Product Identification and Safety Evaluation of Aflatoxin B$_1$ Decontaminated by Electrolyzed Oxidizing Water

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Ⓢ *Supporting Information*

ABSTRACT: In this study with aflatoxin-contaminated peanuts, the effectiveness of electrolyzed oxidizing water (EOW) in the decontamination of aflatoxin B$_1$ was investigated. The aflatoxin B$_1$ content was markedly reduced upon treatment with EOW, particularly with neutral electrolyzed oxidizing water (NEW). The conversion product of EOW treatment was isolated and identified as 8-chloro-9-hydroxy aflatoxin B$_1$ (compound **1**), which is an amphiphilic molecule, in contrast to fat-soluble aflatoxin B$_1$. A mutagenic response study revealed that the number of revertants per plate after treatment of bacterial strains TA-97, TA-98, TA-100, and TA-102 with NEW was within the standard value range. The HepG2 cell viability assay yielded an IC$_{50}$ value of compound **1** approximately 150 mM. This study indicates that EOW had the ability to decontaminate aflatoxin B$_1$, and the conversion product, compound **1**, did not exhibit mutagenic activity or cytotoxic effects.

KEYWORDS: *aflatoxin B$_1$, electrolyzed oxidizing water (EOW), decontamination, 8-chloro-9-hydroxy-aflatoxin B$_1$, mutagenic and cytotoxic responses*

INTRODUCTION

Aflatoxin B$_1$ is the most potent natural hepatocarcinogen, is produced primarily by *Aspergillus flavus* and *Aspergillus parasiticus*, and has been designated as a human liver carcinogen (group I) by the International Agency for Research on Cancer.[1] Aflatoxin B$_1$ has been reported in many food and food materials such as corn, wheat, peanuts, figs, spices, olives, pistachios, and rice.[2−7] It is considered to be an unavoidable source of food contamination by the U.S. Food and Drug Administration (FDA).[8] Controlling the infection by *A. flavus* and *A. parasiticus* and decontaminating food-borne aflatoxins are significant concerns worldwide.[9]

Many current physical and chemical methods to control aflatoxin B$_1$ contamination focus on disinfecting fungi and decontaminating aflatoxin B$_1$, such as exposure of aflatoxin B$_1$ to ammonia vapor at high temperature; treatment of aflatoxin B$_1$ with ozone, sodium bisulphite, calcium hydroxide, and hydrogen peroxide; and the adsorption of aflatoxin B$_1$ with sorbents.[10−14] Most of the proposed methods are not practical because they not only decompose aflatoxin B$_1$ but also deplete the quality of the food. Furthermore, the chemicals employed have carcinogenic and teratogenic attributes as well as residual toxicity.[15−17] For these reasons, consumers tend to be suspicious of chemical additives, necessitating the development of safer and more socially acceptable measures to decontaminate aflatoxin B$_1$.

Previously, we reported that electrolyzed oxidizing water (EOW) exhibited strong antifungal activity against *A. flavus* and a significant reduction of *A. flavus* infection in peanuts. EOW could represent a novel control method that is superior to some physical methods and synthetic chemical fungicides.[18] With the aim of identifying a secure, effective, and energy-saving method to disinfect aflatoxin-producing fungi and decontaminate aflatoxin, we evaluated the effectiveness of EOW in the decontamination of aflatoxin B$_1$.

The two major types of EOW are neutral electrolyzed oxidizing water [NEW, pH 5.0−6.5; high oxidation−reduction potential (ORP), 800−900 mV, high dissolved oxygen and containing available chlorine] and acidic electrolyzed oxidizing water (AcidEW, pH <3.0; high ORP, >1000 mV, high dissolved oxygen and containing available chlorine, which possess specific properties).[19−21] The objectives of this work were to evaluate the ability of EOW to decontaminate aflatoxin B$_1$, isolate and identify the structure of the molecule produced by EOW treatment, and evaluate the mutagenic and cytotoxic properties of the conversion product.

MATERIALS AND METHODS

Chemicals and Safety Precautions. Aflatoxin B$_1$ were obtained from Israel Fermentek Ltd. (Yatziv, Jerusalem, Israel). Acetonitrile and methanol were chromatographic grade (Dikma Technologies Inc., Ontario, Canada). Trifluoroacetic acid (Sigma-Aldrich Co. Ltd., MO) and sodium chloride (Sigma-Aldrich Co. Ltd.) were chromatographic grade. Water, resistance 18.2 MΩ cm, used in the preparation of standard solutions and samples, was obtained from a Millipore Milli-Q-System (Millipore Corp., Billerica, MA). Other chemicals were analytic grade obtained from chemical reagent services.

Handling and decontamination of aflatoxin B$_1$ were performed by mixing with 5% NaClO solutions. Contaminated glassware, vials, tubes, etc. were immersed in the 5% NaClO solutions for 1−2 h and then washed. As a safety precaution, pure aflatoxin B$_1$ reagents were handled in a glovebox or thoroughly controlled safety cabinet in a P2 level facility.

Peanut and Toxigenic Fungus. Peanuts named CA108 of uniform size (0.97 ± 0.20 g) were wound- and rot-free, obtained from the Chinese Academy of Agricultural Sciences (CAAS, Beijing, China),

and kept at 4 °C. The oil content of peanut was 55%, the protein content was 25%, and the oleic acid/linolic acid value was 1.1.

The toxigenic *A. flavus* strain was provided by the Oil Crops Research Institute (OCRI) of CAAS. The fungus was identified by the Microbial Identification System according to standard morphological methods.[22] Fungal cultures were maintained in B. R. Grade Czapek medium (Shuang xuan Microorganism Culture Medium Product Factory, Beijing, China) at 4 °C for further experiments. For fungi activation, spores were taken from slant, transferred to B. R. Grade Rose Bengal medium (Aoboxing Biotech Co. Ltd., Beijing, China), and then incubated for 2−3 days at 30 °C.

Fungal Inoculation. The fungus spores were removed from the Petri dishes with a spatula. A sterile water spore suspension was prepared with approximately 1×10^5 cfu/L conidia, and this suspension was used to elevate the moisture content of the peanut.

The moisture content of the peanut was adjusted to 18%. The peanut was stored in plastic bottles (500 g of peanut per replicate). Bottles were covered with thin polyethylene film (Ultra Lab Co. Ltd., Col Roma, Mexico). Bottles were incubated at 27 °C for 12 and 14 day periods. After the incubation periods, the peanut was put under a 1000 mg/L ethylene oxide gas atmosphere for 5 h, to stop further development of the toxigenic fungus and to avoid the dispersal of viable spores. Finally, the aflatoxin-contaminated peanut was dried to 12.5% moisture capacity (MC).

