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# Adsorption of sterigmatocystin by montmorillonite and inhibition of its genotoxicity in the Nile tilapia fish (*Oreachromis nilaticus*)

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**Abstract**

Sterigmatocystin (Stg) is closely related to the mycotoxin aflatoxin as a precursor in aflatoxin biosynthesis and classified as an IARC Group-2B carcinogen. The aim of this study was to investigate the efficacy of Egyptian montmorillonite (EM), a clay miniral, to adsorb Stg, to test the stability of the resulting complex under different conditions in vitro, and to utilize the Nile tilapia fish as an in vivo model to evaluate the protective effect of EM against Stg-induced toxicity and clastogenicity. In the in vitro study, four concentrations of EM (0.5, 1, 2 and 4 mg/L aqueous solution) and three concentrations of Stg (5, 10 and 50 μg/ml) were tested. The results show that EM had a high capacity of adsorbing Stg at different concentrations tested. The adsorption ranged from 93.1 to 97.8% of the available Stg in aqueous solutions. The complex was stable at different pHs at 37 °C in different organic solvents. An in vivo experiment was conducted to evaluate the ability of EM to prevent the toxicity and chromosomal aberrations induced by Stg in the Nile tilapia fish. Fish received an intragastric dose of EM in corn oil (0.5 mg/kg bw) with or without Stg (1.6 μg/kg bw) twice a week for 4 weeks. Body weight was recorded during dosing, and blood and tissue samples were collected at the end of treatment. Stg residues were determined in fish tissue. The results show that Stg was toxic and clastogenic to fish as indicated by the significant decrease of body weight and the increase in frequencies of micronucleated red blood cells (MN RBC) and chromosomal aberrations in the kidney. The intragastric administration of EM combined with Stg to fish resulted in a reduction of the number of MN RBC and the frequency of chromosomal aberrations in the kidney compared with the group treated with Stg alone. It could be concluded that EM itself was safe and successful in the prevention of Stg toxicity and clastogenicity.


*Keywords:* Sterigmatocystin; Montmorillonite; Fish; Chromosomal aberrations; Clastogenicity

## 1. Introduction

Sterigmatocystin (Stg), a major secondary metabolite of *Aspergillus versicolor* and *Aspergillus nidulans* [1], is closely related to the mycotoxin aflatoxin and a

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precursor in aflatoxin biosynthesis [2]. However, the acute and chronic toxicities of Stg are considerably lower [3]. Stg is carcinogenic in mice (pulmonary adenocarcinomas) and rats (hepatocellular carcinomas) following oral administration [4,5] and is classified as an IARC Group-2B carcinogen (i.e., as possibly carcinogenic to humans). The toxicity of Stg is primarily confined to the liver and kidneys. However, lung tumors were also observed in newborn mice injected by a single subcutaneus dose of 5 mg/kg bw Stg [6]. Stg is one of the predominant contaminating mycotoxins in food and grains in high-incidence areas of malignant tumors in China [7].

Stich and Laishes [8] reported that the activation of the aflatoxins $B_1$, $G_1$, $B_2$, $G_2$, aflatoxicol and Stg by S9 fraction and liver microsomes of several species significantly increases the chromosome-breaking function, lethality, and the DNA-damaging effect. Most of the carcinogenic mycotoxins are genotoxic agents that produce chromosomal aberrations, micronuclei, sister chromatid exchange and chromosomal strand breaks, as well as DNA adducts in rodent and human cells [9]. Because Stg may significantly affect animal and human health, protection of food and feedstuffs from these poisons is in great demand. Several reports have indicated that the phyllosilicate clay, hydrated sodium calcium aluminosilicate (HSCAS), which is currently available as an anti-caking agent for animal feeds, may prevent disease associated with aflatoxicosis in farm animals, including chickens, turkey poults, pigs and minks. These studies have been extensively reviewed by Phillips [10]. Recent studies have shown that the addition of HSCAS, bentonite, or montmorillonite to the aflatoxin-contaminated diets can greatly reduce the bioavailability of toxins in the gastrointestinal tract [11–15] due to the high adsorptive properties of these clays. Up to 85% of toxic effects due to aflatoxins were reversed by the addition of 0.5 g clay/kg contaminated diet, and alterations in serum clinical chemistry profiles indicative of liver damage, teratogenic effects and chromosomal aberrations due to aflatoxin have been prevented [13–16].

