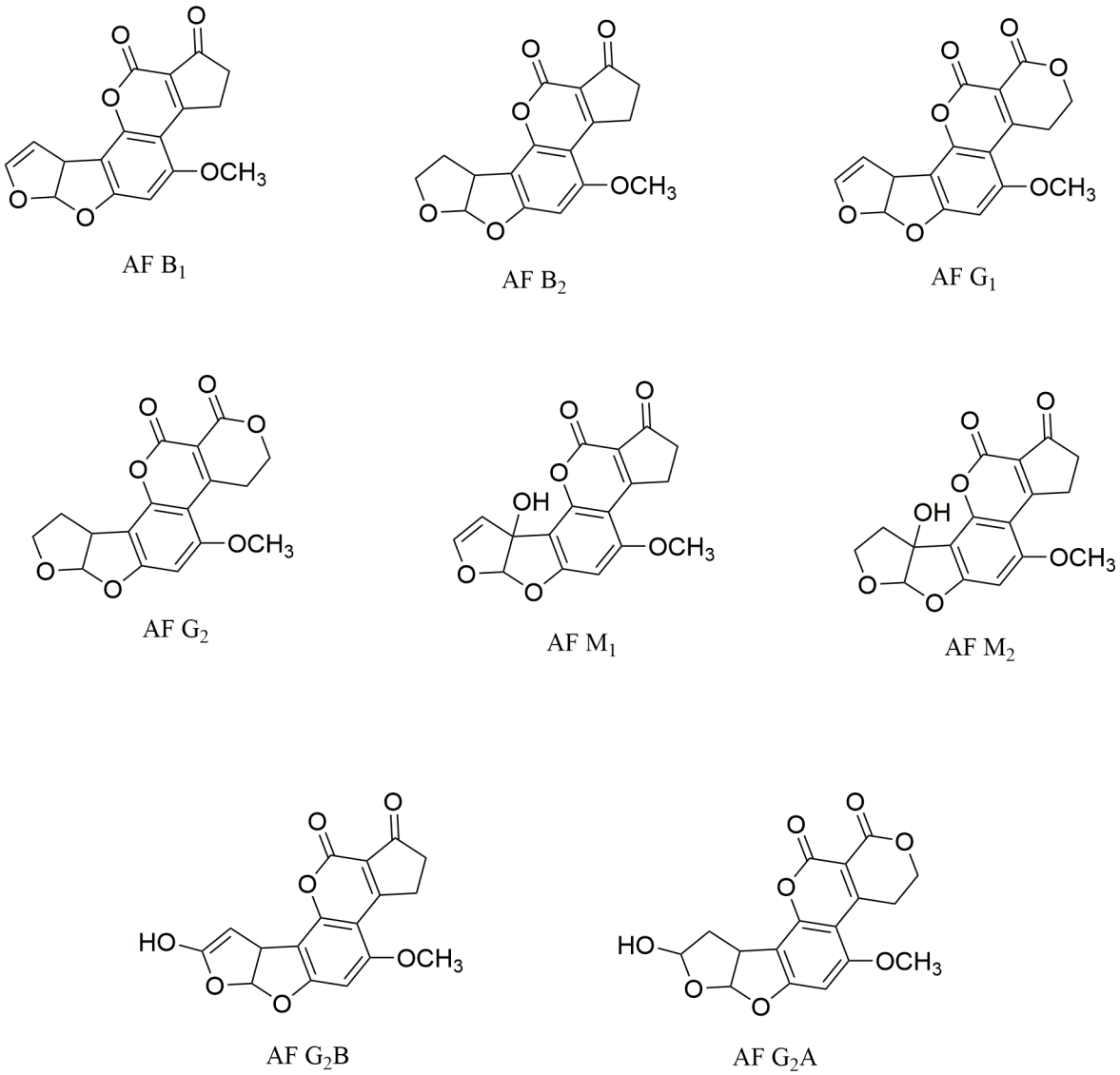

Figure 1: Chemical Structure of different Aflatoxins.