Preparation of EOW. EOW primarily composed of NEW and AcidEW was prepared by two different generators.[18] NEW was prepared using a NEW generator (OSG Company Ltd., Aichi, Japan), and AcidEW was prepared using an AcidEW generator (Sai Ai Environmental Protection and Technology Development Company Ltd., Guangzhou, China). AcidEW and NEW were obtained from the electrolysis of 8.2 mM NaCl and 8.2 mM HCl for 15 min, respectively. Both were used immediately after production.

Five physicochemical parameters of NEW and AcidEW were verified and compared with those of distilled water (DW), tap water (TW), and alkaline electrolyzed oxidizing water (AlkEW). In the assay, the stock NEW and AcidEW were diluted in ambient temperature (23 ± 3 °C) DW to obtain different chlorine concentrations.

Decontamination of Aflatoxin B$_1$-Contaminated Peanuts with EOW. Contaminated peanuts (10 g; particle size, 2−3 mm; nearly 20−30 kernels) were weighed into a 300 mL short-neck flask, and 100 mL of NEW and AcidEW was added to the flask, respectively. The flask was then capped and agitated (150 rpm) with an agitator (Dong Lian Electronic and Technology Development Co. Ltd., Harbin, China) at 40 ± 1 °C for 15 min. In other experiments, the agitation times (0, 5, 10, 20, and 30 min) and concentration of available chlorine (0, 20, 60, 80, and 100 mg/L) were varied by dilution in DW. Reactions were terminated by the addition of 50 mL of freshly prepared, filter-sterilized, pH 7.4, neutral buffer (0.34 M NaH$_2$PO$_4$·H$_2$O and 0.47 M Na$_2$HPO$_4$·12H$_2$O) and drying.[23]

Contaminated peanuts extracts were prepared according to method AOAC 991.31[24] and analyzed by HPLC. The percentage of remaining aflatoxin B$_1$ was calculated as follows:

$$\text{remaining aflatoxin B}_1 \, (\%)$$
$$= \text{remaining aflatoxin B}_1 \text{ amount}/\text{original aflatoxin B}_1 \text{ amount} \times 100\% \quad (1)$$

HPLC Conditions for Determination of Aflatoxin B$_1$ in the Sample Extract by Chemical Derivatization. The contaminated peanuts was prepared and extracted according to the AOAC 991.31 method.[24] For contaminated peanuts without any treatment (control), 20 μL of each preprepared extracted solution was subjected to HPLC. Aflatoxin B$_1$ was identified with Shimadzu HPLC equipment. For the analysis sample prepared, extracting solution was evaporated off under N$_2$, followed by trifluoroacetylation with the same volume of trifluoroacetic acid in a tightly sealed glass vial by vigorous shaking and then incubated for 15 min at room temperature (25 °C) in the dark. It was evaporated off under N$_2$ again, and then, 200 μL of a 1:9 (v/v) mixture of acetone/water was added. The solution was further mixed vigorously, and then, 20 μL of the solution was subjected to HPLC.

Aflatoxin B$_1$ identification was determined by means of Shimadzu HPLC equipment acetic with LC-10ATVP model two pumps (Shimadzu Co., Japan). The column used was 250 mm × 4.6 mm i.d., 5 μm, Venusil MP RP-18 (Shimadzu Co., Japan) with CTO-10ATVP Plus model oven temperature (Shimadzu Co., Japan). Standards as well as samples were injected into a high-performance liquid chromatography (HPLC) and eluted isocratically with a mobile phase of pure water: acetonitrile (65:35, v/v) at a flow rate of 0.8 mL/min at 35 °C. Aflatoxin B$_1$ was fluorometrically detected and identified using a fluorescence detector Waters 474; the excitation and emission wavelengths were 365 and 412 nm, respectively. Aflatoxin B$_1$ was identified by its retention time (Rt), as compared with those for a pure aflatoxin B$_1$ standard solution under identical conditions. Chromatographic analysis was run three times.

Effect of the Form of Available Chlorine in EOW on Aflatoxin B$_1$ Decontamination. A 100 mL NaOCl solution with pH values ranging from 2 to 10 in intervals of 2 was added to flasks containing contaminated peanuts treated for 15 min. Contaminated peanut extracts were prepared and analyzed by HPLC using chemical derivatization described in "HPLC Conditions for Determination of Aflatoxin B1 in the Sample Extract by Chemical Derivatization".

HPLC Conditions for Purification and Determination of the Conversion of Aflatoxin B$_1$ Treated with EOW without Chemical Derivatization. The treatments of aflatoxin B$_1$-contaminated peanut by EOW were analyzed on the HPLC without any chemical derivatization. For the analysis sample prepared, the extracting solution was evaporated off under N$_2$, and then, 200 μL of a 1:9 (v/v) mixture of acetone/water was added. The solution was further mixed vigorously, and then, 20 μL of the solution was subjected to HPLC. Gradient elution was used at a flow rate of 0.4 mL/min with 30% acetonitrile in water from 0 to 30 min, followed by a linear increase in acetonitrile from 30 to 35% between 30 and 45 min. From 45 to 60 min, the acetonitrile increase was linear from 35 to 100%. Eluants were monitored fluorometrically using an excitation wavelength at 365 nm and an emission wavelength at 450 nm. The chromatographic analysis was performed three times for each extract.

Analysis of the Structure of Conversion Products. The molecular formula of the conversion products was analyzed with a Fourier transform ion cyclotron resonance mass spectrometer (FT-ICR-MS) (Bruker Co. Ltd., United States). ^{1}H NMR spectroscopy of the conversion products and aflatoxin B$_1$ was performed with a Bruker Avance-400 equipped with an SGI INDY computer workstation with the program TOPSPIN 2.0 (Bruker) to acquire and process the NMR data. The stock solutions of converted produces were diluted with H$_2$O:MeOH:acetic acid = 49:49:2 (v/v/v), giving a final concentration of converted produces of 10 μM and 20% MeOH, and directly injected into the source region at the rate of 4 μL/min. All ESI mass spectra in positive-ion mode were acquired using a Fourier transform ion cyclotron resonance mass spectrometer (FT-ICR-MS) (Bruker Co. Ltd.). The ^{1}H NMR spectroscopy of conversion produced and the aflatoxin B$_1$ were measured at 25 °C on sealed samples, using a Bruker Arance-400 equipped with an SGI INDY computer workstation using TOPSPIN 2.0 program (Bruker), to acquire and process the NMR data.