On the basis of these earlier findings a variety of other common clay and zeolitic minerals are now being added to feeds as ‘aflatoxin binders’ without appropriate in vitro and in vivo testing. Many of these may be nonselective in their action and may pose significant hidden risks due to interaction with nutrients and other important food-borne chemicals. Research is warranted to establish a sensitive animal model that can be used to compare the efficacy and safety of potential sorbents. The Nile tilapia (*Oreochromis niloticus*) may represent such a model, since this fish is highly susceptible to nutritional deficits and is extremely vulnerable to toxic insult from various chemicals including Stg. Our major objectives in this study were to evaluate the ability of the Egyptian montmorillonite (EM) to adsorb Stg from aqueous solution in vitro, and to test the possible protective effect of EM against Stg-induced cytotoxicity in the Nile tilapia fish as a sensitive model for mycotoxicosis.

## 2. Materials and methods

### 2.1. Chemicals

Sterigmatocystin was purchased from Sigma Chemical Co. (St. Louis, OH). Egyptian montmorillonite (EM) was kindly provided by the Ceramic Department, National Research Center, Dokki, Cairo, Egypt. All reagents were of the highest purity commercially available.

### 2.2. Methods of analysis

#### 2.2.1. In vitro studies

*2.2.1.1. Ability of EM to adsorb Stg in vitro.* Samples of EM were weighed into glass tubes (three replicates per sample) and a specific amount of Stg in aqueous solution was separately added. After a reaction time of 1 h at 25 °C, with mixing at 15-min intervals, all the tubes were centrifuged for 10 min at 1500 × *g*. Three adsorption tests were carried out with varying amounts of Stg. In test 1, 30 ml of a solution containing 5, 10 or 50 μg of Stg/ml were individually prepared before the addition of 0.5, 1, 2 and 4 mg/ml of EM to each concentration of Stg.

*2.2.1.2. The effect of pH on the stability of the sorbent–Stg adsorption complex.* Duplicate samples of EM (0.5, 1, 2, 4 mg) were added to test tubes containing 15 ml of each of the following solutions: solution (1) HCl/water (pH 2); solution (2) distilled water (pH 7); solution (3) 50 mM carbonate/bicarbonate buffer (pH 10). Stg was added at a concentration of 5 μg/ml and then the tubes were incubated for 1 h at 37 °C.

*2.2.1.3. Stability of the EM–Stg complex.* Test samples (0.5 mg) of EM containing 10 μg of adsorbed Stg were added to test tubes and mixed thoroughly with 15 ml of the following solvents: hexane, cyclohexane, benzene, toluene, chloroform, ethyl acetate, 2-propanol, acetone, methanol, and acetonitrile. All solvents were tested at 25 °C. EM was pelleted by centrifugation at 1500 × *g* for 10 min and the aliquots of each supernatant were removed and evaporated to dryness. Stg was determined by HPLC.

*2.2.1.4. Determination of Stg.* Each sample was placed in a 500-ml glass-stoppered Erlenmeyer containing 180 ml $CH_3CN$ and 20 ml 4% KCl solution. The mixture was shaken for 30 min and then filtered through Whatman No. 1 paper. One hundred milliliters of the filtrate was added to 50 ml hexane in a 250-ml separator funnel and shaken. The upper (hexane) layer was discarded. This step was repeated with 50 ml hexane, which was discarded. Twenty-five milliliters $H_2O$ and 50 ml $CHCl_3$ were added to $CH_3CN$–$H_2O$ and shaken. The lower layer was collected in a 250-ml flask. Twenty-five milliliters $CHCl_3$ was added to the aqueous layer and centrifuged at 1500 × *g* for 10 min. The clear lower layer was evaporated to incipient dryness on a steam bath, and the residue was transferred quantitatively with $CHCl_3$ through filter paper into a vial and evaporated to dryness under gentle stream of $N_2$. Stg was determined by HPLC according to the method recommended by the AOAC [17].

*2.2.1.5. HPLC conditions.* HPLC analysis was performed with a Series 1100 binary pump, an autosampler and a column oven (Agilent Technologies, Waldbronn, Germany). Stg was separated by isocratic elution on a 50 mm × 2.1 mm (inside diameter) INERTSIL C18 (5 μm) reversed-phase column (GL Sciences, Inc., Tokio, Japan) maintained at 30 °C. The mobile phase consisted of methanol with 8 mM aqueous ammonium acetate (70:30). The flow rate was 0.2 ml/min, providing a retention time for Stg of approximately 2.3 min. The injection volume was 10 μl.

