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Research Report

Safe and effective degradation of aflatoxins by food-grade culture broth of *Aspergillus oryzae*

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Abstract

Aflatoxins (AFs) are carcinogenic fungal toxins contaminating up to 25% of the global food supply. Over half of the world's population is exposed to unmonitored levels of AFs, mostly aflatoxin B1 (AFB1). Despite numerous efforts over the past 60 years, there are no solutions to remove AFs safely from food. Here, we present a safe and effective AF-degrading product called "D-Tox", a filtered culture broth of *Aspergillus oryzae* grown in a food-grade liquid medium. When 5 ppm of AFB1 is added to D-Tox, ~90% is degraded at 48 and 24 hr at room temperature and 50°C, respectively. Moreover, when varying amounts (0.1 ppm ~ 100 ppm) of AFB1 are added to D-Tox at 100°C, over 95% of AFB1 is degraded in 1 hr, suggesting a nonenzymatic process. Examining degradation of 100 ppm AFB1 reveals that aflatoxin D1 (AFD1) is the major transient degradant of AFB1, indicating that degradation occurs irreversibly by lactone ring hydrolysis followed by decarboxylation. D-Tox further degrades AFD1 to unknown fragmented products. Importantly, the practical application of D-Tox is also demonstrated, as more than 70% of AFB1 is degraded when wheat, corn, and peanuts naturally contaminated with high levels of AFB1 (0.3 ~ 4.5 ppm) are boiled in D-Tox for 1 hr. Additionally, D-Tox can degrade other lactone-ring containing mycotoxins, including patulin and ochratoxin. D-Tox exhibits no cytotoxicity under the conditions tested in MCF-7 breast cancer cell lines. In summary, D-Tox is a safe and effective AF-detoxifying product that can enhance global food safety.

Keywords: aflatoxins, degradation, *Aspergillus oryzae*, broad applications, global food safety

Significance statement

Since the discovery of aflatoxins (AFs) in the early 1960s, numerous efforts have been devoted to controlling or removing AFs. While some of the technologies are used in agricultural fields, none can be directly used in human foods, which can become contaminated with AFs at numerous pre and post-harvest steps. An ideal way to reduce human exposure to highly carcinogenic AFs is by directly degrading them in food before consumption. The food-grade culture filtrate D-Tox can degrade most AFs irreversibly within 1 hr of boiling, providing safe, and effective removal of AFs in food. We envision that D-Tox can be used to eliminate AFs in various foods, including maize, peanuts, wheat, oats, and milk to enhance global food safety and security.

Introduction

Aflatoxins (AFs) are one of the most toxic contaminants that can be present in foods and feeds. Over 4.5 billion people are repeatedly exposed to unmonitored levels of AFs through their daily diet (1). AFs are a group of mycotoxins produced, mainly by the ubiquitous soil fungus *Aspergillus flavus*, that threaten global food safety by contaminating up to 25% of the world's food supply (2). Among AFs, aflatoxin B1 (AFB1; Fig. 1a) is the most predominant one with extremely high carcinogenicity and toxicity. AFs, mostly AFB1, are projected to cause up to 28% of the 550,000–600,000 new liver cancer cases diagnosed each year globally (3). Furthermore, consuming AFs-contaminated food is associated with stunted growth in children, damage to the immune system, and high mortality rates, especially in developing countries (3, 4). With climate change, AF producing fungi will expand their growth regions and will infect more crops, leading to an increased burden of AF contamination in foods worldwide (5, 6). Even at trace levels, e.g. 20 ppb (parts per billion μg/L; the U.S. FDA action level), AFs can be dangerous, and foods contaminated with higher amounts of total AFs are not fit for human consumption (7, 8). AFs also pose a

Competing Interest: All authors declare the existence of a financial competing interest. All authors are stockholders of SkyAngel Bio Co, which filed the US and Korean patents of the D-Tox technology.
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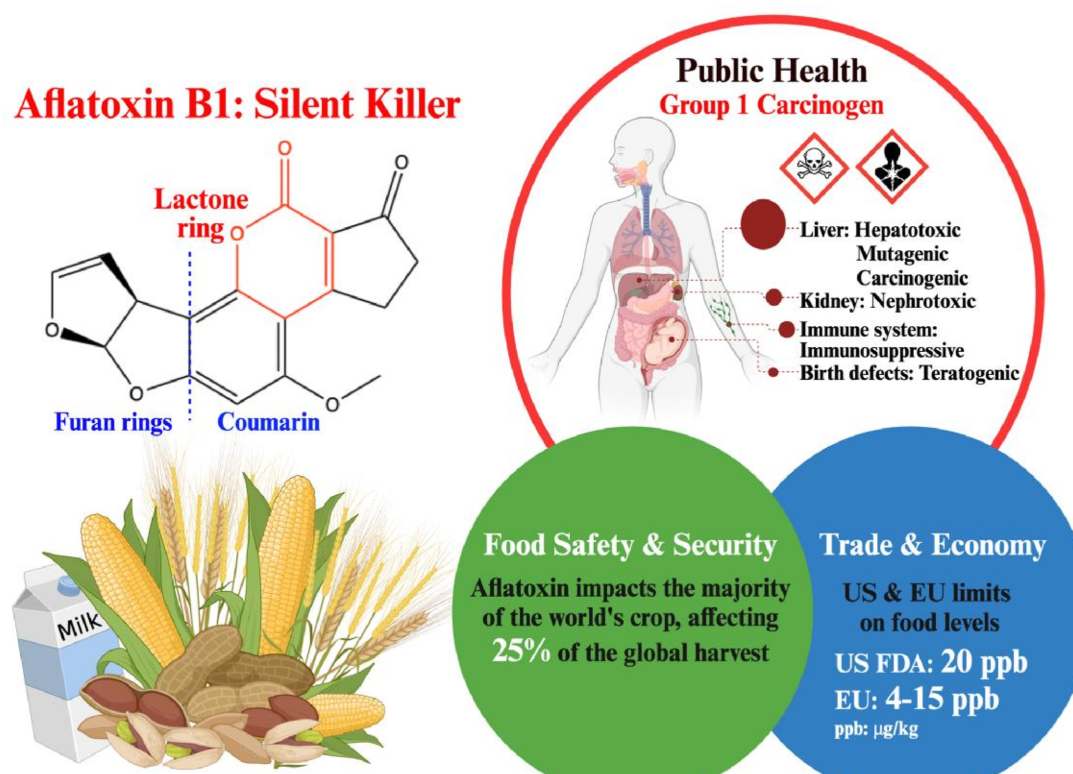

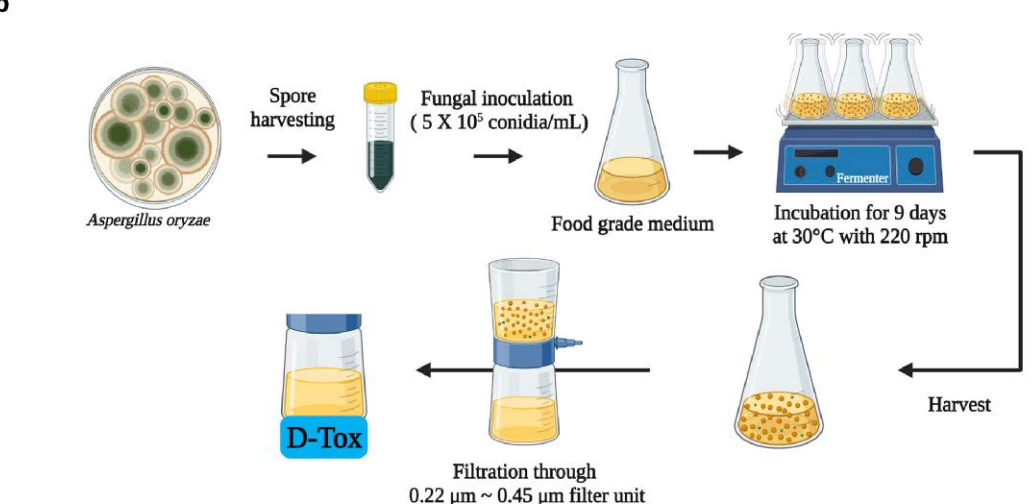

Fig. 1. Schematic presentation of AFB1's global impacts and production of D-Tox. (a) AFB1's impacts on public health, food safety, security, trade, and economy, along with its chemical structure featuring a lactone ring. (b) The production process of D-Tox. It was created with Biorender.com. Structure of AFB1 was drawn using Chemdraw program.

significant economic burden, causing an estimated 25% or more of the world's food crops to be destroyed annually (WHO) (2, 4).