Mutagenesis Assays *Salmonella typhimurium* **Tester Strain.** TA-97, TA-98, TA-100, and TA-102 were obtained from Dr. B. N. Ames (University of California, Berkeley, CA).[25] For the micro-suspension procedure, bacteria were grown overnight in an Erlenmeyer flask to approximately $1 \times 10^9 - 2 \times 10^9$ cells/mL. The cells were collected by centrifugation (4500g, 10 min, 4 °C) and resuspended in ice-cold phosphate-buffered saline (0.2 M PBS, pH 7.4). Rat-liver mix S9 (metabolic enzymes) was obtained from the Beijing Centres for Disease Control and Prevention Centre (Beijing, China) and contained 40 mg/mL protein. The concentration of S9 in the mix was 300 g/mL.

For the microsuspension assay, ingredients were added in the following order to 12 mm × 75 mm sterile glass culture tubes on ice: 0.5 mL of S9 mix, 0.1 mL of concentrated bacteria in PBS, and 0.02 mL of aflatoxin B$_1$ (5, 10, 50, and 100 ng/tube) or 0.02 mL of EOW-treated aflatoxin B$_1$ extract. The mixture was incubated in the
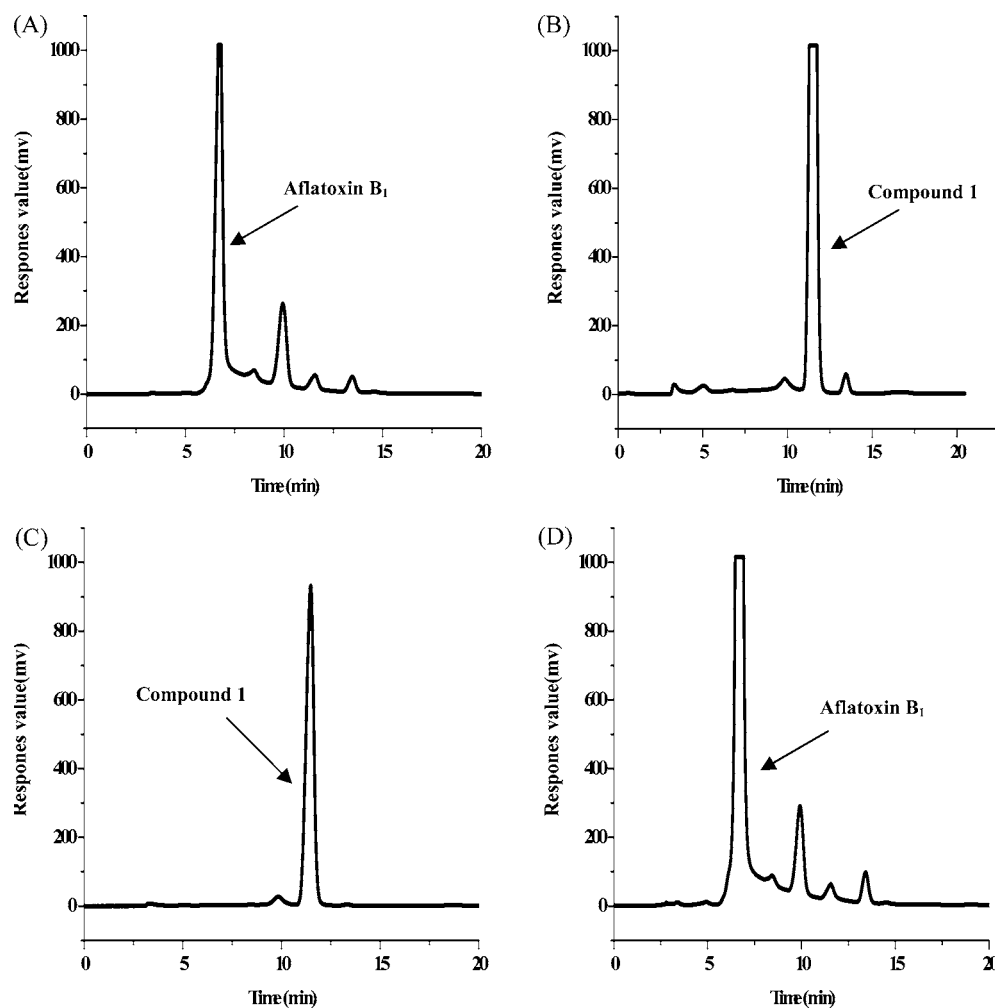
Figure 1. HPLC analysis of the changes in aflatoxin B_1 fluorescence peak height after exposure to the treatment solution. (A) Control, (B) aflatoxin B_1 exposed to NEW, (C) aflatoxin B_1 exposed to AcidEW, and (D) aflatoxin B_1 exposed to AlkEW.

dark at 37 °C with rapid shaking (1500 rpm). After 20 min, the tubes were placed in an ice bath. Tubes were removed one at a time, and 2 mL of molten top agar containing 0.5 mM histidine and biotin was added. The combined solutions were vortex-mixed and poured onto minimal glucose plates. Plates were incubated at 37 °C in the dark for 48 h, after which the number of revertant colonies was counted. Strain markers and bacterial survival were routinely monitored for each experiment. Each treatment was tested in triplicate for each independent experiment.

In Vitro Cytotoxicity Examination. The cytotoxicity was measured with the MTT assay. [26] Liver cancer cells (HepG2) in the exponential growth phase were cultured at a density of 1×10^4 cells/well in a 96-well plate. After treatment with various concentrations of aflatoxin B_1 or compound **1** (conversion product formed after aflatoxin B_1 exposure to EOW) for 48 h, MTT solution (5.0 mg/mL in phosphate-buffered saline) was added (20.0 μL/well), and the plates were incubated for another 4 h at 37 °C. Purple formazan formation in the plates was read on a microplate reader (Bio-Tek Co. Ltd., United States) at 570 nm. Cells without aflatoxin B_1 or compound **1** were used as the control. Assays were performed in three independent experiments. The percentage of cytotoxicity was calculated as follows:

$$\text{cell viability (\%)} = (\text{OD of experimental well/OD of control well})$$
$$\times 100\% \qquad (2)$$

The cytotoxicity was assessed by plotting cell viability versus compound concentration (on a $\log_{10}$ X-scale), followed by polynomial curve fitting and determination of the IC_{50} with the Origin 7.5 software package.