*2.2.2. In vivo studies*

*2.2.2.1. Fish.* Two-month-old Nile tilapia (Oreachromis nilaticus) fish weighing 90 ± 10 g were purchased from EL-Ibrahimia Fish Farm (Sharkia, Egypt) and transported in a large plastic water container supplied with battery aerators as a source of oxygen. Fishes were maintained on ad libitum standard fish diet (free from Stg) at the Animal House, Faculty of Veterinary Medicine, Cairo University (Giza, Egypt). After an acclimation period of 1 week, the fish were divided over five experimental groups (8 fish/group) and each group was placed in a fish aquarium containing de-chlorinated tap water. The water was circulated 15 times a day, the average water temperature was 14.5 ± 3.7 °C, and the pH was in the range 7.17–8.19.

*2.2.2.2. Experimental design.* Fish received intragastric doses of Stg for 4 weeks (2 doses/week) as follows: group 1, untreated control; group 2, treated with Stg (1.6 μg/kg body weight dissolved in corn oil); group 3, treated with EM alone; group 4, treated with EM plus Stg; and group 5, treated with corn oil alone. Fish treated with EM alone or in combination with Stg were given an amount of the sorbent equivalent to 0.5% of the estimated maximum daily intake of feed dissolved in corn oil. Mortality rate was recorded daily, whereas body weight gain was recorded twice a week (during dosing) throughout the treatment period. Micronuclei were measured in blood cells and chromosomal aberrations were carried in kidney cells. The kidney is considered a target organ for Stg.

### 2.3. Determination of Stg in edible tissues of fishes

All fish were removed from the water and left to die, then kept at 4 °C for the determination of Stg in muscle tissues according to the method described in AOAC [17].

### 2.4. Preparation of samples for cytogenetic studies

*2.4.1. Micronucleus test*

A drop of blood from the gills was mixed with a drop of foetal calf serum on a glass slide and air-dried. The slide was fixed in methyl alcohol for 5 min, then stained with 5% Giemsa (5%) for 10 min. Two thousands erythrocytes were examined for each fish for the

determination of the percentage of RBCs that contained micronuclei [18].

### 2.4.2. Chromosomal preparation

The samples were exposed to different treatments for chromosomal preparation according to the method described by Al-Sabti [19] with the following modifications:

1- Each live fish was injected intramuscularly with 0.01 ml colchicine (0.05%) and kept in a well-aerated plastic bag for 3 h.
2- The anterior kidney (head kidney) was excised and cut into fine particles in 5 ml isotonic solution (NaCl 0.9%) and centrifuged at 1000 × *g* for 10 min.
3- The supernatant fluid was discarded and 5 ml of hypotonic solution (KCl 0.5%) were added, mixed and kept for 30 min then centrifuged at 1000 × *g* for 10 min.
4- A fixative (5 ml cold mixture of 1:3 glacial acetic acid and methyl alcohol) was added to the specimen and left overnight at 4 °C.
5- Two changes of the same fixative were applied with centrifugation and removal of the supernatant fluid each time, then the sediment was suspended in a small amount of the fixative.
6- The solution was spread on glass slides and gently flamed and dried.
7- The slides were stained for 40 min with 10% Giemsa stain and 100 metaphase spreads from each specimen were examined.

### 2.5. Statistical analysis

All data for body weight gain were statistically analyzed using the General Linear Model Procedure of the Statistical Analysis System [20]. The significance of the differences among treatments was determined by Waller–Duncan *k*-ratio [21]. All statements of significance were based on probability of $P \leq 0.05$. Data for micronucleus test and chromosomal aberration were subjected to statistical analysis with the $\chi^2$-test [22].

## 3. Results

Results of the in vitro studies clearly indicated that EM was capable of adsorbing Stg from aqueous solutions. Table 1 shows that EM had a very high affinity for Stg and the adsorption ability was not significantly affected by the EM concentration. Addition of 0.5 mg/L of EM resulted in the binding of 96.5% of Stg at the level of 5 μg/ml, and the adsorption rate reached 93.1% at the level of 50 μg/ml. Nonetheless, the binding capacity was insignificantly affected by the level of Stg in aqueous solutions. The evaluation of the stability of the EM–Stg adsorption complex at different pHs at 37 °C showed that the complex was stable at pH 2, 7 and 10 (Fig. 1). Moreover, less than 10% of Stg bound to EM at different test concentrations could be extracted by various organic solvents (Table 2).