The furan and coumarin rings are essential for the toxicity of AFs (Fig. 1a). Therefore, breaking the double bond in the furan ring and/or the lactone ring in the coumarin structure could potentially diminish the AF toxicity (9). Nevertheless, AFs are remarkably stable, remaining largely intact even following exposure to high-temperature treatments during food processing. Approaches to address AF contaminations aimed at directly degrading AFs, whether before or after harvest, have demonstrated limited success (10). To address this critical global problem, we have developed a novel food-grade AF-degrading product, named "D-Tox", by utilizing the Generally Recognized as Safe (GRAS) fungus *Aspergillus oryzae*, which has long been used to produce various foods and beverages. We have produced D-Tox by culturing this safe fungus in a liquid medium composed of edible components and filtering the resulting culture broth (Fig. 1b). This D-Tox can effectively degrade AFB1 at low, moderate, and high concentrations, leading to the degradation of over 70~95% of AFB1 in vitro and in food. This study presents the production, characterization, and application of D-Tox, which promises to greatly enhance global food safety and security.

Results

D-Tox effectively degrades AFs in vitro

Culturing *A. oryzae* in a medium comprising glucose, nitrate salts, and trace elements (minerals) for 9 days at 30°C, 220 rpm yielded a notably potent D-Tox. When 5,000 ppb AFB1 was spiked into this initial D-Tox and incubated at 50°C, it was able to degrade more than 90% of AFB1 within 24 hr, whereas at 25°C, 89% of AFB1 was degraded at 48 hr (Fig. 2a). Furthermore, at 100°C, D-Tox degraded 76.5 ± 6.3% and 95.3 ± 1.7% of 1,000 ppb AFB1 after 30 and 60 min of boiling, respectively, resulting in 43.6 ppb ± 16.0 ppb after 60 min. For 500 ppb AFB1, degradation rates were 69.9 ± 3.4% and 94.2 ± 2.4% after the same boiling durations, yielding 25.2 ppb ± 8.4 ppb after 60 min. Similarly, with 100 ppb AFB1, D-Tox degraded 68.9 ± 3.3% and 90.6 ± 4.8% after 30 and 60 min, respectively, resulting in 7.1 ppb ± 3.3 ppb after 60 min. Lastly, for 50 ppb AFB1, D-Tox demonstrated degradation rates of 66.2 ± 8.1% and 95.2 ± 5.0% after the same boiling durations, resulting in 2.1 ppb ± 2.1 ppb after 60 min (Fig. 2b). Each condition was tested in triplicate across three independent experiments, resulting in a total of nine replicates ($n = 9$). These results indicate that D-Tox degradation in vitro across multiple flasks is consistent and shows low variation. These results suggest that D-Tox can be effectively used to degrade AFB1 over a broad temperature and AFB1 concentration range, with maximum activity obtained at boiling. To test whether dilutions of D-Tox retained efficacy, it was mixed with distilled water and examined for AFB1 degradation ability. After boiling for 60 min, as low as a 25% solution of D-Tox in water could effectively degrade 500 ppb AFB1 to less than 20 ppb, the US FDA action level (Fig. 2c). This indicates that simple dilutions of D-Tox could be cost-effective and practical. In addition to AFB1, D-Tox also degrades AFB2, AFG1, and AFG2 at varying rates (Fig. 2d). The discrepancy in degradation effectiveness between boiling alone in control and D-Tox treatment for AFG1 and AFG2 can be attributed to the inherent structural differences among AFs. Research by Cho et al. (11) and Elizalde-González et al. (12) have demonstrated that the six-membered lactone ring in AFG1 and AFG2 renders them more susceptible to oxidation and degradation compared to AFB1 and AFB2 in water contained methanol solution. This structural instability likely contributes to the observed higher rate of degradation of G AFs compared with B AFs.

To explore broader applications beyond household settings, the long-term stability and efficacy of D-Tox were evaluated through

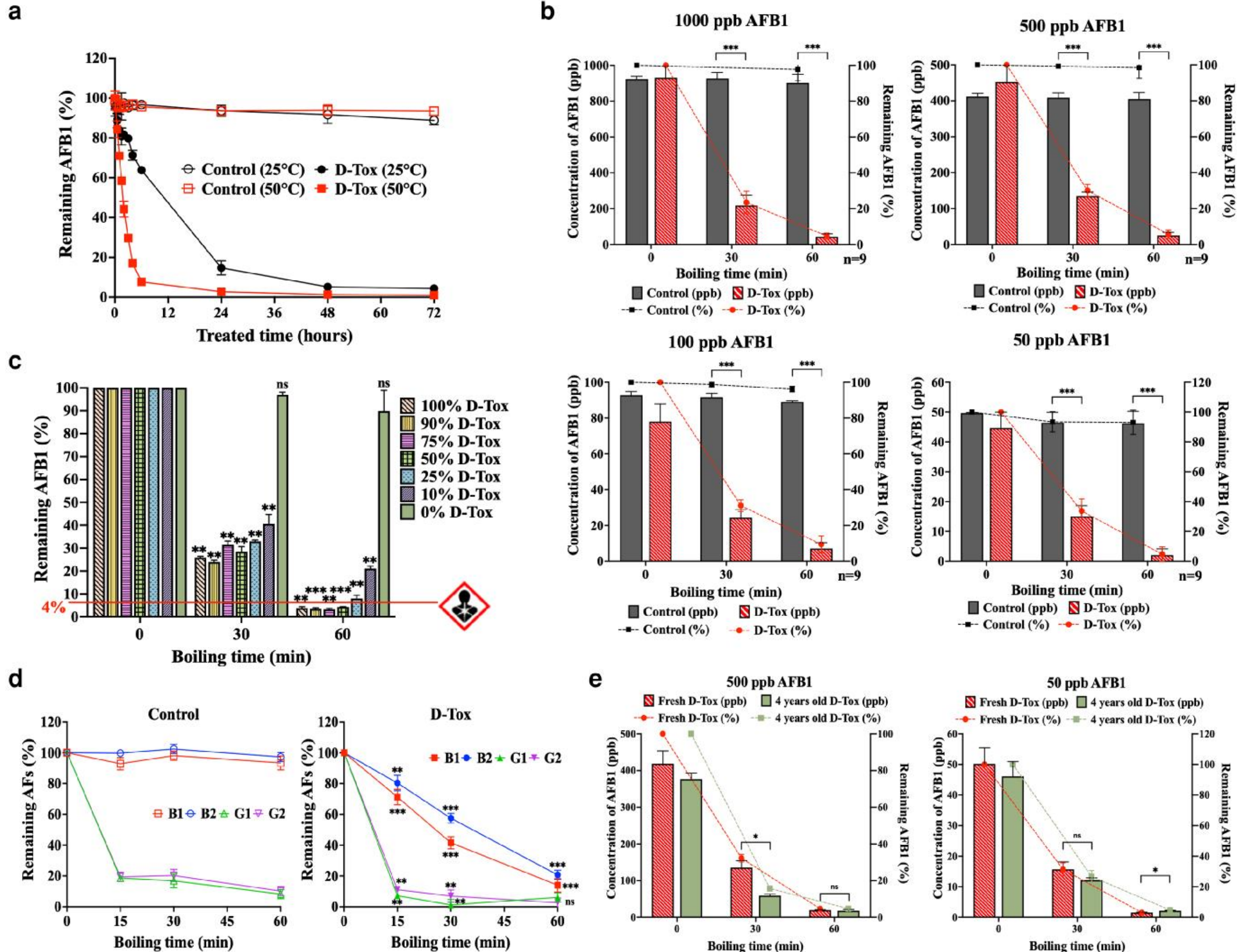

Fig. 2. Degradation of AFs by D-Tox. (a) Determination of AFB1-degrading activity of D-Tox at 25°C and 50°C (5,000 ppb AFB1 spiked). (b) AFB1-degrading activity of D-Tox at 100°C with 1,000 ppb, 500 ppb, 100 ppb, and 50 ppb AFB1, $n = 9$. (c) Degradation of 500 ppb AFB1 in different % of D-Tox at 100°C. (d) D-Tox-mediated degradation of AFs (1,000 ppb AFB1, AFB2, AFG1, and AFG2) at 100°C. (e) Degradation of 500 ppb and 50 ppb AFB1 in fresh D-Tox and 4-year-old D-Tox. * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$; ns, not significant.

Table 1. Composition of sodium nitrate and trace element solutions.