Statistical Analyses. Analysis of variance (ANOVA) and Duncan's multiple range tests (at $P \leq 0.05$) were performed to analyze statistical differences and discriminate between means. Values are shown as the means of two different tests with triplicate treatments per experiment.

RESULTS AND DISCUSSION

Physicochemical Parameters of EOW. The physico-chemical properties of NEW, AcidEW, AlkEW, TW, and DW, mainly including pH, ORP, dissolved oxygen (DO), electrical conductivity (EC), and available chlorine concentration (ACC). NEW was subacidic (pH 5.6 ± 0.5), and AcidEW was strongly acidic (pH 2.5 ± 0.1). By contrast, AlkEW was strongly alkaline (pH 11.6 ± 0.2), had a low level of DO, and had an extremely low ORP. Available chlorine was almost negligible in AlkEW. In other words, NEW and AcidEW are highly oxidative, whereas AlkEW is highly reductive. The parameters of both of these solutions were also distinctly different from those of TW and DW. However, the values of these parameters, in particular the available chlorine, which functions as an oxidant, were dependent on the concentration of electrolyte, the electrolytic efficiency, and the length of the electrolysis time.

HPLC Assessment of the Decontamination of Aflatoxin B_1 in the Sample Extracts. Figure 1 shows that the trifluoroacetylated aflatoxin B_1 peak with derivatization changes after exposure to the treatment solution. DW treatment of aflatoxin B_1-contaminated peanuts was used as a control.

The peak height of trifluoroacetylated aflatoxin B_1, which eluted at 6.9 min (Figure 1A), changed with different treatment solutions. Although the original concentrations of aflatoxin B_1 in the serial treatments are consistent with the control, the results show that there were significant discrepancies after exposure to different treatment solutions. AcidEW (Figure 1B) and NEW (Figure 1C) completely abolished the peak for trifluoroacetylated aflatoxin B_1. Although the peaks eluting at 9.8 and 11.5 min have not yet been assigned, the peak at 11.5 min (compound **1**) is likely to be the main conversion product formed after aflatoxin B_1 exposure to NEW and AcidEW. However, AlkEW has no effect on aflatoxin B_1 (Figure 1D).

Treatment of Aflatoxin B_1-Contaminated Peanuts with EOW. HPLC analysis confirmed that the toxin in the contaminated peanuts was aflatoxin B_1, at a concentration of 95.9 ± 2.9 μg/kg. This aflatoxin B_1 concentration (approximately 100 μg/kg) is similar to the potential average concentration in commercial peanuts destined for consumption.[27−30]

AcidEW and NEW containing various ACC exhibited different efficiencies with respect to aflatoxin B_1 decontamination in 15 min (Figure 2A). As the ACC level increased, the remaining

aflatoxin B_1 percentage in peanuts treated with AcidEW and NEW decreased significantly; when the ACC reached 60 mg/L in NEW and 80 mg/L in AcidEW, the remaining aflatoxin B_1 percentage was less than 10%. After treatment of aflatoxin B_1-contaminated peanuts for a variety of exposure times (0, 5, 10, 15, 20, 25, and 30 min), there were differences in the ability of AcidEW and NEW to decontaminate aflatoxin B_1-contaminated peanuts (Figure 2B). As the exposure time increased, the remaining aflatoxin B_1 percentage decreased significantly; when the exposure time reached 10 min for NEW and 15 min for AcidEW, the remaining aflatoxin B_1 percentage was approximately 10%. These results indicated that AcidEW and NEW clearly react with aflatoxin B_1 in contaminated peanuts, and both types of EOW can decontaminate aflatoxin B_1.

In an additional experiment, 50 mL of NaOCl solution in a series of pH values between 2 and 10 in intervals of 2 pH units was prepared to identify differences in AcidEW- and NEW-mediated decontamination (Figure 3A). As the amount of aflatoxin B_1 decreased, the amount of compound **1** increased simultaneously. Compound **1** was the main conversion product produced by aflatoxin B_1 treated with EOW. When the pH reached 4, the compound **1** content reached its maximum, while the remaining aflatoxin B_1 content reached its minimum. The form of ACC present at the various pH values was an important factor in the conversion of aflatoxin B_1 to compound **1**. The form of ACC present at the different pH values is shown in Figure 3B. As the pH changes, ionic chlorine undergoes the following reactions:

$$2Cl^- \xrightarrow{+2e} Cl_2 \tag{3}$$

$$Cl_2 + H_2O \rightarrow HOCl + H^+ + Cl^- \tag{4}$$

$$HOCl \rightarrow H^+ + OCl^- \tag{5}$$
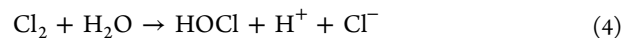
Between pH 10 and pH 8, the ClO^- ion (reaction 4) was the main form of available chlorine.[31] The available chlorine usually exists in the form of ClO^-, which is found in the NaClO dilute solution, confirming the conclusion. As the pH decreases, reaction 4 reverses. The available chlorine compounds derived from the ionic forms of ClO^- are converted to the HOCl. At approximately pH 4, the percentage of HOCl reaches its maximum. The pH values of NEW ranged from 5 to 6.5. Therefore, HOCl was the main form of ACC. As the pH decreases further, the primary form of ACC was partially converted to Cl_2 gas (reactions 2 and 3). The pH values of AcidEW ranged from 2 to 3. Therefore, the available chlorine was HOCl and Cl_2 gas.

Efforts were made in several countries to find an economically acceptable way of decontamination of aflatoxin B_1 into nontoxic products using different chemicals, such as ammonia, sodium hydroxide, and calcium hydroxide, etc., used particularly for decontamination of aflatoxin.[32] Although such treatment reduces nearly completely the aflatoxin concentration, these chemicals cause losses of some nutrients.