Table 1
The in vitro adsorption capacity of EM for Stg

| EM concentration (mg/L) | Stg concentration (μg/ml) | | |
|---|---|---|---|
| | 5 (%) | 10 (%) | 50 (%) |
| 0.5 | 96.5 | 95.2 | 93.1 |
| 1 | 96.8 | 96.4 | 94.2 |
| 2 | 97.6 | 96.5 | 94.6 |
| 4 | 97.8 | 96.6 | 95.4 |

In the in vivo study, a total of 40 Nile tilapia fish were divided into five treatment groups. Two out of eight fish (25%) treated with Stg alone died between days 16 and 23 (after 4 and 5 doses). No death occurred in any of the other groups. The effects of the sorbent (EM) and Stg on the average body weight gain of the fish are depicted in Fig. 2. Fish treated with Stg alone showed a significant decrease in body weight gain, whereas, fish treated with corn oil or with EM, alone or in combination with Stg, were comparable with the controls. Analysis of Stg residues in edible tissues of

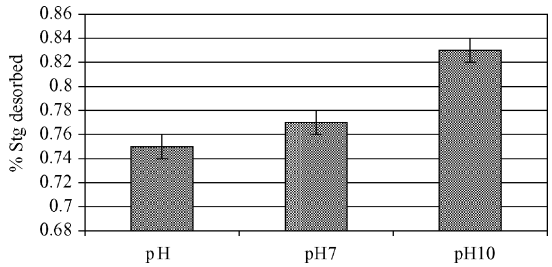


Fig. 1. Effect of pH on the stability of EM–Stg complex at 37 °C.

Table 2
Effect of various solvents on the stability of the EM–Stg adsorption complex

| Solvent | Percentage of total Stg desorbed[a] | | | |
|---|---|---|---|---|
| | A | B | C | D |
| Hexane | 0.41 | 0.42 | 0.39 | 0.40 |
| Cyclohexane | 0.18 | 0.19 | 0.18 | 0.19 |
| Benzene | 0.86 | 0.84 | 0.85 | 0.84 |
| Toluene | 0.29 | 0.28 | 0.27 | 0.28 |
| Chloroform | 8.76 | 7.94 | 7.69 | 8.68 |
| Ethyl acetate | 0.30 | 0.31 | 0.29 | 0.31 |
| 2-Propanol | 1.23 | 1.24 | 1.24 | 1.25 |
| Acetone | 7.13 | 7.12 | 6.87 | 7.03 |
| Methanol | 7.13 | 7.11 | 7.12 | 6.98 |
| Acetonitrile | 1.03 | 1.04 | 1.02 | 1.04 |

A, B. C and D are the levels of EM (i.e., 0.5, 1, 2 and 4%, w/v).
[a] Data are expressed as mean% of Stg desorbed (from two replicate assays per treatment).

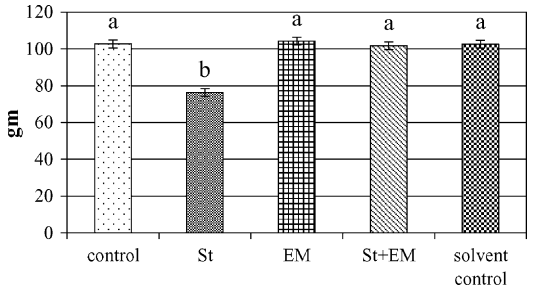


Fig. 2. Effect of Egyptian montmorillonite on body weight gain in fish treated orally with Sterigmatocystin (1.6 μg/kg bw twice a week) for 4 weeks.

fish in different groups showed significantly higher concentrations of Stg in fish treated with Stg alone than in those treated with Stg plus EM or in controls (Fig. 3).

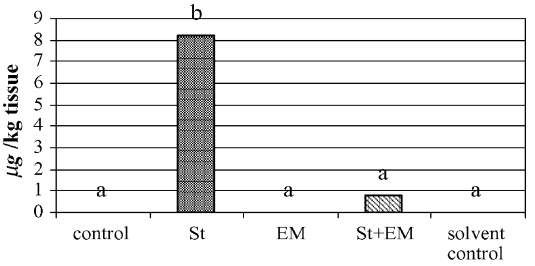


Fig. 3. Toxin residue in fish muscles treated with Egyptian montmorillonite with or without Sterigmatocystin.