20× Sodium nitrate solution (per liter)	(g)
Sodium nitrate ($NaNO_3$)	120.0
Magnesium sulfate·heptahydrate ($MgSO_4 \cdot 7H_2O$)	10.4
Potassium chloride (KCl)	10.4
Potassium phosphate (KH_2PO_4)	30.4
1,000× Trace element solution (per liter)	
Zinc sulfate·heptahydrate ($ZnSO_4 \cdot 7H_2O$)	22.0
Boric acid (H_3BO_3)	11.0
Manganese chloride·tetrahydrate ($MnCl_2 \cdot 4H_2O$)	5.0
Ferrous sulfate·heptahydrate ($FeSO_4 \cdot 7H_2O$)	5.0
Cobalt chloride·pentahydrate ($CoCl_2 \cdot 5H_2O$)	1.6
Copper sulfate·pentahydrate ($CuSO_4 \cdot 5H_2O$)	1.6
Ammonium molybdate·tetrahydrate ($(NH_4)_6Mo_7O_{24} \cdot 4H_2O$)	1.1
Disodium salt of ethylenediaminetetraacetic acid (Na_2EDTA)	50.0

a comparison between fresh and 4 years aged D-Tox. Our result revealed no significant difference between the two variants when exposed to 500 ppb AFB1 after a 60 min boiling period, with degradation abilities of approximately 95.3 ± 0.2% and 95.2 ± 0.7% for fresh and aged D-Tox, respectively (Fig. 2e; left). Furthermore, at a lower AFB1 concentration of 50 ppb, both fresh and four-year-old D-Tox exhibited remarkable reductions, resulting in concentrations of only 1.6 ppb ± 0.1 ppb and 2.2 ppb ± 0.1 ppb, respectively, following the same boiling duration (Fig. 2e; right). The comparable performance between fresh and aged D-Tox variants underscores the reliability of D-Tox over a prolonged period. These results have significant implications for food safety practices, offering a viable solution to enhance AFB1 reduction in domestic settings.

Requirement of iron sulfate for the D-Tox activity

To determine the minimal essential composition for the D-Tox activity, a series of elimination experiments was carried out. Briefly, we cultured *A. oryzae* in the medium lacking each of the trace elements and nitrate salts (see Table 1 for composition) and tested each culture filtrate's ability to degrade AFB1 upon 60 min boiling. As shown in Fig. 3a, the absence of ferrous sulfate (iron sulfate) led to a significant loss of D-Tox activity, indicating that iron sulfate is necessary for the AFB1-degrading activity of D-Tox. The presence of zinc sulfate in the culture medium was also needed for D-Tox activity due to its requirement for fungal growth; thus, a low concentration of zinc sulfate was sufficient to produce D-Tox in the presence of iron sulfate.

We explored the optimal conditions for D-Tox production by cultivating *A. oryzae* in eight different culture media, including D-Tox medium, YPD, CZ, TSB, MRS, LB, M17, and MEB, all maintained under identical conditions of 30°C and 220 rpm for 9 days. The results showed that D-Tox produced in a medium containing glucose, nitrate salts, and trace elements (minerals) exhibited greater potency compared to those produced in other media (Fig. 3b). Furthermore, we tested media with various glucose-to-nitrate salt ratios. The most effective D-Tox production occurred in a medium comprising 20 g glucose, 10 mL nitrate salts, 1 ppm (0.001 g) zinc sulfate, and 10 ppm (0.01 g) iron sulfate per liter (Fig. 3c).

To test whether D-Tox production varied between fungal strains, 10 different *A. oryzae* strains were cultured separately in the optimized culture conditions. All 10 D-Tox preparations exhibited over 90% AFB1-degrading activity, suggesting that D-Tox activity is not strain-dependent and any *A. oryzae* strain can be used to produce D-Tox (Fig. 3d).

Identification of aflatoxin D1 as the major and transient degradant of AFB1

To test our hypothesis that D-Tox degrades AFB1 by cleaving the lactone ring, 100 ppm (5,000 times the FDA action level 20 ppb) of AFB1 was subject to D-Tox at 100°C and its degradation was monitored at a 10-min interval using high-performance liquid chromatography (HPLC). As shown in Fig. 4a, coupled with the reduced AFB1 peak, a new peak appeared. Fourier transform mass spectrometry (FTMS) of the D-Tox-treated AFB1 sample showed high intensity for an ion peak at *m/z* 287.09, which closely aligns with the m/z ion peak of aflatoxin D1 (AFD1), a nontoxic, nonfluorescent degraded product of AFB1 (13, 14). In contrast, the control AFB1 sample displayed the same intensity of the ion peak at m/z 313.06, corresponding to the intact AFB1 (Fig. 4b). To further validate the hypothesized AFB1 to AFD1 degradation process, direct infusion using quadrupole time-of-flight (QTOF) mass spectrometry (MS) was conducted. As shown in Fig. 4c, AFB1 and AFD1 showed empirical formulae of $C_{17}H_{12}O_6Na^+$ with a sodium adduct $[M + Na]^+$ at m/z 335.05 and $C_{16}H_{14}O_5Na^+$ with sodium adduct $[M + Na]^+$ at m/z 309.07, respectively. These results indicate that D-Tox-mediated degradation of AFB1 involves hydrolysis of the lactone ring leading to $C_{17}H_{14}O_7$, followed by decarboxylation resulting in $C_{16}H_{14}O_5$, AFD1 (Fig. 4d). The intensified hydrolysis of the lactone ring at 100°C can be attributed to principles of chemical kinetics. Elevated temperatures typically enhance the reactivity of chemical reactions by increasing the average kinetic energy of the involved molecules. Consequently, molecules move more rapidly and collide with greater energy, resulting in more frequent collisions between AFB1 molecules and D-Tox. This heightened collision frequency accelerates the hydrolysis of the lactone ring in AFB1, thereby enhancing its degradation efficiency. Thus, at 100°C, the supplied energy may expedite the degradation process, leading to more efficient lactone ring hydrolysis that AFD1 further degraded into unknown products, as D-Tox degrades AFB1 safely. These results indicate that D-Tox degrades AFB1 irreversibly and permanently.

D-Tox effectively degrades AFB1 in wheat, corn, peanuts, ginseng, and oil

As D-Tox is found to degrade up to 100 ppm of AFB1 in vitro, we assessed the real-world applicability of D-Tox. Commercially packaged wheat, corn, and peanuts were purchased from the local store and naturally contaminated with AFB1 by inoculating with a highly toxigenic *A. flavus* NRRL3357 strain. We found that AFB1 levels were 870.2 ± 98.7 ppb, 368.5 ± 10.4 ppb, and 606.7 ± 12.9 ppb in wheat, corn, and peanuts, respectively (Fig. 5a). During the initial stages of the food experiment testing, we explored different ratios, such as 10 g of corn samples in 150 and 200 mL (w/v), leading to degradation abilities of approximately 55 and 70%, respectively. However, an enhancement in degradation ability was observed upon increasing the volume of D-Tox to 250 mL (resulting in a total of 20 grams in 500 mL D-Tox), yielding AFB1 degradation abilities ranging from 70 to 90%. This discovery indicates that D-Tox necessitates adequate capacity to interact with AFB1 in crop samples to achieve optimal degradation. Twenty grams of each sample were immersed in 500 mL of D-Tox and subjected to either 60 min of boiling in D-Tox or 24 hr of soaking in D-Tox at room temperature (RT). As shown in Fig. 5a, 91.9 ± 1.4%, 76.6 ± 3.6%, and 72.3 ± 3.3% of AFB1 in wheat, corn, and peanuts were removed, respectively, after boiling 60 min in D-Tox, and 69.3 ± 5.9%, 77.1 ± 3.3%, and 77.3 ± 2.8% of AFB1 in wheat, corn, and peanut were removed, respectively, after

soaking 24 hr in D-Tox at RT. We then performed *A. flavus* NRRL3357 infestation using fresh produce, yielding 1,115 ppb, 872 ppb, and 4,570 ppb of AFB1 levels in wheat, corn, and peanuts, respectively. Upon 60 min boiling of 20 g of each sample in 500 mL of D-Tox, 94.0 ± 0.1%, 77.2 ± 2.4%, and 75.1 ± 0.8% of AFB1 were degraded in wheat, corn, and peanuts, respectively ($P < 0.001$). Next, to test the potential reusability of D-Tox, each previously used D-Tox solution was re-used to treat the samples with 60 min boiling, and 84.8 ± 0.3%, 66.8 ± 0.8%, and 56.5 ± 4.0% of AFB1 were degraded in wheat, corn, and peanuts, respectively (Fig. 5b), suggesting that D-Tox can be re-used with only a minor loss in efficacy. The varying effectiveness of D-Tox in degrading AFB1 in different crops observed in Fig. 5a and b may be attributed to the surface characteristics of each crop. D-Tox could potentially interact more effectively with wheat compared to peanuts and corn due to the larger surface area available for interaction. Wheat may provide a larger surface area per unit volume for D-Tox to come into contact with AFB1, facilitating more efficient degradation. In contrast, the larger size and structure of peanuts and corn may limit the accessibility of D-Tox to AFB1, resulting in relatively lower degradation rates.