The effect of oxidizing agents was also studied.[33,34] Such treatment is too drastic for grain destined for food uses. It should be mentioned that some indigestible adsorbents may adsorb the aflatoxin, so that they are not absorbable in the digestive tract. Such adsorbents are used in some feed supplements.[5−37] The possibility of adsorption of important micronutrient is a potential disadvantage of such methods of detoxification. Nevertheless, some adsorbents are commercially used in feed supplements. Although the different methods used at present are to some extent successful, they have big
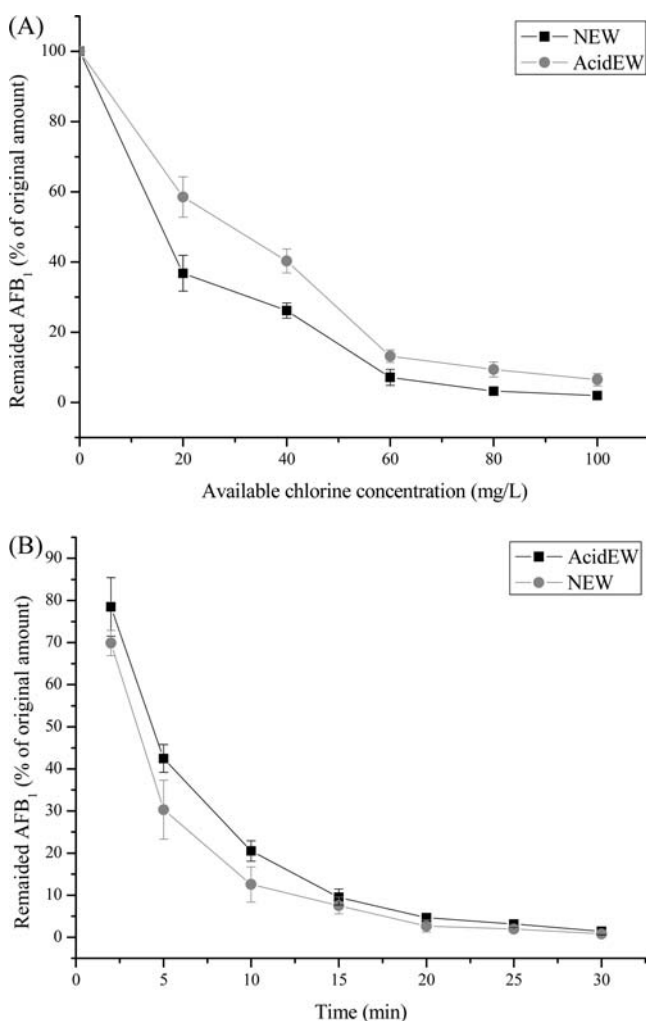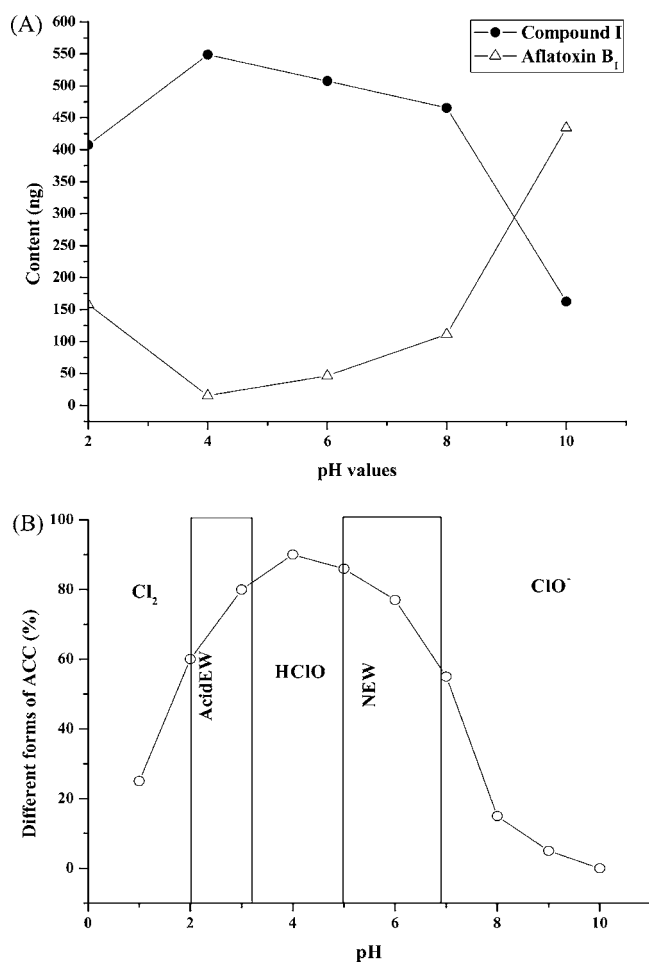
Figure 2. Decontamination of aflatoxin-contaminated peanuts by different EOW treatments. (A) Exposure to EOW of different ACC in 15 min and (B) different exposure times. The remaining aflatoxin B_1 content was determined by HPLC and plotted. Ordinate, relative amount of remaining aflatoxin B_1 (percent); abscissa, different treatments. Each plot represents the mean of three measurements.

Figure 3. Effect of the available chlorine form in EOW on aflatoxin B₁ decontamination. (A) Change in the aflatoxin B₁ and compound **1** contents at different pH values in 15 min and (B) the form of ACC present at different pH values.

disadvantages with limited efficacy and possible losses of important nutrients and normally with high costs.

AcidEW has limited potential for long-term applications due to its strong acidity and Cl_2 gas content. Dissolved chlorine (Cl_2) can rapidly escape due to volatilization, decreasing the concentration of available chlorine and reducing the effective-

ness of the solution over time. Len et al.[38] have indicated that AcidEW can create a human health and safety issue and that the strong acidity of AcidEW may adversely affect equipment and surfaces by causing corrosion and may also be phytotoxic to plants. At pH 5−6.5, the main form of ACC in NEW was HOCl. NEW will be gradually restored to common water, thus making it a safer and more socially acceptable measure for decontaminating aflatoxin B₁.

Structure of the Main Conversion Product. The conversion product in the sample extract was isolated and identified by liquid chromatography−mass spectrometry (LC-MS) in an experiment without chemical derivatization. The chemical formula of compound **1** was identified by high-resolution Fourier transform ion cyclotron resonance mass spectrometer (HR-FT-ICR-MS) shown in Figure 4. In the positive mode, an $[M + Na]^+$ ion peak at m/z 387 (relative intensity, 5.6×10^6) was obtained. HR-FT-ICR-MS gave a $[M + Na]^+$ ion peak at m/z 387.02 (calcd, 387.02), consistent with a molecular formula of $C_{17}H_{13}NaClO_7$. These data demonstrated that HOCl (the main ACC in EOW) underwent addition to aflatoxin B₁ ($C_{17}H_{12}O_6$). The sensitivity for compound **1** was very low in the negative mode. The difference in sensitivity between the positive and the negative modes indicated that compound **1** is positively charged. The chemical formula of compound **1** was $C_{17}H_{13}ClO_7$. According to the identified molecular formula, the structure of the conversion product confirms that EOW eliminates aflatoxin B₁ not by destroying the fundamental ring system of aflatoxin B₁ but rather by modifying its structure.