The dosage was 4 μg/kg body weight/day.[54] Another study investigated the teratogenic effects of maternal aflatoxin exposure on infant growth in the Gambia. Maternal aflatoxin-albumin (AF-alb) levels were strong predictors of growth, with higher exposure linked to a 0.8 kg decrease in weight and a 2 cm reduction in height by the age of one year. The findings highlight the critical need for interventions to mitigate aflatoxin exposure during pregnancy.[55]

Detection of Aflatoxin

Due to their chemical stability, most mycotoxins can withstand processing, storage, and even cooking at temperatures as high as

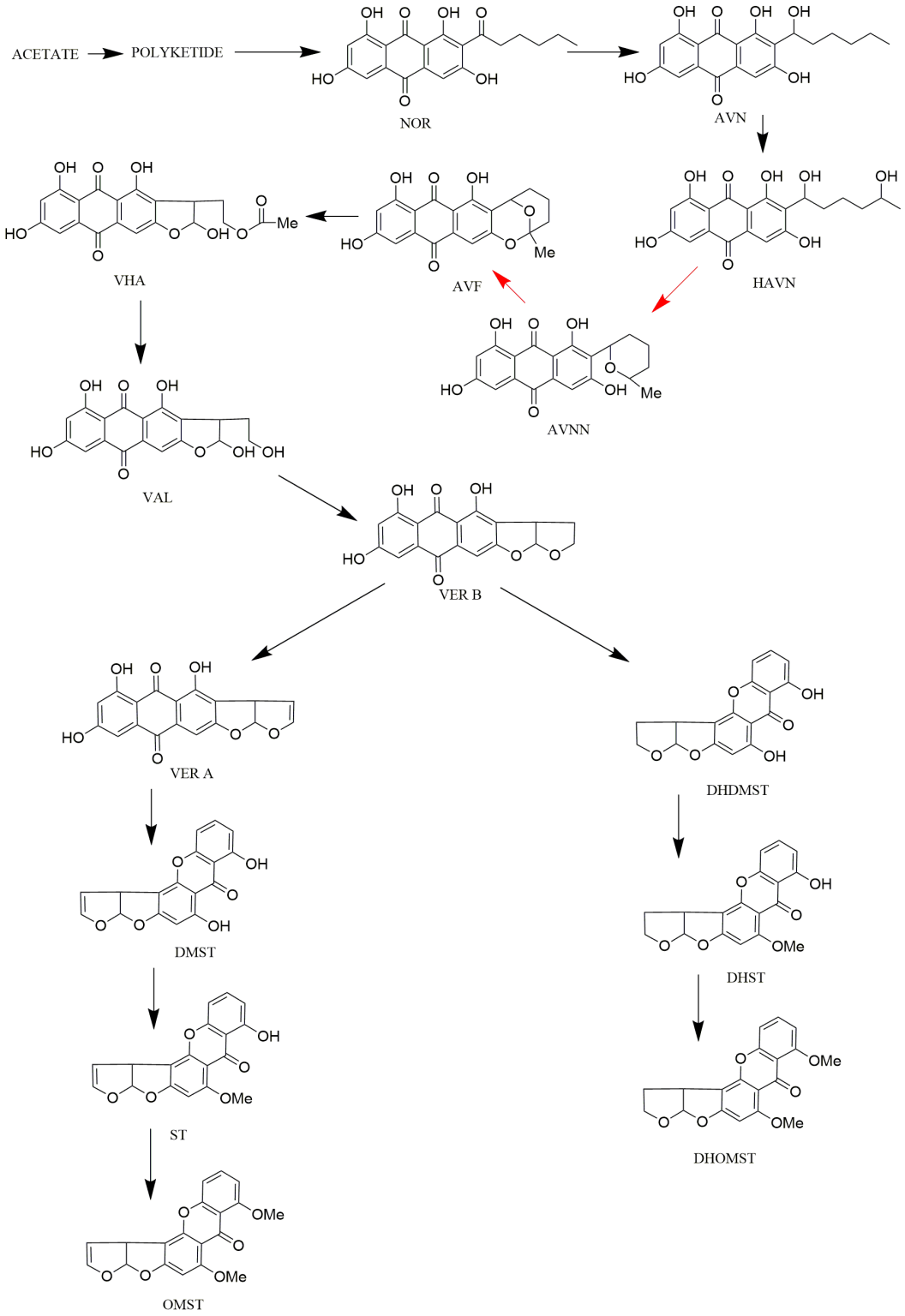

Figure 2: Biosynthesis of Aflatoxin.

those used to make breakfast cereal or bread. Because of this, it's critical to prevent the circumstances that give rise to mycotoxin production, which is not always feasible or accomplished in real-world situations. In stored goods, fungi typically grow in small, irregular patches rather than throughout the entire material.[56] Consequently, it's critical to establish a methodology to guarantee that it accurately represents the entire consignment if a sample is removed for examination. Generally, reports on the mycotoxin content of grab samples have shown them to yield very low estimations. The method used to obtain the initial sample could account for around 90% of the errors in mycotoxin testing. It is considered challenging to obtain a sample from feed or grain that will yield a relevant result in mycotoxin studies, as mycotoxins are not always evenly distributed in grains or mixed feeds. Since most mycotoxins are harmful at very low concentrations, so highly sensitive with validated procedures are needed to detect them.[57] Identifying aflatoxin contamination with precision is essential for maintaining food safety and public health. Currently, several analytical techniques are used in practice to determine aflatoxins. The review paper aims to give a practical guide that outlines global regulations about aflatoxin levels and analytical techniques for determining aflatoxin levels in various food and feed matrices. This guide will assist in selecting the most suitable method to effectively control aflatoxin contamination quickly and sensitively.