We then also examined the applications of D-Tox in different types of edible products. As shown in Fig. 5c, upon 60 min boiling in 250 mL D-Tox, 98.3 ± 0.1% of AFB1 present in 10 g of dried ginseng was degraded, resulting in residual concentration of 5.6 ppb ± 0.3 ppb AFB1 ($P < 0.001$; Fig. 5c). In addition, as edible oils can be contaminated by AFB1 during pre and post-harvest periods (15), we tested the applicability of D-Tox in oil by adding freeze-dried D-Tox (1%, w/v) to the peanut oil containing 500 ppb. These oil samples were kept in a boiling water bath for 60 min and 56.0 ± 2.1% of AFB1 in the peanut oil was found to be degraded by D-Tox (Fig. 5d; $P < 0.001$). In summary, D-Tox can be used to reduce high levels of AFB1 in various foods that can be commonly contaminated with AFs.

Degradation of other lactone-ring containing mycotoxins and safety of D-Tox

Patulin, aflatoxin M1 (AFM1), and ochratoxin A (OTA) are other mycotoxins that contain a lactone ring. Patulin primarily affects apple products, OTA is a frequent contaminant in rice, coffee, grapes, and wine (16), while AFM1 predominantly contaminates milk products sourced from animals that have consumed feed contaminated with AFB1 (17). To test the possibility of D-Tox-hydrolyzing the lactone ring in other mycotoxins, 500 ppb of patulin, AFM1, and OTA, respectively, was added to D-Tox solution and boiled for 30 ~ 60 min. As shown in Fig 6a–c, D-Tox degraded 100.0 ± 0.0% of patulin in 30 min and 83.0 ± 3.0% of AFM1 in 60 min. However, only 18.9 ± 1.4% of OTA was degraded upon 60 min of boiling in D-Tox. When considering the structural differences among patulin, AFM1, and OTA (as illustrated in Fig. 6), it becomes apparent that simple structure mycotoxins,

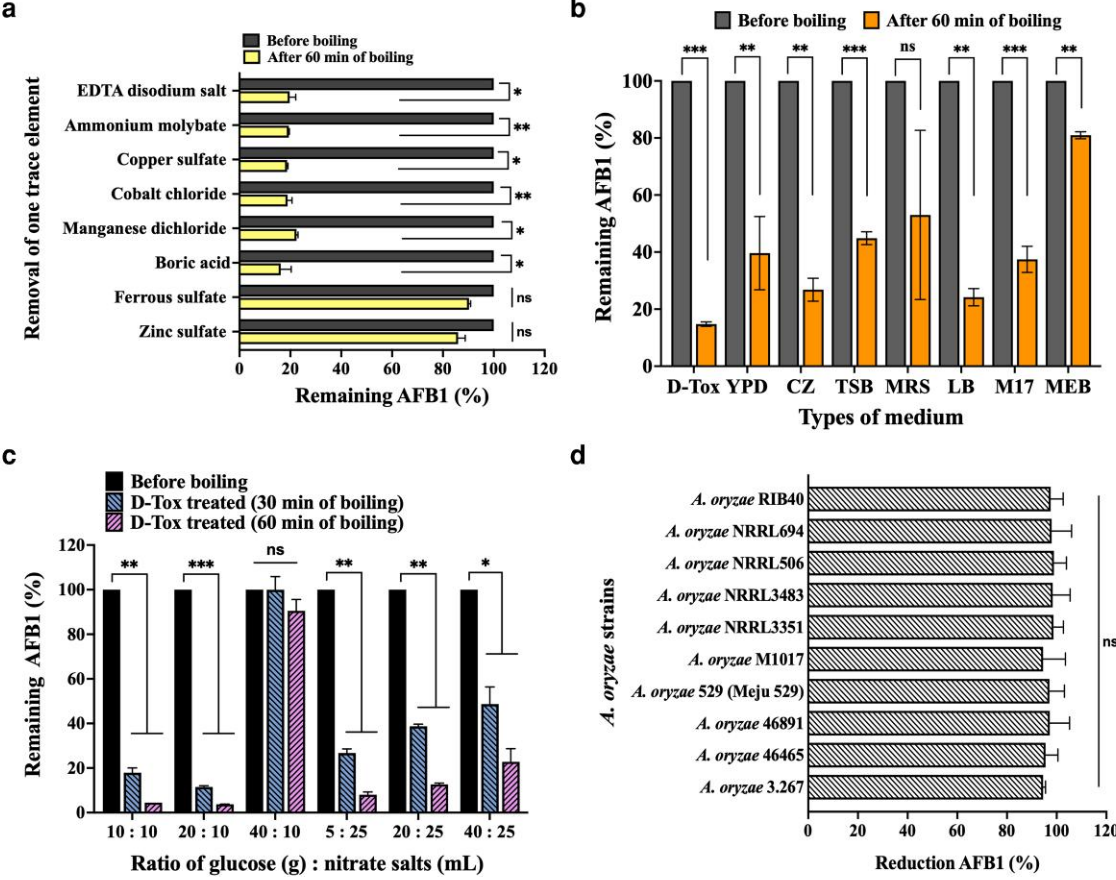

Fig. 3. AFB1-degrading activity of D-Tox requires iron sulfate. (a) Determination of AFB1-degrading activity of D-Tox upon removal of each trace element. (b) Determination of AFB1-degrading activity of D-Tox based on each medium. (c) Determination of the optimal ratio of glucose (in grams) to nitrate salts (in milliliters). (d) Assessment of D-Tox's ability to degrade AFB1 based on *A. oryzae* strains. All experiments were performed at 100°C with 1,000 ppb AFB1. * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$; ns, not significant.