The current study indicated that Stg treatment resulted in a significant increase ($P \leq 0.01$) in the frequency of micronucleated erythrocytes (MN RBC), whereas treatment with EM alone had no significant effect on the number of MN RBC. Fish treated with Stg plus EM showed a significant decrease in the number of MN RBC although these values were still higher than in the negative control group. The addition of EM to Stg resulted in a 60.9% reduction of the number of MN RBC compared with the group treated with Stg alone (Table 3).

The structural aberrations studied in the present work included centromeric attenuation, gaps/breaks, deletions and fragmentation. It is clear that treatment with Stg alone resulted in a significant increase in chromosome aberrations, mainly chromatid and chromosome breaks, in kidney tissues. No significant differences in chromosomal aberrations were observed in the group treated with EM alone compared with controls. The present results also indicate that the percentage inhibition of chromosomal aberrations resulting from EM treatment was 38.4% (Table 4).

Table 3
Effect of Egyptian montmorillonite (EM) on sterigmatocystin (Stg)-induced micronuclei in the tilapia fish

| | Groups | No. of RBCs examined | Mean values of MnRBCs/2000 RBCs ± S.D. |
|---|---|---|---|
| Mean values ± S.D. | 1. Control | 8000 | 3.5 ± 0.5 |
| | 2. Stg alone | 8000 | 36.75 ± 3.49 |
| | 3. EM alone | 8000 | 6.5 ± 1.1 |
| | 4. Stg + EM | 8000 | 16.5 ± 2.29 |
| | 5. Solvent control | 8000 | 5.25 ± 0.82 |
| $\chi^2$-values | Between 1 and 2 | 54.93** | |
| | Between 1 and 3 | 1.8 | |
| | Between 2 and 4 | 15.40* | |

Inhibition % of EM = 60.9%.
* Significant at $P < 0.05$.
** Significant at $P < 0.01$.

Table 4
Effect of Egyptian montmorillonite (EM) on Sterigmatocystin (Stg)-induced chromosomal aberrations in fish

| Groups | Total aberrations | Structural aberrations | | | | | | Polyploidy | Total aberration without gaps |
|---|---|---|---|---|---|---|---|---|---|
| | | Gaps | Chromosomal breaks | Deletion | Fragment | Centromeric attenuation | Centric feusion | | |
| 1. Control | 1.5 ± 0.5 | 0.25 ± 0.43 | 0 | 0.25 ± 0.43 | 0.25 ± 0.43 | 0.75 ± 0.43 | 0 | 0 | 0.25 ± 0.43 |
| 2. Stg alone | 29.75 ± 1.47 | 4.75 ± 1.29 | 8.75 ± 0.43 | 3.0 ± 0.70 | 0.702.5 | 3.0 ± 0.70 | 2.5 ± 0.5 | 4.0 ± 0.703 | 33.0 ± 0.70 |
| 3. EM alone | 8.75 ± 0.43 | 1.25 ± 0.82 | 1.5 ± 0.5 | 1.5 ± 0.5 | 0.75 ± 0.43 | 0.75 ± 0.43 | 0.75 ± 0.43 | 0.75 ± 0.43 | 7.5 ± 0.86 |
| 4. Stg + EM | 19.0 ± 0.70 | 3.25 ± 0.82 | 5.0 ± 0.70 | 25.0 ± 0.70 | 2.0 ± 0.702.5 | 2.75 ± .43 | 2.75 ± .43 | 2.75 ± .43 | 15.75 ± 0.82 |
| 5. Solvent control | 4.5 ± 0.5 | 1.25 ± 0.43 | 1.25 ± 0.43 | 0.430.25 | 0.75 ± 0.43 | 1.25 ± 0.43 | 0 | 0.75 ± 0.43 | 30.25 ± 0.43 |
| Between 1 and 2 | 51.0* | 8.1* | 17.5** | 4.6* | 3.68* | 4.44* | 7.5* | 6.5* | 42.97** |
| Between 1 and 3 | 10.25* | 1.3 | 3.0* | 1.78 | 0.5 | 0.8 | 0.5 | 3.5* | 8.92* |
| Between 2 and 4 | 6.0* | 1.06 | 2.04 | 0.4 | 0 | 0.46 | 2.13 | 0.36 | 4.19* |

Inhibition % of EM = 38.4%.