Analytical Techniques

As mycotoxins produced by the fungus are toxic in nature with low Molecular Weight (MW<1000) there isn't a single accepted method for their analysis and/or detection because of their complex chemical makeup.[56,58] The majority of techniques rely on proper extraction and cleanup. Polar protic solvents can

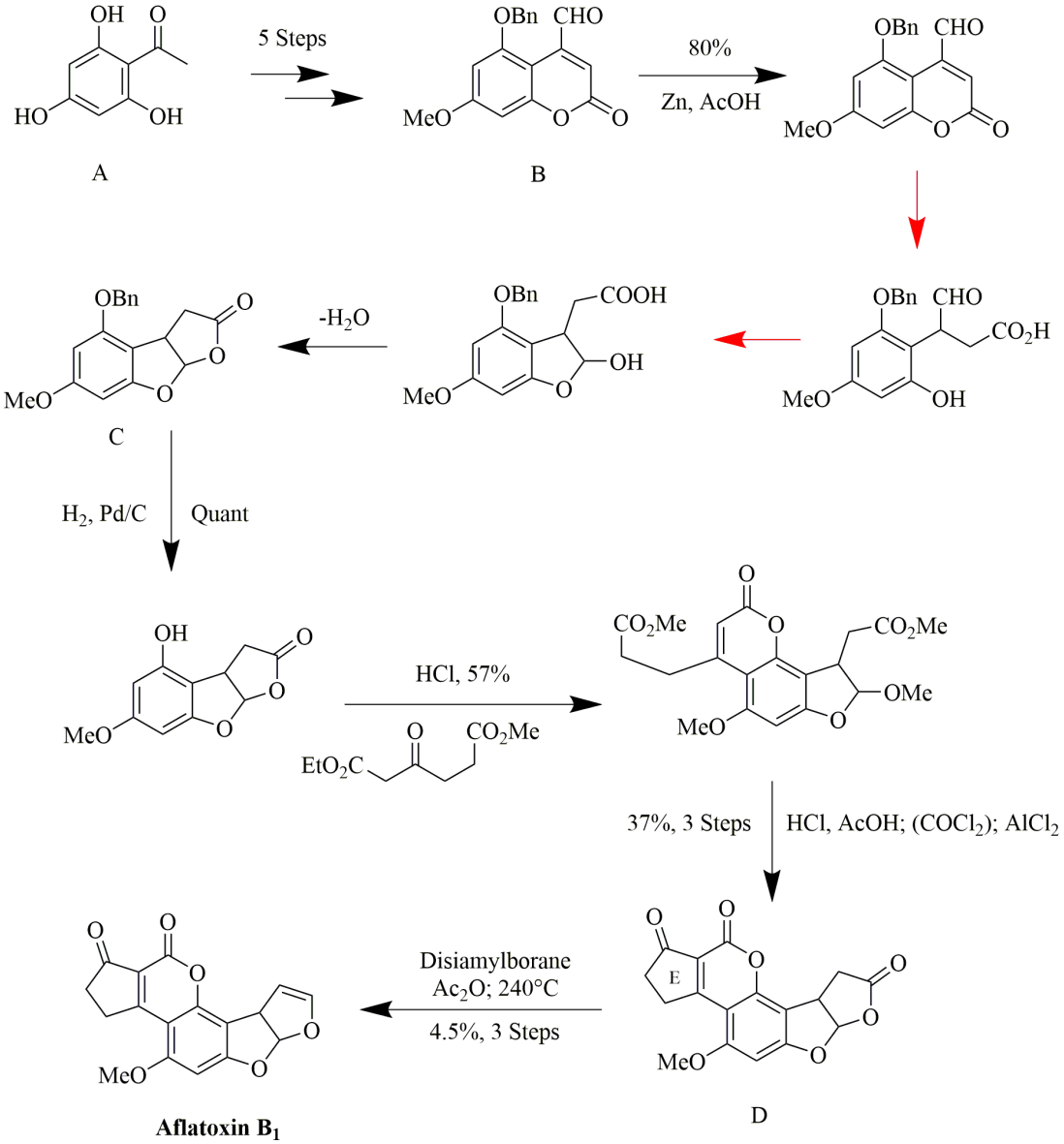

Figure 3: Synthesis of Aflatoxin B_1.