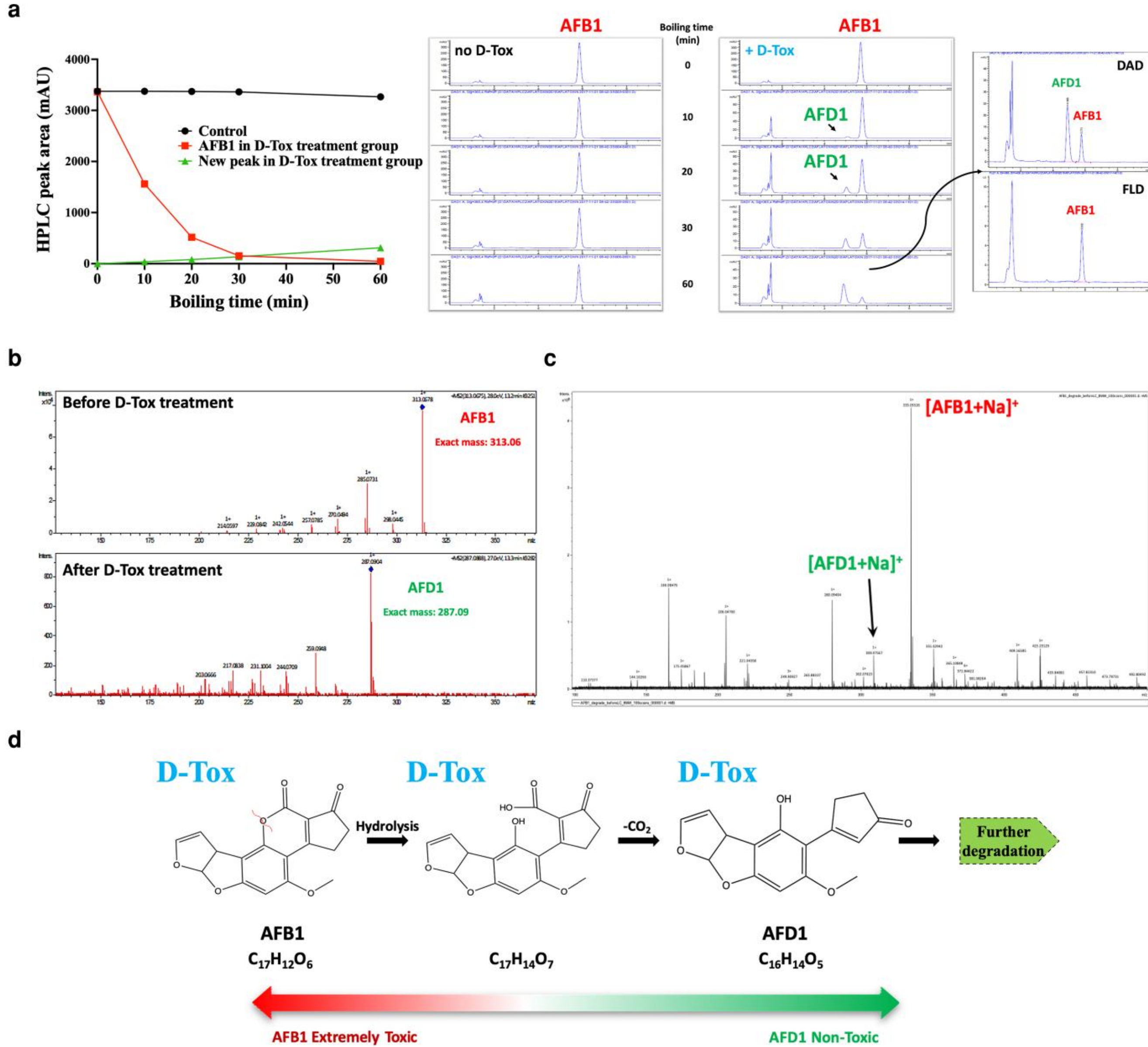

Fig. 4. Irreversible and permanent degradation of AFB1 by D-Tox. (a) AFB1 (100 ppm) degradation kinetics and HPLC chromatographs showing a new peak (AFD1), which lacks fluorescence. DAD: 365 nm, FLD: $\lambda_{excitation} = 365$ nm and $\lambda_{emission} = 450$ nm (32). (b) Confirmation of AFB1 and AFD1 empirical formula through FTMS. (c) Validation of the empirical formula of AFB1 and AFD1 in their sodium adduct through QTOF direct infusion. (d) Proposed mechanism of D-Tox-mediated irreversible degradation of AFB1 involving lactone ring hydrolysis followed by decarboxylation. Chemical structures were generated using ChemDraw.

such as patulin, tend to be more readily degraded than complex ones. This is because simple structure mycotoxins typically have fewer chemical bonds and functional groups, making their reactive sites more accessible to degradation agents, such as D-Tox. Therefore, the relatively low degradation percentage of OTA (18.9%) can be attributed to several factors related to its chemical structure. OTA possesses a relatively stable structure and exhibits resistance to heat and acid compared to other mycotoxins due to its chemical composition (18). Unlike AFs, OTA has a larger cyclic lactone ring and additional substituents such as the phenylalanine moiety and chlorine atom (19), which can impede ring-opening reactions. Therefore, in terms of D-Tox degradation, patulin exhibits a higher degradation rate relative to AFM1 and OTA, based on the ascending order of structural complexity (patulin < AFM1 < OTA). Consequently, these results suggest that D-Tox might be effectively used for apple products (patulin) and milk (AFM1) with limited applicability for OTA degradation in wine, coffee, and rice.

To assess the preliminary toxicity of D-Tox, a tissue-culture-based cell viability assay was carried out employing the breast cancer cell line MCF-7, which is highly sensitive to xenobiotics due to its elevated division rate. Two sets of D-Tox samples, along with their corresponding controls, were evaluated. One set comprised samples without AFB1 and heat treatment (Fig. 6d; top), while the other set contained 500 ppb of AFB1 and underwent heat treatment through boiling process (Fig. 6d; bottom). The cells were exposed to varying concentrations (10 ∼ 1000 µg/mL) of lyophilized culture medium (control), D-Tox, and 2.5 µM of doxorubicin (control for 50% cell viability), then cultured for 72 hr. In the top section of Fig. 6d, even at

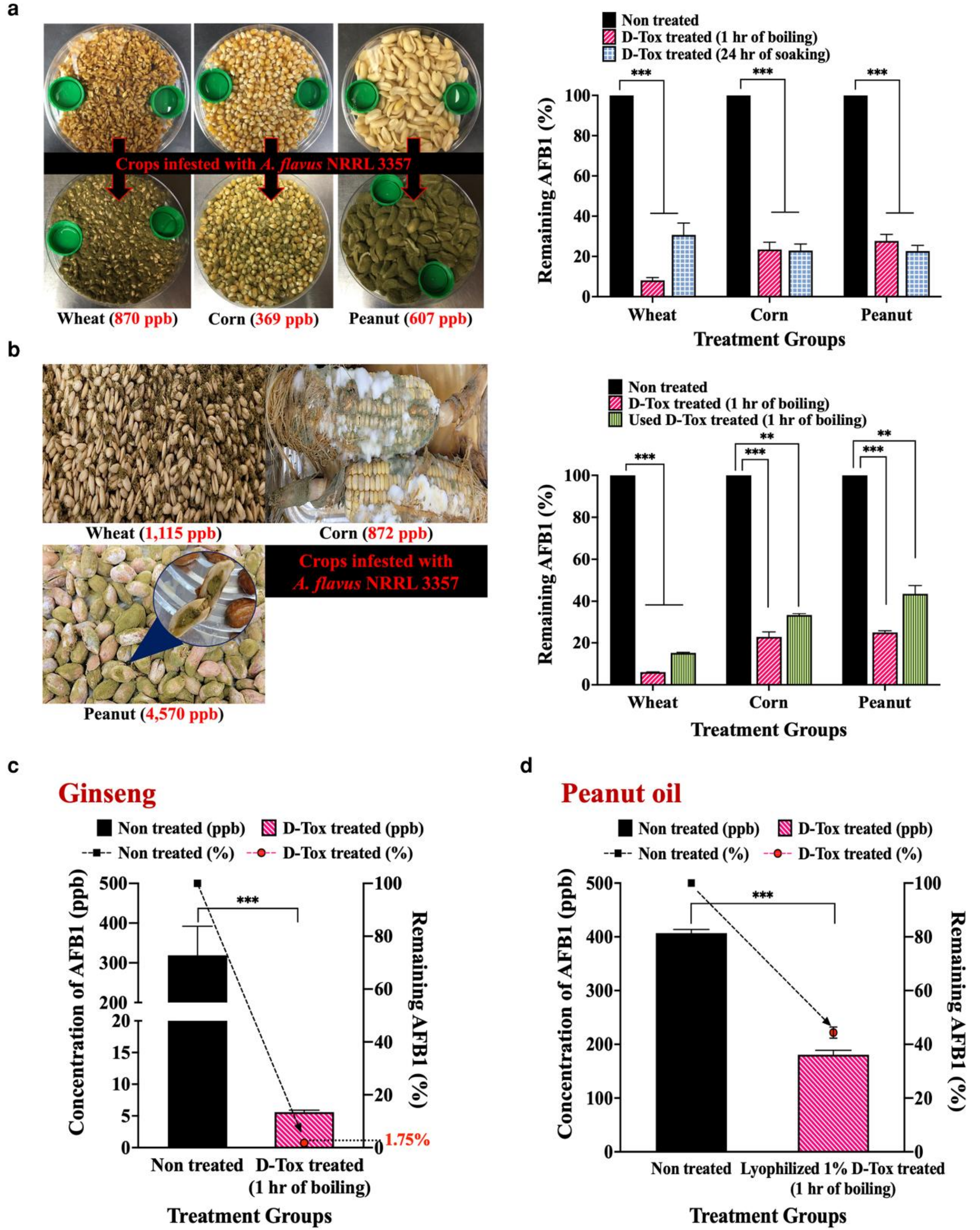

Fig. 5. Application of D-Tox in various foods. Degradation of high levels of AFB1 in wheat, corn, and peanut (a) after 1 hr of boiling and 24 hr of soaking in D-Tox at RT and (b) after 1 hr of boiling in fresh D-Tox and re-used D-Tox. (c) Reduction of 500 ppb of AFB1 spiked in 10 g ginseng after 1 hr of boiling in D-Tox. (d) Degradation of 500 ppb of AFB1 in peanut oil by 1% lyophilized D-Tox. * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$.