## 4. Discussion

Stg is one of the predominant contaminating mycotoxins in foodstuffs and grains of high-incidence area of malignant tumors and is the precursor of aflatoxin $B_1$. From this regard, we evaluated the ability of EM to bind Stg in vitro and in vivo. Results of the in vitro study indicated that the binding capacity of EM to Stg reached 98% and the adsorption complex was stable under different conditions of pH and temperature (Table 1 and Fig. 1). Furthermore, less than 10% of the Stg bound to EM could be extracted by various organic solvents. This may be due to the complex structure of montmorillonite, which increases the adsorption of organic compounds in each of its layers [23]. Carroll [24] reported that phyllosilicates are composed of layers of lattice silicates and chain silicates. These silicates are essentially composed of repeating layers of: (1) divalent or trivalent cations (e.g., aluminas) held in octahedral coordination with oxygen and hydroxyl groups, and (2) silicas that are tetrahedrally coordinated with oxygen and hydroxyl groups. In general, EM may possess three types of active binding sites: (1) those located at basal planes within interlayer channels, (2) those located on the surface, and (3) those located at the edges of clay particles. In an earlier report, we indicated that EM was effective in preventing the toxic effects of aflatoxins [15]. Studies on rat bone marrow showed that chromatid gaps and chromatid breaks were the most frequent types of aberration observed with aflatoxin [13,25,26]. Abdel-Wahhab et al. [13,14] reported that HSCAS and bentonite are effective in the protection against aflatoxin $B_1$ and prevent its toxic and clastogenic effects as was reflected by ameliorating the alterations in serum biochemical parameters and suppressing chromosomal aberrations. Mayura et al. [12] reported that HSCAS clay acts as an aflatoxin enterosorbent that tightly and selectively binds this mycotoxin in the gastrointestinal tract of animals, thereby decreasing its bioavailibilty and subsequent toxicity.

In the present study, fish were treated orally with 1.6 μg Stg/kg body weight twice a week for four successive weeks resulting in an overall cumulative dose of 12.8 μg/kg bw, which was comparable with previous doses reported in the literature [27,28]. The selected dose of EM was based on the current in vitro results. Treatment with Stg resulted in 25% mortality of the fish and significantly decreased body weight gain, whereas these parameters were comparable with those of controls in the fish treated with EM alone (Fig. 3).

Sivakumar et al. [1] reported that treatment of rats with Stg-contaminated diets resulted in the generation of free radicals, which imposed depletion of antioxidants. Tian et al., [29] reported that the DNA-Stg adduct was detected in specimens of cancerous tissues and/or peri-cancerous tissues. These results clearly indicate that Stg is a potent toxigenic agent. In the current study, Stg treatment resulted in a significant increase in the frequencies of MN RBC and chromosomal aberrations in fish. The clastogenicity of Stg may be related to the formation of adducts that disturb DNA replication, resulting in chromosomal aberrations.

Our results show that EM did not have negative impact on the overall health of the fish as indicated by the final body weight. Moreover, it protected the fish from the clastogenic effects of Stg. Addition of EM to Stg resulted in a significant decrease in the number of MN RBC and chromosomal aberrations. These findings are in line with earlier reports that the basic mechanism appears to involve sequestration of Stg in the gastrointestinal tract and chemisorption (i.e., tight binding) to EM, which results in a reduction in toxin bio-availability. Stg is closely related to the mycotoxin aflatoxin and, as a precursor in aflatoxin biosynthesis [2], it contains the dihydrofurobenzofuran system. Phillips et al. [30] reported that the intact dicarbonyl system in aflatoxin $B_1$ is essential for optimal adsorption by HSCAS and suggested that aflatoxin reacts at multiple sites on HSCAS clay surfaces (especially those within the interlayer region). The in vivo adsorption of Stg in the gastrointestinal tract of fish by EM was confirmed by the determination of Stg residues in fish tissues. The concentration of Stg in the edible tissues was significantly reduced in fish treated with EM plus Stg. These findings suggest a strong Stg-binding capacity of EM resulting in a decrease in the bioavailability of Stg in the gastrointestinal tract and subsequent reduction in the distribution to different organs.

In conclusion, the results of this study indicate that EM has a high affinity for Stg in vitro, forming an adsorption complex that was stable under different pHs at 37 °C. Moreover, the stability of this complex was very high when extracted with different organic solvents. Furthermore, the present data show the effectiveness and safety of EM in reducing the bioavailability of Stg in the gastrointestinal tract and prevention of its

clastogenicity in fish. Consequently, the fish may be useful as a sensitive in vivo model to investigate the efficacy and safety of other potential mycotoxin binders.

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