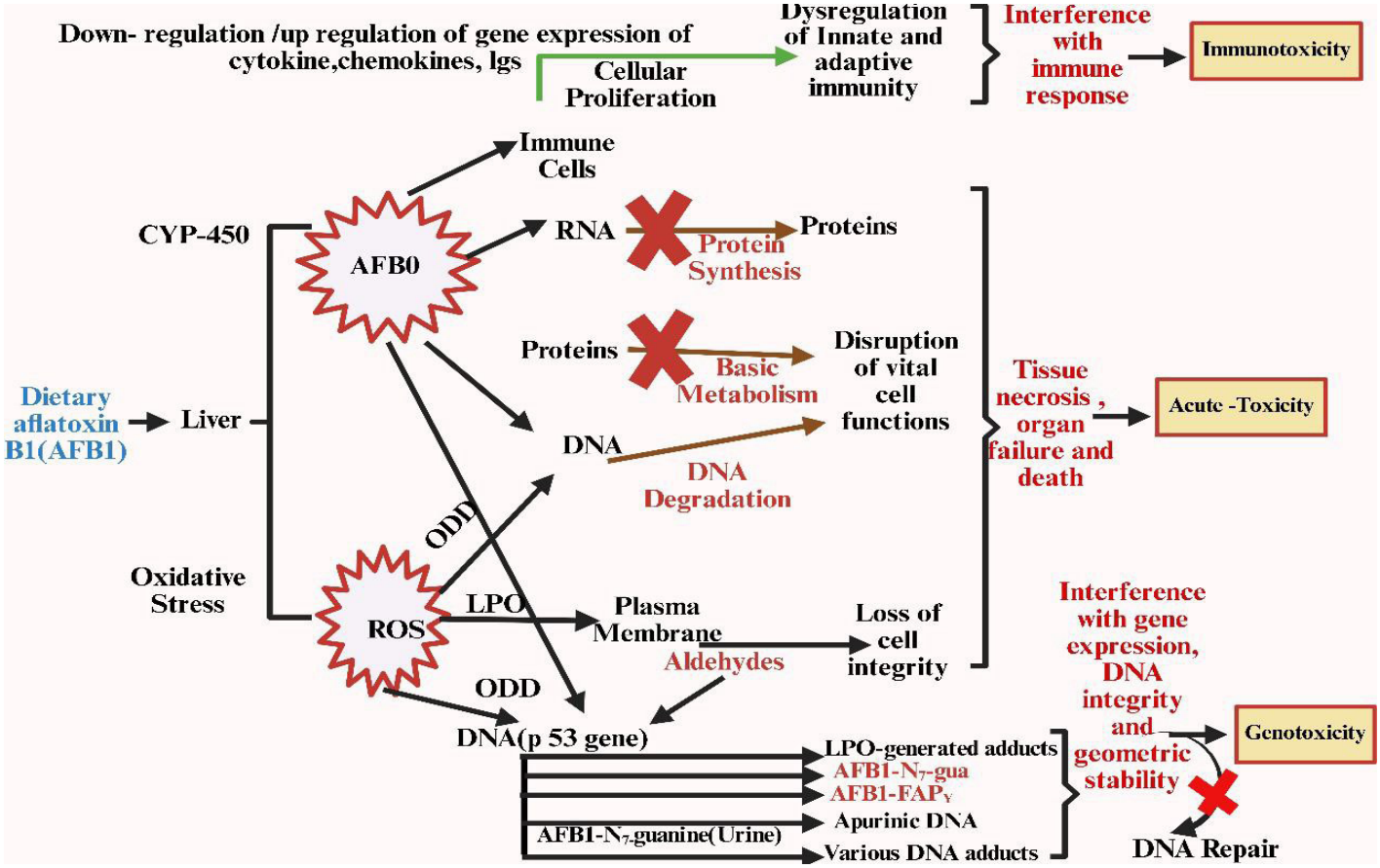

Figure 4: Mechanism of Aflatoxins.

dissolve aflatoxins, including methanol, acetone, chloroform, and acetonitrile. Therefore, these organic solvents, methanol, acetonitrile, or acetone, combined in various ratios with small amounts of water, extract aflatoxins. After aflatoxins are extracted, a cleanup is typically performed.[59-63] Making a sample is one of the most crucial processes in identifying mycotoxins. It could take up to two-thirds of the total analysis time and have a major impact on the precision and accuracy of the findings. Liquid and Solid-Phase extraction procedures are the most often utilized clean-up techniques employed in aflatoxin analysis.[23] The isolation of Aflatoxin can be carried out by using different techniques like Thin Layer Chromatography (TLC), High-Performance Liquid Chromatography (HPLC), Gas Chromatography (GC), Electrophoresis, and ELISA Technique, etc.