250 µg/mL of D-Tox, cells exhibited high viability levels, while viability started to decline at 125 µg/mL of nonfermented culture medium containing 20 g/L of glucose. Previous research has indicated that MCF-7 cells are sensitive to glucose, with high doses capable of inducing apoptosis (20). However, it is important to note that corn sugar (commonly known as D-glucose or dextrose), as per FDA regulations (21 CFR 184.1857), is recognized as generally safe for direct addition to human food (GRAS). Therefore, as a food ingredient, glucose is not inherently cytotoxic. In the second group, the control group retained

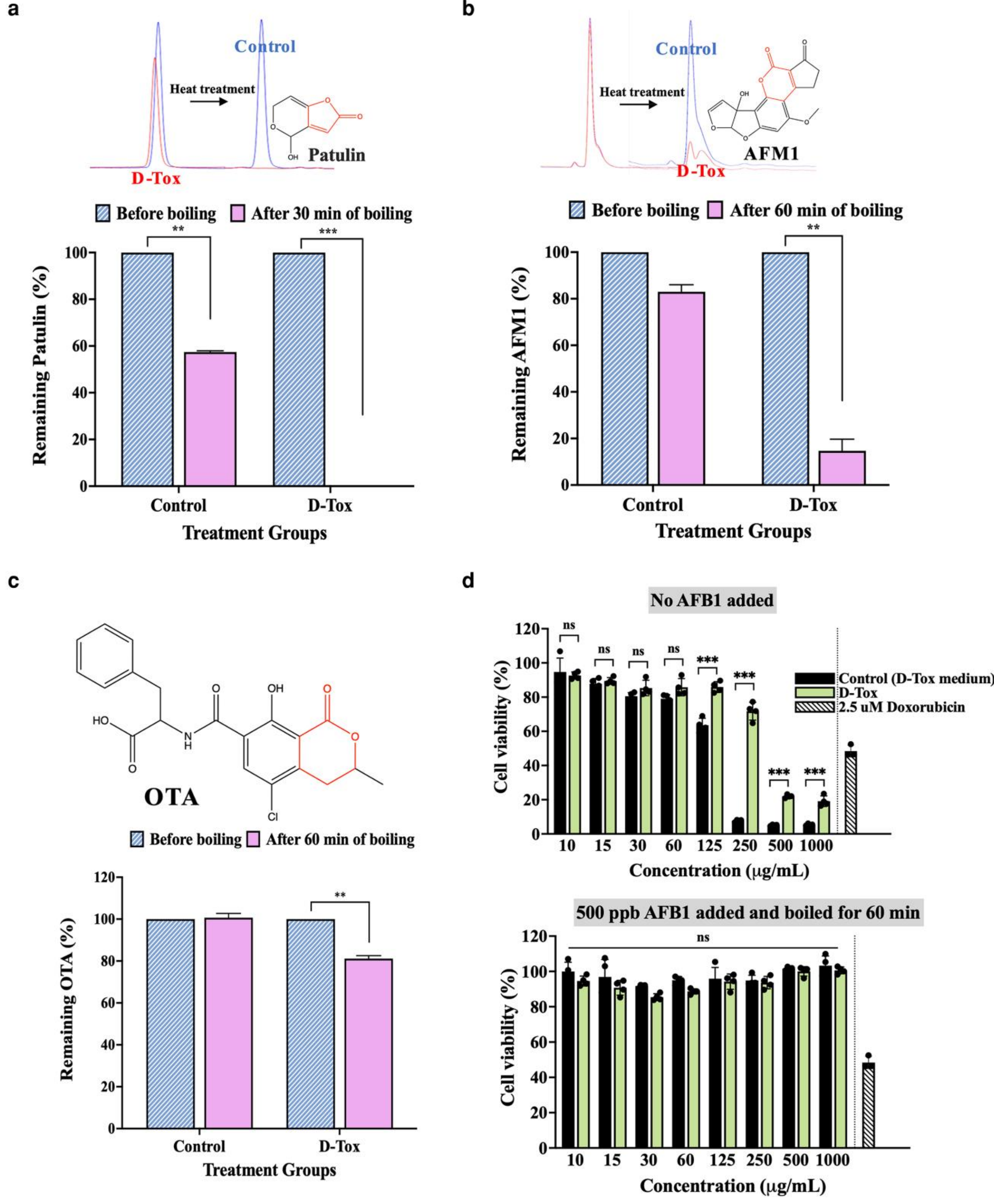

Fig. 6. Degradation of other lactone-ring-containing mycotoxins and the nontoxic nature of D-Tox. (a) D-Tox-mediated degradation of patulin (500 ppb) in 30 min of boiling. (b) Degradation of AFM1 (500 ppb) by D-Tox in 60 min of boiling. (c) Degradation of OTA (500 ppb) by D-Tox in 60 min of boiling. (d) Viability of the MCF-7 cells with varying amounts of freeze-dried control and D-Tox without AFB1 added (top) and 500 ppb AFB1 added and boiled for 60 min (bottom). * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$; ns, not significant. Chemical structures were generated using Chem Draw.

approximately 450 ppb of AFB1, whereas the D-Tox group effectively degraded and eliminated all traces of AFB1 after 60 min of boiling. Irrespective of concentration and the presence of AFB1, treatment with D-Tox and its control, combined with heat treatment, did not significantly impact the viability of MCF-7 cells (Fig. 6d; bottom).

Regarding the observed decrease in cytotoxicity in the glucose-containing medium in the presence of AFB1: Post-boiling, glucose or its degradation products may interact with AFB1, potentially forming adducts or altering its chemical structure. This interaction could influence the toxicological properties of AFB1. While specific research on AFB1–glucose interactions reducing

toxicity is limited, previous study on other mycotoxin like fumonisin B (FB) suggested that FB-glucose adducts can mitigate toxicity in swine. For example, reducing sugars such as D-glucose and D-fructose can inhibit FB's primary amino group, thereby modifying its toxicity profile (21). Nevertheless, further research is needed to elucidate the exact mechanisms involved. These results support the idea that D-Tox, a culture broth of the GRAS fungus *A. oryzae* grown in a food-grade medium, is a novel fermented food product that can effectively and safely degrade AFs and patulin in food and beverage.

Discussion

Since the discovery of AFs in the early 1960s, numerous attempts have been made to reduce AF levels in food and feed due to their extreme toxicity and carcinogenicity (22). These methods include physical processes such as gamma irradiation and UV treatment, chemical treatment employing acids, bases, ammonization, and ozonation, and biological approaches using microorganisms and their products (4). However, none of these methods are suitable for the effective and practical elimination of AFs in food products. Inarguably, the most practically successful method thus far is a preharvest control employing nonaflatoxigenic strains, e.g. Afla-guard or Afla-safe, that compete out the naturally present aflatoxigenic *A. flavus* isolates in the field (23, 24).

However, it is important to note that AF contamination can occur in fields, during transportation, and storage in commercial and residential conditions. For example, during 2012 ∼ 2015 in Romania, 44 out of 97 crop samples obtained from private cereal traders and food manufacturers were contaminated with AFs, with some reaching up to 82.94 ppb, surpassing the E.U. regulatory limit (EC 2006) of 4–15 ppb for total AFs (2, 25). Therefore, the best way to reduce human exposure to AFs would be to remove/detoxify AFs right before consumption. However, due to the stability of AFs, both pre and post-harvest mitigation strategies have only achieved marginal success (10, 26).

D-Tox is the first product that can effectively eliminate most AFs in food through a straightforward boiling process, thereby addressing the longstanding global challenge of the AF problems. As described, D-Tox can degrade 72 ∼ 94% of high levels (368 ppb ∼ 4,570 ppb) of AFB1 in artificially contaminated wheat, corn, and peanuts. These concentrations are significant as they mirror the levels of naturally occurring AFs contamination in specific regions. According to the E.U.'s RASFF (Rapid Alert System for Food and Feed) notifications, levels of total AFs in the U.S. food and feed products exported to the E.U. were 28 ppb ∼ 220 ppb (2, 27). Moreover, among approximately 10,199 feed samples collected globally from January to June 2023, Asia exhibited the highest incidence of AFs contamination, with an average positive level of 33 ppb and a maximum of 1,392 ppb, followed by Europe, Latin America, and North America. Additionally, a study by Benkerroum (28) reported AFs contamination prevalence in regions like Southeast Asia and Sub-Saharan Africa, where levels exceed regulatory limits, with AFs levels ranging from 0.1 to 32,328 ppb (32 ppm) in peanuts in Kenya. AFs ranging from 0 to 12,256 ppb (12 ppm) were detected in peanuts from Thailand, while Uganda revealed the highest levels with a maximum of 3,760 ppb and an average of 66.5 ppb. This highlights the urgency of addressing AFs contamination in crop samples from these regions. Therefore, we anticipate that D-Tox, owing to its stability, can be used to eliminate AFs in maize, rice, wheat, barley, oats, sorghum, and milk (AFM1). Several D-Tox application methods can be envisioned, including soaking/misting crops with D-Tox, cooking plant-based foods with D-Tox (cooking process), or adding a lyophilized D-Tox tablet to milk. For instance, degrading AFs in 500 g of maize may require approximately 5 to 6 L of D-Tox solution, depending on factors such as the surface area of the crop in contact with the D-Tox. Similarly, for wheat, a lower quantity of D-Tox may be needed due to its larger surface area per unit volume, facilitating more efficient degradation of AFs.