Comparing the ¹H spectroscopic data of compound **1** and aflatoxin B₁ further indicated that most of the ¹H signals of compound **1** were similar to those of aflatoxin B₁, except for atoms 8 and 9 (Table 1 and Figure 5). The chemical shifts of H (δ_H) in C_8−H and C_9−H of structure **1** were 6.49 and 5.51, respectively, while the chemical shifts of H (δ_H) in C_8−H and C_9−H of structure **2** were 5.78 and 4.71, respectively. There were significant changes in the H (δ_H) chemical shifts and ¹H peak style of C_8−H and C_9−H between structure **1** and structure **2**, indicating that the formation of the conversion product affected the C_8−H and C_9−H double-linked carbon structure **1**. We noticed that the H (δ_H) chemical shifts of structure **1** and structure **2** in C_{18}−H were 6.81 and 6.80 due to two oxygen atoms added to same carbon atom. If the −OH and −Cl groups, respectively, added to C-8 and C-9, the H (δ_H) chemical shift of structure **2** in C_8−H should also be similar
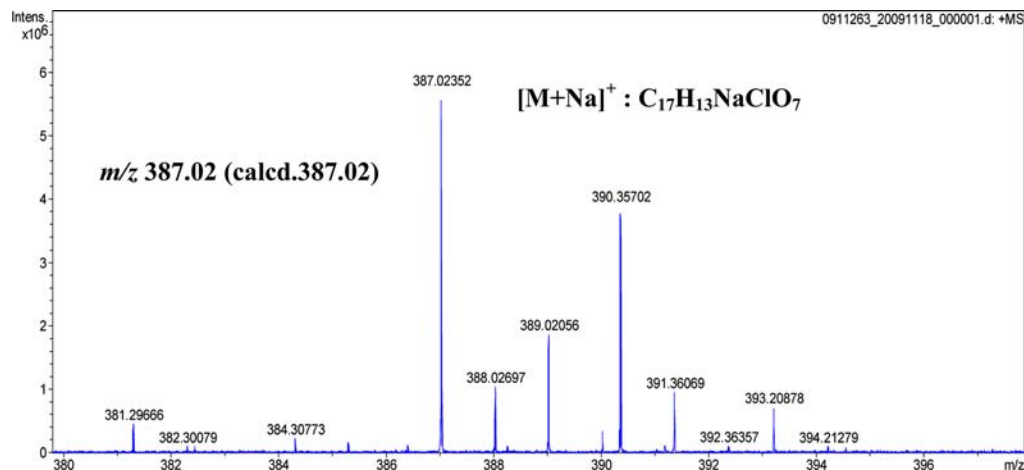

Figure 4. HR-FT-ICR-MS to analyze the structure of compound **1**.

Table 1. ^{1}H NMR Data for Aflatoxin B$_1$ and Compound 1 (400 MHz)^a

atom no.	the chemical shift of H (δ_H)	
	aflatoxin B$_1$^{b}	compound 1^c
2	3.40, m	3.52, m
3	2.67 (t, J = 5.4 Hz)	2.71 (t, J = 5.7 Hz)
5	6.45, s	6.64, s
8	6.49 (dd, J = 2.7, 2.2 Hz)	5.78, s
9	5.51 (dd, J = 2.7, 2.2 Hz)	4.71, s
18	6.81 (d, J = 7.1 Hz)	6.80 (d, J = 6.1 Hz)
19	4.77 (ddd, J = 7.1, 2.7, 2.2 Hz)	4.5 (d, J = 6.1 Hz)
23/25	3.95, s	4.01, s

^{a}The atom no. in structures of aflatoxin B$_1$ and compound 1 according to Figure 5. Key: m, multiple peaks; ddd or t, triple peaks; dd, double peaks; s, single peak; and J, coupling constant. ^{b}Solvent CDCl$_3$. ^{c}Solvent CD$_3$COCD$_3$.

with the H (δ_H) chemical shift of structure **1** and structure **2** in C$_{18}$−H due to two oxygen atom added to same carbon atom. However, the other H (δ_H) chemical shift of structure **2** in C$_8$−H was significantly different from the H (δ_H) chemical shift in C$_{18}$−H, confirming that the −OH group was not bonded to the C-8 atom but to the C-9 atom. Thus, the −Cl and −OH groups were added to C-8 and C-9, respectively. The results indicated that the conversion product structure **2** is 8-chloro-9-hydroxy-aflatoxin B$_1$ (compound **1**). The formation of chlorohydrins in compounds containing double bonds upon addition of hypochlorous acid is well-known from organic chemistry.[39] Winterbourn et al.[40,41] also showed the formation of chlorohydrins in biological systems.

With respect to the toxicity, carcinogenicity, and biological metabolism of aflatoxin B$_1$, recent studies strongly support the conversion of aflatoxin B$_1$, a potent hepatocarcinogenic mycotoxin, to a highly reactive ultimate carcinogen, aflatoxin B$_1$-8,9-oxide, in rat liver.[42,43] The most conclusive evidence supports the formation of an RNA-aflatoxin adduct by microsomal oxidation of aflatoxin B$_1$ in the presence of RNA. Acid hydrolysis of the DNA- and RNA-bound derivatives of aflatoxin B$_1$ formed in vivo in the rat liver released a major portion of the bound aflatoxin B$_1$ as the dihydrodiol. The hepatocarcinogenicity of aflatoxin B$_1$ is greatly reduced when the 8,9-double bond is hydrogenated to yield AFB$_2$. Aflatoxin B$_1$ has the strongest toxicity and carcinogenicity, followed by G$_1$, with B$_2$ and G$_2$ being weaker. Aflatoxin B1 was also the main constituent, followed by B$_2$, G$_1$, and G$_2$. In our research, we found that the EOW was likely to attack the double bond between C$_8$ and C$_9$. Aflatoxins B$_1$ and G$_1$ all contain the C-8

and C-9 double bond. We hypothesized that the −OH and −Cl groups were added to C-8 and C-9 of aflatoxin G$_1$, respectively.