Chromatographic Techniques

Chromatographic techniques are pivotal in identifying and quantifying aflatoxins across various sample types. Due to their exceptional sensitivity, TLC and HPLC are the most common techniques applied for routine aflatoxin analysis. However, they often require extensive sample preparation, skilled operators, and expensive equipment, which can limit their accessibility, particularly in developing regions.[64] Gas Chromatography (GC) was first utilized for detecting aflatoxin B_1 in 1981 and later expanded to identify multiple aflatoxin types, including B_1, B_2, G_1, and G_2. However, GC's popularity has declined due to the availability of less labor-intensive and more cost-effective alternatives.[65] High-Performance Liquid Chromatography (HPLC) has emerged as a widely adopted technique for analyzing organic compounds, including aflatoxins, in various food matrices. Modern HPLC systems coupled with detectors such as Fluorescence (FLD), Ultraviolet (UV), Photodiode Array (PDA), and Mass Spectrometry (MS) offer enhanced sensitivity and specificity.[66] HPLC-FLD is particularly favored due to its superior detection capabilities, leveraging the natural fluorescence of aflatoxins, although post-column derivatization using reagents like trifluoroacetic acid and potassium bromide is often required to enhance fluorescence for certain aflatoxins.[67] Recent applications of HPLC include the analysis of aflatoxin M_1 in dairy products, achieving Limits of Detection (LOD) as low as 0.003 µg/kg in milk and recovery rates of up to 83%, and the detection of aflatoxins in animal feed with LODs of 2 µg/kg for aflatoxins B_1 and G_1 whereas 0.64 µg/kg for B_2 and G_2, with recovery rates ranging from 73.6% to 88%.[68,69] LC-MS/MS has emerged as a preferred method for aflatoxin analysis for its high sensitivity, selectivity and ability to detect multiple mycotoxins in diverse food matrices without requiring derivatization for fluorescent enhancement.[70,71] A comprehensive international study involving 23 organizations validated the technique's

reliability for simultaneous quantification of 12 mycotoxins, including aflatoxins B_1, B_2, G_1, G_2 and M_1 in various foods such as spices, various nuts, milk powder, cereals, dried fruits and baby foods. The study reported excellent repeatability, reproducibility, and trueness, highlighting its suitability for regulated mycotoxin analysis, including products for infants.[72] Additionally, Al-Taher *et al.*, demonstrated recovery rates between 70% and 130% across grains like rice, barley, oats, and mixed cereals, with relative standard deviations under 20%, ensuring robust quantification.[73] Furthermore, Deng *et al.*, reported Detection Limits (LOD) of 0.1-2.0 µg/kg and Quantification Limits (LOQ) of 0.3-5.0 µg/kg for aflatoxins and other mycotoxins in dried seafood, confirming the technique's versatility across food products.[74]

Immunochemical methods

This type techniques have become an efficient alternative to usual chromatographic methods for aflatoxin analysis due to their simplicity, rapidity, high sensitivity, and minimal pre-analytical requirements.[75,76] Enzyme-Linked Immunosorbent Assays (ELISA) and Lateral Flow Immunoassays (LFIA) are widely used. ELISA, developed in the 1960s, is praised for its high sensitivity, detecting aflatoxins with recoveries of 85-108% and detection limits as low as 27.5 ng/L for aflatoxin M_1 in milk[77] and 0.128 µg/L for aflatoxin B_1 in lotus seeds.[78] Despite its advantages, ELISA may suffer from cross-reactivity and false positives.[79] LFIA, known for its portability and simplicity, employs antibody-antigen interactions on paper-based test strips and supports multiplexed analysis of mycotoxins. Recent developments include gold nanoparticle-based LFIAs capable of detecting aflatoxins at minute levels as 0.5 µg/kg in soybean-based foods[80] and simultaneous analysis of aflatoxin B_1, ochratoxin A, and zearalenone in various samples with detection limits of 0.10-0.13 µg/kg.[81] While versatile and cost-effective, immunoassays require optimization of antibody stability and labeling materials to ensure accuracy.[82] Other immunochemical methods, like radioimmunoassays and fluorescence polarization immunoassays, have limited applications due to complexity or safety concerns.[83,84]