Furthermore, due to its noncytotoxic nature and the mechanisms of AFs degradation, we expect that D-Tox can be safely used in various food applications. As described, D-Tox-mediated degradation of AFB1 occurs by hydrolysis of the lactone moiety followed by decarboxylation, resulting in the formation of AFD1 as the primary transient degradant, which in turn is further degraded. The stability of D-Tox activity upon multiple prolonged boiling and autoclaving twice indicates that the key functional D-Tox molecule(s) is a nonprotein and nonenzyme-based compound. These characteristics of D-Tox distinguish it from other biological technologies that rely on probiotic bacterial and/or yeast strains for AF detoxification through binding, enzymatic degradation, and nutrient competition (29, 30). Additionally, the reusability of D-Tox and its superior capability of degrading over 95% of 100 ppm (5,000×higher than the US FDA action level 20 ppb) of AFB1 in 60 min of boiling provides additional advantages of D-Tox over other technologies. D-Tox can degrade other fungal toxins containing lactone rings, including patulin, AFM1, and OTA. This broad-spectrum detoxification capability of D-Tox suggests that it might be used as a multifaceted solution in mitigating the risks associated with other lactone ring-containing molecules, e.g. macrolide antibiotics and pyrethroids, for potential applications in environmental remediation of excessive antibiotics.

Finally, we envision that D-Tox can be commercially produced in both solution and tablet forms, enhancing its practicality for diverse applications. For this, the identification and concentration of the D-Tox molecule in powder or tablet forms would be an important next step for the practical application of D-Tox in real-world conditions from household to production settings. Its application at the household level could provide a practical solution for individuals in AF-prone regions to lower AF levels in their food supply. On a larger scale, D-Tox may also have implications for food production and safety industries, offering a cost-effective and efficient method for aflatoxin remediation in food processing facilities and agricultural settings thereby offering a promising avenue for safeguarding the quality of the agrifood systems in the world.

Materials and methods

Fungal strains and culture conditions

Aspergillus oryzae NRRL 3483 was used to produce D-Tox and *A. flavus* NRRL 3357 was used as a high AFB1 producer. Fungal strains were grown on potato dextrose agar medium composed of 4 g potato starch, 20 g glucose, and 15 g agar in 1 L of distilled water and incubated for 4 days at 30°C. Asexual spores (i.e. conidia) were then harvested with sterile 0.1% Tween-80 solution and counted using a hemocytometer. Conidia suspension was stored at 4°C and used within 2 weeks of preparation.

Preparation of media for D-Tox determination

To determine the optimal medium for D-Tox assessment, we prepared several media with distinct compositions. Yeast Peptone Dextrose (YPD) medium was prepared by dissolving 20.0 g glucose, 10.0 g yeast extract, and 20.0 g peptone per liter of distilled water.

For Czapek (CZ) medium, 10.0 mL CZ concentrate (containing 30.0 g sodium nitrate, 5.0 g potassium chloride, 5.0 g magnesium sulfate heptahydrate, and 0.1 g iron sulfate heptahydrate per 100 mL), 30.0 g sucrose, and 1.0 mL trace element solution (containing 0.5 g copper sulfate pentahydrate and 0.1 g zinc sulfate heptahydrate per 100 mL) were mixed in distilled water, adjusted to 1 L. Tryptic Soy Broth (TSB) medium consisted of 2.5 g glucose, 17.0 g pancreatic digest of casein, 3.0 g peptic digest of soybean, 2.5 g dipotassium phosphate, and 5.0 g sodium chloride per liter of distilled water. De Man, Rogosa & Sharpe (MRS) medium was prepared by mixing 20.0 g glucose, 5.0 g yeast extract, 10.0 g peptone, 2.0 g disodium phosphate, 0.1 g magnesium sulfate, 5.0 g sodium acetate, and 1.0 g polysorbate 80 per liter of distilled water. For Luria–Bertani (LB) medium, 5.0 g yeast extract, 10.0 g tryptone, and 10.0 g sodium chloride were dissolved in distilled water, adjusted to 1 L. M17 medium was composed of 2.5 g yeast extract, 5.0 g beef extract, 5.0 g soy peptone, 5.0 g pancreatic digest of casein, 19.0 g disodium-β-glycerophosphate, 0.25 g magnesium sulfate, and 0.5 g ascorbic acid per liter of distilled water. Lastly, Malt Extract Broth (MEB) medium was prepared by dissolving 6.0 g glucose, 1.8 g maltose, 1.2 g yeast extract, and 6.0 g malt extract per liter of distilled water. Liquid media were then autoclaved for 20 min at 121°C and 15 PSIG for further sterilization.

Preparation of D-Tox

To prepare the initial D-Tox medium, 10.0 g D-glucose, 50 mL of 20× sodium nitrate solution, and 1.0 mL of 1,000× solution of trace elements were added to 600 mL distilled water in a 1,000 mL volumetric flask. The mixture was then stirred thoroughly for 20 min and adjusted to a final volume of 1,000 mL, with the final pH adjusted to 6.5 using sodium hydroxide. This liquid medium was then autoclaved for 20 min at 121°C and 15 PSIG. The procedure for preparing the 20× sodium nitrate solution and the 1,000× solution of trace elements is detailed in Table 1 as described in Pontecorvo et al. (31) *A. oryzae* NRRL 3483 conidia were inoculated into a culture liquid medium (150 mL) at the final concentration of 5×10^5 conidia/mL and incubated for 9 days at 30°C with 220 rpm shaking. The cell-free culture fermentate (D-Tox) was obtained by filtering through four layers of Miracloth (MilliporeSigma, USA) and a 0.22 μm PES filter unit (Thermo Scientific, USA). D-Tox was subsequently stored at 4°C throughout the experiments.

Preparation of AFs and HPLC conditions

The powder standards of individual AFs (AFB1, AFB2, AFG1, AFG2, and AFM1) were purchased from Sigma Chemical Co. (USA). Standard solutions of individual AFs were prepared in acetonitrile and stored at 4°C in amber glass vials. The levels of AFs were quantified by HPLC according to Official Methods of Analysis of AOAC International (32) using an Agilent 1100 system equipped with a Zorbax Eclipse XDB-C18 4.6 mm ×150 mm, 3.5 μm column. All AFs were detected simultaneously using two detectors connected in series, diode array (DAD), and fluorescence (FLD). Samples were monitored at a wavelength of 365 nm for UV detection and at 365 nm excitation and 450 nm emission for FLD detection. The samples were eluted with a mobile phase of water (H_2O)/methanol (CH_3OH)/acetonitrile (CH_3CN) (50:40:10) at a flow rate of 0.8 mL/min. AFM1 was detected at wavelength (FLD [$\lambda_{excitation} =$ 365 nm and $\lambda_{emission} = 435$ nm]) with a mobile phase of water (H_2O)/isopropyl alcohol (C_3H_8O)/acetonitrile (CH_3CN) (80:12:8) at a flow rate of 1.0 mL/min. All solvents were of HPLC grade, and all AF peaks were integrated using ChemStation software (Agilent, USA).

Preparation of patulin/OTA and determination of the HPLC analysis conditions

The powder standards of patulin and OTA were purchased from Sigma Chemical Co. (USA) and prepared in ethyl acetate and acetonitrile, respectively, at a final concentration of 10 μg/mL according to the Official Methods of Analysis of AOAC International (32). Both mycotoxins were quantified by the HPLC system and separation column mentioned previously. Patulin was detected at a wavelength of 276 nm with a mobile phase of 1% acetic acid in water (H_2O)/methanol (CH_3OH) (95:5) at a flow rate of 0.8 mL/min. OTA was detected at wavelength (FLD [$\lambda_{excitation} = 333$ nm and $\lambda_{emission} = 460$ nm]) with a mobile phase of water (H_2O)/acetonitrile (CH_3CN)/acetic acid (CH_3COOH) (99:99:2) at a flow rate of 1.0 mL/min. All solvents were of HPLC grade, and all peaks were integrated using ChemStation software (Agilent, USA).