Aflatoxin B$_1$ 8,9-epoxide is a strong candidate as the ultimate reactive and carcinogenic metabolite of aflatoxin B$_1$. Goeger and Hsie[44] reported that the mutagenicity of AFB$_2$ was 500 times less than that of aflatoxin B$_1$, due to the lack of a double bond between C$_8$ and C$_9$. For the same reason, the toxicity of aflatoxin G$_2$ was also less than that of aflatoxin G$_1$. The toxicity of compound **1** was partially confirmed by the mutagenicity assay. Therefore, it is key to identify the stereostructure of compound **1** produced by EOW treatment of aflatoxin B$_1$ and its physical and chemical properties. It is also important to evaluate the structural conversion to aflatoxin B$_1$ that leads to the change in its toxicity, mutagenicity, and carcinogenicity and whether compound **1** can form the highly reactive ultimate carcinogen, aflatoxin B$_1$-8,9-epoxide, via biological metabolism.

Physicochemical Properties of Compound 1. The results indicated that the conversion product compound **1** is more stable than the conversion product 8-hydroxy-9-chloro-aflatoxin B$_1$. The lipid/water partition coefficient (Log P) of compound **1** is −0.06 ± 0.52. These physicochemical characteristics also suggest that the molecular structure is amphiphilic, in contrast to fat-soluble aflatoxin B$_1$. The loss of the double bond in the bisfuran ring of aflatoxin B$_1$ and the amphiphilic nature of compound **1** may not readily be transmitted across biomembranes.

In our studies, NEW and AcidEW eliminated the contamination of aflatoxin B$_1$ on food materials such as peanuts and corn, among others. However, the EOW-mediated decontamination of aflatoxin B$_1$-contaminated materials may require more time than the elimination of pure aflatoxin B$_1$, particularly in food materials with surface wrinkles and damage. Oomori et al.[45] documented that the effect of EOW could be reduced by the presence of organic materials, including proteins and amino acids. The active components of EOW (e.g., available chlorine, etc.) may react with organic materials and reduce the level of decontamination.[46] Treatment of the whole peanut may be less effective than that of the peanut kernel because toxins deposited inside whole kernels are less likely to be exposed to EOW treatment than toxins in small peanut particles, although this possibility remains under investigation.

Mutagenic Response of the _S. typhimurium_ Test Strains TA-97, TA-98, TA-100, and TA-102 to Aflatoxin B$_1$ Treated with EOW. In the mutagenic response study, the observed numbers of natural revertants/plate of the bacterial strains TA-97, TA-98, TA-100, and TA-102 were approximately 127 ± 12, 35 ± 4, 135 ± 22, and 242 ± 18, respectively (DMSO, negative control). The response is considered mutagenic when an agent or a sample doubles the number of
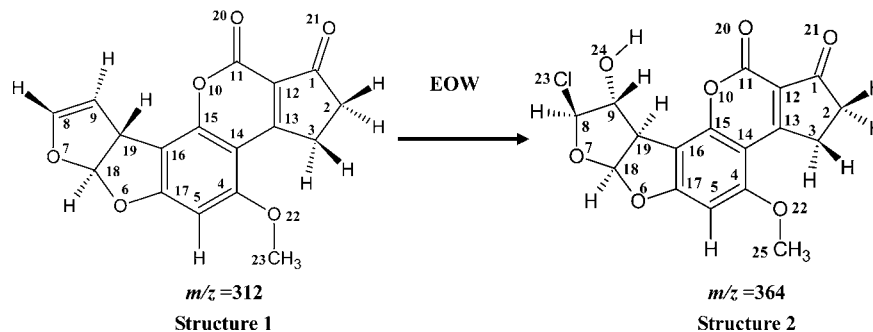

Figure 5. Structures of **1** and **2** formed with EOW.

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Table 2. Mutagenic Response of *S. typhimurium* Test Strains TA-97, TA-98, TA-100, and TA-102 to Aflatoxin B$_1$ Treated with EOW

treatments	no. of revertants plate^{-1} mean $\pm$ standard deviation			
	TA-97	TA-98	TA-100	TA-102
negative control[a]	127 $\pm$ 12 d	35 $\pm$ 4 d	135 $\pm$ 22 d	242 $\pm$ 18 e
positive control[b]	1332 $\pm$ 187 b	104 $\pm$ 11 c	408 $\pm$ 11 c	448 $\pm$ 23 c
positive control[c]	2248 $\pm$ 147 a	496 $\pm$ 23 a	828 $\pm$ 79 a	832 $\pm$ 45 a
untreated extract[d]	936 $\pm$ 127 c	330 $\pm$ 42 b	504 $\pm$ 45 b	756 $\pm$ 85 b
AcidEW-treated extract[e]	300 $\pm$ 56 d	96 $\pm$ 13 c	162 $\pm$ 13 d	352 $\pm$ 15 d
NEW-treated extract[f]	166 $\pm$ 23 d	54 $\pm$ 3 d	100 $\pm$ 11 d	321 $\pm$ 23 d

[a]DMSO. [b]Aflatoxin B$_1$ at 50 ng/tube. [c]Aflatoxin B$_1$ at 100 ng/tube. [d]Treated with TW. [e]Treated with AcidEW at ACC 1800 ng/tube. [f]Treated with NEW at ACC 1800 ng/tube. Means with same letter are not significantly different ($p < 0.05$).
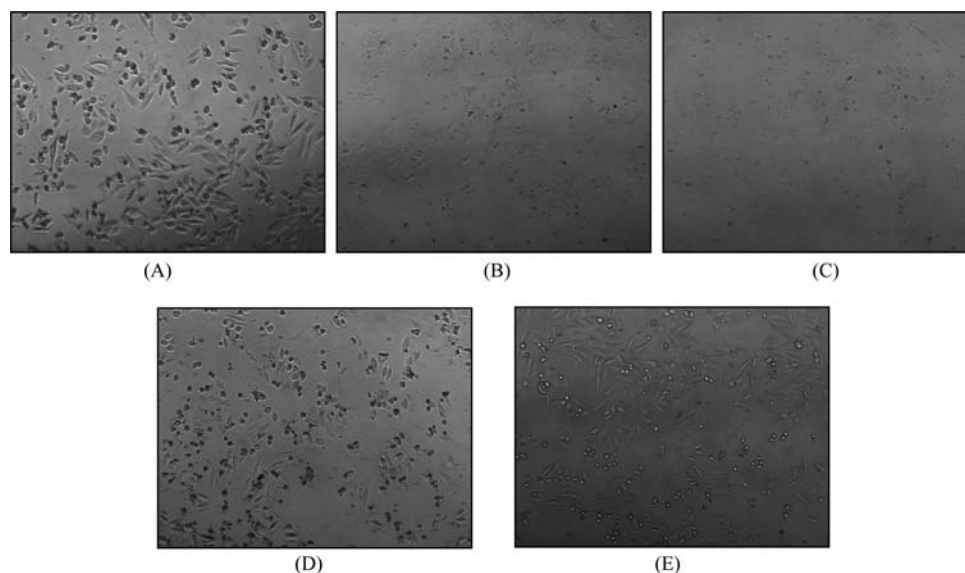
Figure 6. Effect of different concentrations of aflatoxin B$_1$ and compound **1** on cell morphology. (A) Control, (B) aflatoxin B$_1$ (0.120 μM), (C) aflatoxin B$_1$ (1.20 μM), (D) compound **1** (0.120 μM), and (E) compound **1** (1.20 μM).