Spectroscopic Method

Spectroscopic techniques such as Near-Infrared (NIR), Raman, fluorescence, and Hyperspectral Imaging (HSI) are gaining prominence as non-destructive methods for aflatoxin detection in food products. These techniques require minimal sample preparation and enable rapid identification of contaminated items within large batches, making them valuable for quality control in food processing.[85,86] For instance, Fourier-transform NIR reflectance spectroscopy achieved 100% accuracy in distinguishing aflatoxin-contaminated figs,[87] while HSI demonstrated classification accuracies of over 95% for maize kernels contaminated with low aflatoxin levels (<20 ppb).[88] Imaging methods like color imaging have proven effective for peanut screening, achieving up to 100% accuracy[89] and laser-based in-line sorting technology has detected aflatoxin B_1 with 99% specificity in peanut processing at speeds of 3.2 tons/hr.[90] Additionally, laser-induced fluorescence spectroscopy classified pistachios with low aflatoxin B_1 levels with over 91% accuracy.[91] Despite these advantages, these techniques are primarily suitable for screening rather than quantitative analysis, as their performance depends heavily on food matrices and lacks multi-toxin detection capability. Nonetheless, ongoing research focuses on improving rapid on-site detection methods to enhance their applicability, particularly in large-scale production and resource-limited settings.[92]

Cultural Method

Aspergillus species, such as *Aspergillus flavus* and *Aspergillus parasiticus*, are commonly grown on selective media in aflatoxin detection techniques to screen for aflatoxin generation. Although less frequently employed for quantitative analysis, this technique is useful for preliminary screening and qualitative evaluation of food and feed samples that Aflatoxin contaminates. The sample is initially inoculated into appropriate growth media that facilitate the growth of aflatoxin-producing fungus in culture methods for aflatoxin detection. Potato Dextrose Agar (PDA), which is frequently used, is added with particular nutrients and inhibitors to encourage the growth of fungi and the formation of aflatoxin while inhibiting the growth of other microorganisms.[93] Following inoculation, the plates are incubated in an environment with regulated humidity and temperature that promotes fungal growth. Aflatoxin-producing fungi can have distinct spore structures when examined under a microscope and unique colony morphologies throughout time, such as olive or yellow-green colors. Some analytical and biological techniques can be used to verify aflatoxin formation. The two primary cultural methods used to identify aflatoxins generated by these isolates are the fluorescence of these fungi create and the apparent color of the pigments the colonies make.[94]

Blue Fluorescence Method

Aflatoxin synthesis by Aspergillus species cultivated on appropriate media has been identified by the development of qualitative culture methods utilizing the blue fluorescence of aflatoxin. Solid media like coconut agar and potato dextrose agar are used in several techniques. Using the proper solvents, materials suspected of having aflatoxins are usually extracted to separate the aflatoxins from the matrix in this diagnostic process. After that, the extracted solution is exposed to UV light, which excites the aflatoxin molecules and causes them to exhibit blue fluorescence. The UV light typically has a wavelength of about 365 nanometers. Fluorescence detection instruments, such as a fluorometer or a UV-vis spectrophotometer fitted with a fluorescence detector can be used to visually see or measure the intensity of the blue fluorescence released by the aflatoxins.[95]

Yellow Pigmentation Method

Identifying and measuring aflatoxins based on their distinctive yellow pigmentation is known as "yellow pigment aflatoxins detection." When aflatoxins are found in food or feed samples, they can occasionally cause noticeable yellow pigments to appear, which can be a sign of their existence. Samples contaminated with aflatoxin may show yellow discoloration, which skilled workers can see firsthand. This visual examination can be used to determine possible aflatoxin contamination in samples as a first screening method.[95]

Ammonium Hydroxide Vapor-Induced Color Change

With this technique, an individual colony is cultivated in the middle of a Petri dish that has medium like potato dextrose agar in it. 1-2 drops of a strong ammonium hydroxide solution were added to the interior of the lid after the dish was inverted. Colonies produced by aflatoxin rapidly develop plum-red undersides when the bottom of the Petri dish is flipped over the ammonium hydroxide-containing lid. The undersides of colonies that are not generating aflatoxins essentially do not change in color.[95]

Strategy of Prevention

Aflatoxins are extremely hazardous secondary metabolites that can contaminate various crops, including maize, peanuts, tree nuts, and spices. Certain molds, mainly *Aspergillus* species, produce them. Programs to control mycotoxin levels will benefit the region's health and economy. Many developing nations are increasingly aware that lowering mycotoxin levels in food will improve local populations' long-term health and international trade. Aflatoxin contamination prevention measures are essential for maintaining food safety, public health, and economic stability, especially in areas where aflatoxin exposure is common. Mycotoxin researchers have developed numerous ways to combat aflatoxin generation in food, which are mentioned below.[94,96]

Education and Extension

Since a greater portion of the public, especially the educated, are unaware of the threat that mycotoxins bring to the economy and public health, the issue is confined to the scientific community. So, every country's National Agency for Food and Drug Administration and Control should take some initiative to conduct programs to educate the peoples.