Degradation of AFB1 by D-Tox in vitro

In total, 10 μL aliquots of varying concentrations of AFB1 in acetonitrile were added to 10–20 mL of D-Tox to yield 50 ppb, 100 ppb, 500 ppb, 1,000 ppb, 5,000 ppb, and 100 ppm of AFB1, respectively. Degradation of 50 ∼ 5,000 ppb AFB1 was carried out at 25°C and 50°C for 72 hr, and at 100°C for 1 hr. At a concentration of 100 ppm AFB1, D-Tox-mediated degradation was monitored at 10-min intervals upon boiling. At each specified time point, 1 mL aliquots were withdrawn and filtered using a 0.45 μm Teflon syringe filter unit (Thermo Scientific, USA) for subsequent HPLC analysis to assess the levels of remaining AFB1 (%). As a control, either distilled water or fungus-free medium was used with equivalent concentrations of AFB1. Both the D-Tox and control experiments were carried out in triplicate, and the remaining % of AFB1 was calculated using the following equation (A).

$$\text{Remaining AFB1 (\%)} = 100 - \frac{\textit{Initial concentration} - \textit{Concentration after treatment}}{\textit{Initial concentration}} \times 100 \quad (A)$$

Degradation of total AFs and other mycotoxins by D-Tox in vitro

Ten μL aliquots of mycotoxin (AFB1, AFB2, AFG1, AFG2, AFM1, patulin, or OTA) in acetonitrile were added separately to 10–20 mL of D-Tox to obtain a final mycotoxin concentration of 1,000 ppb (AFB1, AFB2, AFG1, and AFG2) or 500 ppb (AFM1, patulin, or OTA). The reaction mixtures were incubated at boiling temperature (100°C) for 60 min with samples collected at zero min (before treatment), after 30 min, and after 60 min of boiling. The samples were treated similarly to total AFs, and the residual levels of AFs were determined using the calculation outlined previously in Eq. (A).

Degradation of AFB1 by D-Tox in food samples

Food crop samples (wheat, corn, and peanut) were washed with sterile distilled water for 2 min and inoculated with conidia of highly toxigenic *A. flavus* NRRL 3357. This was done by combining 2 mL of a 5×10^5 conidia/mL spore suspension with 200 ∼ 300 g of food crop samples in an aluminum foil tray. The mixture was manually stirred after 2 days of incubation at 30°C, and the crops were then incubated at 30°C for 7 days. Following this, crop samples were washed three times with sterile distilled water, ensuring the complete removal of *A. flavus*. The samples were then dried under gentle airflow to eliminate moisture. Twenty grams of crop samples were subjected to treatment in 500 mL of D-Tox

through a 24 hr soaking or 1 hr of boiling. Additionally, another 20 grams of crop samples underwent treatment with 500 mL of both fresh and recycled D-Tox at boiling temperature for 1 hr.

Degradation of AFB1 by D-Tox in ginseng and peanut oil samples

In both ginseng and peanut oil samples, an AFB1 standard was spiked to achieve a final concentration of 500 ppb. For the ginseng experiment, 500 µL of 10 ppm AFB1 was spiked into 10 g of dried ginseng to reach a final concentration of 500 ppb. Subsequently, the 10 g of ginseng was treated in 250 mL of D-Tox and boiled for 1 hr. Additionally, in the peanut oil experiment, 20 mL of D-Tox solution was freeze-dried using a lyophilizer (Yamato, USA), yielding approximately 36 mg of freeze-dried powder, yielding 1.8 mg per 1 mL of D-Tox solution. As 10–20 mL of D-Tox solution was used in the experiments to test its ability to degrade AFs, 500 mg of freeze-dried D-Tox powder (equivalent to 14 mL D-Tox solution) was mixed with peanut oil containing 500 ppb AFB1. Samples from both reactions were collected at zero min (before the boiling process) and after 60 min of boiling.

Extraction of AFB1 from food samples

After treatment with D-Tox, crops were dried overnight under gentle air to eliminate the moisture.

The AFB1 extraction and analysis were carried out according to the manufacturer's protocol using an immunoaffinity Aflatest Vicam Column (Vicam, USA). Briefly, 10 g of ground crops were added to 20 mL of extraction solution (80% methanol), and vortexed for 2 min. The mixtures were filtered through Whatman No.4 filter paper (Whatman plc, UK). Then, 5 mL of the filtrate was combined with 20 mL of sterile distilled water and passed through a micro-fiber filter (Whatman plc, UK). Ten mL of the filtrate were passed through an immunoaffinity Aflatest Vicam Column (Vicam, USA). A clean-up process was carried out using 10 mL of sterile distilled water. To elute AFB1, 1.5 mL of methanol was passed through the immunoaffinity column into 10 mL conical tubes. These eluates were then combined with 1.5 mL of acetonitrile: water solution in a ratio of (1:4) for subsequent HPLC analysis described earlier. The remaining percentage of AFB1 was calculated using Eq. (A).

Extraction of AFB1 from ginseng and peanut oil

The extraction of AFB1 from 10 g of ginseng was performed using the same procedure outlined in the section "Extraction of AFB1 from food samples". After peanut oil was subjected to 1 hr boiling, 2 mL of peanut oil was mixed with 2 mL of chloroform and stirred for 5 min. The mixtures underwent centrifugation at 5,000 rpm for 5 min. The resulting chloroform layer was transferred to a glass vial and dried using gentle airflow. After overnight drying, 1 mL of the HPLC mobile phase for AFB1 (H_2O: CH_3OH:CH_3CN, 50:40:10) was added to the vials, mixed, and subsequently filtered using a 0.45 µm Teflon syringe filter unit (Thermo Scientific, USA). The sample was then subjected to HPLC analysis to determine the remaining percentage of AFB1 using Eq. (A).

LC-MS/MS analysis of AFB1 degraded products

The degraded product of AFB1 was analyzed using high-resolution FTMS and QTOF MS (Bruker Daltonics, Billerica, MA). D-Tox suspensions containing the degraded aflatoxin were first extracted using ethyl acetate and then reconstituted with 0.5% acetic acid in water after evaporation of the organic solvent under gentle air. Conditions for FTMS analysis of AFs included using CH_3CN as the mobile phase, a skimmer voltage of 20 V, octopole RF amplitude set at 350 Vpp, and collision cell RF at 1,000 Vpp. Data were collected in positive mode within the range of 100–500 m/z with an average scan 200 m/z. The ion accumulation time was optimized at 5 ms. The collision cell voltage was set to −4.0 V. The final identification of an unknown compound was based on the accurate mass measurement of parent and fragment ions, as well as other useful MS/MS spectrum information (13).

Cell viability assay

Two sets of D-Tox groups, each with their corresponding controls, were prepared: one set of samples without AFB1 and another set containing 500 ppb AFB1 and subjected to boiling for 1 hr. D-Tox along with controls were lyophilized using a freeze dryer (Yamato, USA) in preparation for cell cytotoxicity test assay. A mammalian cell cytotoxicity assay was conducted by the Cancer Pharmacology Lab at the University of Wisconsin-Madison. The lyophilized samples were reconstituted in sterile molecular biology-grade water to achieve a final concentration of 100 mg/mL. The samples were dispensed onto white 384-well plates (Corning 3765, Thermo Fisher, USA) at final concentrations ranging from 10 to 1000 μg/mL using an Echo 650 acoustic liquid handler (Beckman Coulter, USA). MCF7 cells (breast cancer cell line) were plated per well (50 μL final volume) into 384 well plates using a multidrop liquid handler (Thermo Fisher, USA). The cells were then incubated with the samples for 72 hr. Toxicity was assayed using the CellTiter-GLO (Promega, USA) reagent following the manufacturer's protocol. Doxorubicin, a chemotherapy drug, was used at a concentration of 2.5 μM as a positive control.

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Author Contributions

Conceptualization: A.F.A, D.C., and J-H.Y.; Investigation: A.F.A., and D.C.; Writing-Original Draft: D.C.; Writing-Review & Editing: D.C., A.F.A., and J.-H.Y.; Funding Acquisition: J.-H.Y.; Supervision: A.F.A., and J.-H.Y. All authors have read and approved the final version of the manuscript.

Data Availability

All data and materials are presented in this manuscript. Fungal species and strains in this study are available through the Agricultural Research Service in the United States Department of Agriculture (https://nrrl.ncaur.usda.gov/).

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