spontaneous or natural revertants/plate.[47] For these test strains treated with NEW, the ranges are 90−180, 30−50, 100−200, and 240−320 revertants/plate, respectively. It is clear that the spontaneous reversion values fall within the range of standard values. The mutagenicity assay demonstrated that the conversion product from peanuts treated with NEW did not exhibit mutagenic activity as compared with untreated aflatoxin B$_1$-contaminated peanut (Table 2).

For AcidEW, the bacterial strain reversion values of TA-97 and TA-98 were slightly higher than the standard values, while the reversion values of the other strains fell within the standard value range. AcidEW contains available chlorine main in the form of HOCl and Cl$_2$ gas. Dissolved chlorine (Cl$_2$) can induce some mutagenicity in the test strains. The NEW- and AcidEW-treated samples, which can reduce aflatoxin B$_1$ fluorescence almost completely, did not produce any more revertants than did the negative control (DMSO). These results showed that none of the decontaminated peanuts exhibited signs of mutagenicity against the *Salmonella* tester strains.

However, untreated aflatoxin B$_1$-contaminated samples (100 ng/tube approximately) exhibited mutagenic activity in the presence of rat liver S9 mix, as did the positive control at 50 and 100 ng/tube. With respect to the mutagenic response of the *S. typhimurium* tester strains TA-97, TA-98, TA-100, and TA-102 to aflatoxin B$_1$ treated with EOW, the possible effect of EOW treatment on the nutritional and organoleptic quality of

the peanut should be evaluated in future experiments. The mutagenic and toxicity response of compound **1** indicated that it has minor potential to induce a new safety risk from the contaminated peanut when aflatoxin B$_1$ is decontaminated with EOW.

Cytotoxic Effects of Compound 1 on Cell Growth, Viability, and Morphology of the HepG2 Cell Line. After the incubation (12−24 h) of cell suspensions with medium in the presence of different compound **1** and aflatoxin B$_1$ concentrations (0.006−1.20 μM) at 5% CO$_2$ and 37 °C, viability was assessed by the MTT assay at 570 nm. The data with the same concentrations of aflatoxin B$_1$ (0.006−1.20 μM) were compared. The polynomial dose−response curves of the tested compounds are programmed. The plot of cell viability (Y-scale) versus compound concentration (on a log$_{10}$ X-scale) was fit by a polynomial curve, and the IC$_{50}$ was determined. All curves have an R^2 greater than or equal to 0.95. Compound **1** was found to be less cytotoxic than aflatoxin B$_1$ after 48 h of exposure, even at the maximum compound **1** concentration of 1.20 μM. None of the test concentrations (0.006−1.20 μM) had any effects on the cell line when compared with the controls. The viability is 90−100% at 570 nm. The examined IC$_{50}$ value of compound **1** is near 150 mM. When cells were exposed to low concentrations of aflatoxin B$_1$ (0.006 μM, the safety limit in the European Union), their viability decreased to 70−80% at 570 nm. When cells were exposed to aflatoxin B$_1$

(0.120 μM), their viability diminished to 1−2% at 570 nm. The determined IC_{50} value of aflatoxin B_1 is less than 0.015 μM. The IC_{50} value of 150 mM for compound **1** is much greater than that of aflatoxin B_1, 0.015 μM. Given its high IC_{50} value, compound **1** may be considered nontoxic.

The normal morphology of the HepG2 cell is visible in the control (Figure 6A). The cell grows adherently and has normal cell morphology, with sharp edges. Figure 6B shows HepG2 cells 48 h after the addition of a low concentration of aflatoxin B_1 as observed under an inverted microscope. As compared with the control shown in Figure 6A, the cell morphology changed markedly, including reduced cell size, loss of original morphology, a large amount of cell debris, and near death of the cell. The damage to the cell increased with high concentrations of aflatoxin B_1. There are no surviving cells upon exposure to high concentrations of aflatoxin B_1 (1.20 μM) (Figure 6C). Compound **1** had little or no effect on the morphology of cells at low or high concentrations, as shown by the presence of regular morphology, sharp edges, no cell death, limited cell debris, and little reduction of cell size (Figure 6D,E). The mutagenic and toxicity response of compound **1** indicated that it has minor potential to induce a new safety risk in aflatoxin B_1-contaminated peanuts decontaminated with EOW. Cell-based biological tests to assess the toxicity and mutagenicity of compound **1** support the feasibility of its application. However, this is an in vitro assay and hence limited in correlation with in vivo studies. Further animal toxicity studies are required to confirm the protective effect of EOW treatment against chronic aflatoxin B_1 toxicity, and this study demonstrates the prospects for using EOW to decontaminate aflatoxin B_1 in food and feed materials.

ASSOCIATED CONTENT

Ⓢ Supporting Information

Highest occupied molecular orbital and lowest free molecular orbital of compound **1** (Figure S1), dose−response curves of compound **1** and aflatoxin B_1 cytotoxic response as tested with MTT assays analyzed at 570 nm (Figure S2), and physicochemical parameters of the different types of water (Table S1). This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

ABBREVIATIONS USED

EOW, electrolyzed oxidizing waters; NEW, neutralized electrolyzed oxidizing water; AcidEW, acidic electrolyzed oxidizing water; AlkEW, alkaline electrolyzed oxidizing water; ACC, available chlorine concentration; ROS, reactive oxygen species; ORP, oxidation−reduction potential; ●OH, OH radical; DO, dissolved oxygen; EC, electrical conductivity

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