Seminars and Workshops

Workshops and seminars offer a forum for sharing findings and learning about the activities occurring in other labs. Scientists could evaluate each other's work from the past and present and narrow down the study topics for the future. There should be more of these gets-together in both urban and rural locations.

Adoption of agronomic practices

Agronomic practices like proper irrigation management, crop rotation and pest management are crucial in preventing aflatoxin contamination by addressing factors contributing to crops fungal growth and toxin production.

Early harvesting

It has been suggested that early harvesting lowers the chance of aflatoxin infection. Although most farmers are conscious about the importance of harvesting early, labor shortages, erratic weather patterns, financial pressures, and the fear of theft, rodents, and other animals frequently force farmers to harvest earlier or on wrong time, which can reduce the risk of aflatoxin contamination.

Rapid drying

Since all scenarios leading to aflatoxin contamination involve failure to maintain preserved items at a safe level of moisture, quick drying of cultivated products to low moisture is frequently stressed among recommendations for managing the mycotoxin problem. Dry grains do not require as much water activity to grow, so they retain longer and are protected from mold and insects.

Sanitation

Basic hygienic precautions against storage deterioration include removing the remnants of past harvests and eliminating infected agricultural leftovers. Aflatoxin levels were seen to decrease when stocks were cleaned before loading in fresh harvests.

Smoking

Smoking is also an significant method of protecting maize against invasion by fungi. Smoking is a preservation technique that involves exposing food products to smoke produced from burning wood or other organic materials. The heat and smoke generated during this process can inhibit fungal growth and reduce aflatoxin contamination in the smoked products.

Synthetic chemicals

Use of chemicals such as pesticides and fungicides are often used to lower the contamination of aflatoxin with foods. The farmers' poor educational background often leads to pesticide misuse.

Use of plant products

It has been demonstrated that certain traditionally beneficial plants have fungitoxic qualities. *Aethiopia aethiopica, Xylopia gratissimum, Cinnamum verum, Monodera myristica, Syzigium aromaticum*, and *Piper nigrum* are effective in preventing the development of sorbic acid, which is a precursor in the route leading to the creation of aflatoxin. It was shown that the essential

oils of 16 different aromatic plants considerably decreased the productin on of aflatoxin and inhibited the growth of a toxigenic *A. flavus* by PDA diffusion assay.[97]

CONCLUSION

Mycotoxin contamination of crops remains a serious financial issue for the global agriculture and food industries. The mycotoxins that are created by fungus have the potential to reduce agricultural yield and quality, as well as induce a range of symptoms in animals that come into contact with them. Aflatoxin poisoning is a widespread issue that affects both people and animals globally, particularly in underdeveloped countries with low incomes. The metabolites, aflatoxin-8,9-epoxide and the Reactive Oxygen Species (ROS) they produce, can harm human and animal health. These consequences include hepatotoxicity, teratogenicity, mutagenicity, and carcinogenicity, which can lead to the development of cancer.[12] Additionally, aflatoxins cause the reduction of humoral and cell-mediated immunity, which leaves people vulnerable to infectious illnesses. Furthermore, they result in the malabsorption of several nutrients, which impairs immune function, causes malnutrition, stunts growth, and nutritional deficiencies. To effectively address the adverse impacts of aflatoxins on general public health and the economy, more study is needed to clarify several crucial features of these pervasive, extremely toxic pollutants.[68] This will improve our understanding of their toxicity-producing patterns and incidence in foods. To understand the regulatory mechanisms of aflatoxin production, scientists are investigating its mechanisms. Research has shown that the roles played by the enzymes in each stage of aflatoxin biosynthesis can aid in understanding the regulatory mechanisms governing the development of aflatoxin. Finding natural products with inhibitory properties against fungal growth and aflatoxin creation will be easier with a deeper comprehension of the mechanisms governing gene control on aflatoxin biosynthesis. In due course, we will be able to create fresh and efficient methods to eliminate aflatoxin contamination, ensuring a safer, healthier, and longer-lasting supply of food and feed. To complete the aflatoxin biosynthesis puzzle, enzymatic steps that are missing from the process must be identified. Even if preventing aflatoxin contamination is the main objective, contamination is nonetheless inevitable in some environments due to specific temperature and humidity levels. Consequently, there has been a greater drive to find efficient decontamination techniques that may be applied commercially. A number of treatments have previously found commercial use, including air separation and screening, dilution of contaminated grain, roasting, specific chemical treatments, and the use of binding agents. The global food and agriculture industries will continue to face mycotoxin-related issues until more dependable, affordable, and commercially viable techniques become widely accessible. Furthermore, prevention is possible if the public health issue brought on by aflatoxins is fully understood. Aflatoxin exposure reduction initiatives necessitate the provision of adequate funding and cooperation between the public health and agricultural sectors, as well as local, regional, national, and international governments.

ACKNOWLEDGEMENT

We thank GITAM (Deemed to be a University), Visakhapatnam, Andhra Pradesh, India, for providing the necessary facilities for this research.

FUNDING

This study was supported by GITAM School of Pharmacy, GITAM (Deemed to be University), Visakhapatnam, Andhra Pradesh, India.

CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

ABBREVIATIONS

AF: Aflatoxin; **NOR:** Norsolorinic Acid; **VER B :** Versicolorin B; **AVN:** Averantin; **VER A:** Versicolorin A; **HAVN:** 5-hydroxy Averantin; **DMST:** Demethylsterigmatocystin; **AVNN:** Averufanin; **DHDMST:** Dihydrodemethylsterigmatocystin; **ST:** Sterigmatocystin; **AVF:** Averufin; **VHA:** Versiconal Hemiacetal acetate; **DHST:** Dihydrosterigmatocystin; **VAL:** Versiconal; **OMST:** O-methylsterigmatocystin; **DHOMS:** Dihydro-O-methylsterigmatocystin; **AFBO:** Aflatoxin B1-exo-8,9-epoxide; **NA:** Nucleic Acids; **ROS:** Reactive Oxygen Species; **LPO:** Lipid Peroxidation; **ODD:** Oxidative DNA Damage; **Acr:** Acrolein; **Cro:** Crotonaldehyde; **Acet:** Acetaldehyde; **HNE:** 4- Hydroxy-2-Nonenal; **uFA:** Unsaturated Fatty Acids; **IL1β:** Interleukin 1β, **IL6:** Interleukin 6; **TNFα:** Tumour Necrotizing Factor α; **P-dG:** Cyclic Propano-Deoxyguanosine; **Igs:** Immunoglobulins; **PDA:** Potato Dextrose Agar.

CONSENT FOR PUBLICATION

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SUMMARY

Food crops, including cereals, nuts, and dairy products, can become contaminated by aflatoxins, which are dangerous mycotoxins mostly generated by *Aspergillus flavus* and *Aspergillus parasiticus*. Aflatoxin B1 (AFB1) is the most powerful, with mutagenic, teratogenic, hepatotoxic, and carcinogenic effects. Through the gastrointestinal and respiratory systems, aflatoxins are absorbed. In the liver, they are broken down by cytochrome P450 enzymes, creating DNA-binding adducts that fuel cancer, especially

hepatocellular carcinoma. A polyketide route containing many enzyme conversions drives their production. Aflatoxin persists in food chains and poses serious health concerns to humans and animals because of its stability under heat and storage settings. Detection techniques include spectroscopic (fluorescent, NIR, hyperspectral imaging), immunochemical (ELISA, lateral flow immunoassays), chromatographic (HPLC, LC-MS/MS), and cultural approaches. Good agronomic methods, early harvesting, quick drying, and appropriate storage conditions are also part of prevention efforts. Controlling aflatoxin infection requires chemical decontamination, natural antifungal treatments, and food safety laws. Policy-driven treatments and awareness efforts are essential, particularly in poor nations where aflatoxin exposure is common. Future studies will concentrate on developing new decontamination strategies, comprehending the genetic control of aflatoxin manufacturing, and improving detection technologies. To assure food safety and reduce health risks worldwide, addressing aflatoxin contamination necessitates a multidisciplinary approach that combines innovation, regulatory actions, and public health campaigns.

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Cite this article: Banerjee A, Ghosh A, Nandi A, Saha D, Sahoo SK, Kundu A, *et al*. Aflatoxin: A Prevalent Toxin in the Demesne of Existence. Pharmacog Res. 2025;17(3):740-54.