RESEARCH ARTICLE

Efficacy of Kaolin and Bentonite Clay to Reduce Aflatoxin M₁ Content in Contaminated Milk and Effects on Milk Quality

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ABSTRACT

Mycotoxins contamination in milk products represents a major problem for milk industries. Aflatoxin M₁ (AFM₁) is very stable and resists any heat treatments as pasteurization and sterilization methods. The aim of this study was to determine the level of aflatoxin M₁ in fifty raw milk samples collected from different dairy shops in Kafr El-Sheikh governorate in Egypt. We also evaluated the efficacy of detoxification methods of AFM₁ in milk by using natural clay as Kaolin and Ca-bentonite. The milk survey study revealed that AFM₁ was detected in all the examined raw milk samples with mean value of 10.7±0.89 ppb, which exceeded the Egyptian standard and European Union limit in raw milk. Then we artificially contaminated raw milk samples with AFM₁ standard and then added three different concentrations of Kaolin and Ca- bentonite (5gm, 10gm, 20 gm), separately. The obtained results showed a significant reduction and detoxification in AFM₁ concentration by Kaolin and Ca- bentonite treatments comparing with non-treated milk. The percent AFM₁ detoxification rate by using kaolin and Ca- bentonite clay was 86.1 to 93.3% and 93.7 to 97.7%, respectively without any changes in nutritional constituents of milk. Moreover, the Ca-bentonite clay revealed a tendency to the comprehensive removal of AFM₁ by increasing the quantity of clay added to the tested milk. These results indicate efficacy and safe usage of kaolin and Ca-bentonite clay to detoxification and reduce the amount of AFM₁ in raw milk and consequently; minimize its dangerous effect on the public health.

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INTRODUCTION

Milk is considered to be a perfect natural food for consumers of all age groups due to its high nutritional value. It is high in protein and a valuable source of calcium, vitamins, and antioxidants (Zelueta *et al.*, 2009). Also, milk has the greatest potential demonstrated for introducing aflatoxins M₁ (AFM₁) into human diet. The frequency of occurrence of AFM₁ in commercially available milk and dairy products, the high intake of these products by human population, especially by infant and young children and its probable carcinogenic effect (Rwangwises and Rwangwises, 2010). Mycotoxins are a group of naturally occurring secondary metabolites which are mainly produced by the filamentous fungi (Iqbal *et al.*, 2011). Among them, aflatoxins (AFs) are the most toxic and carcinogenic class and mainly produced by *Aspergillus flavus*, *Aspergillus parasiticus* and rarely by *Aspergillus nomius*. They can contaminate food, cereals, vegetable, fruits and cattle feed (Asi *et al.*, 2012).

Mycotoxins represent a major problem for food industries affecting productivity, welfare and health and are also a permanent risk concerning food safety for humans and animals (Brayden, 2012). Aflatoxins are most commonly known for causing acute or chronic liver disease according to the exposed doses, they are also

considered as immunosuppressive, mutagenic, carcinogenic, teratogenic and hepatotoxic (Flores-Flores *et al.,* 2015; Naseem *et al.,* 2018). Aflatoxin B$_1$ (AFB$_1$) is the most common form present in contaminated foods. Consumption of AFB$_1$ contaminated feed by lactating animals lead to formation and release of AFM$_1$ in milk. AFM$_1$ is formed in the liver and excreted from the mammary glands in the milk of animals consumed contaminated feed with AFB$_1$, these toxins are considered the most dangerous threat for humans especially for children and elderly who fed milk and dairy products (Gürbay *et al.,* 2010). The World Health Organization and International Agency for Research on Cancer (IARC) changed AFM$_1$ classification from group 2 to group 1 human cancer-causing agent (IARC, 1993); as it is recognized to be hepatotoxic and carcinogenic (Rahimi and Karim 2008; Ghazani, 2009).

The AFM$_1$ level in milk may vary according to development level of the country, climatic conditions and geographic location, so, it is important to determine its levels in milk produced in different locations to protect consumers from its harmful effects (Picinin *et al.,* 2013). In the European Union (EU), 50 ng/L is the maximum level of AFM$_1$ in liquid milk, whereas 500 ng/L is considered the maximum level of AFM$_1$ for United States and most of Asian countries' regulations (Anukul *et al.,* 2013).

Detoxification of aflatoxins is the requisite of the hour since their incidence in food is continuously posing threats to both live being health and economics all over the world. Various strategies for their decontamination from feed stuff resources have been involving physical, chemical and biological means (Basappa and Shantha 1996). The stability of AFM$_1$ is not affected considerably neither by heat treatments i.e. pasteurization and sterilization used in dairy industry nor during processing and storage of numerous dairy products (Rokhi *et al.,* 2013). The greatest way to control the existence of AFB$_1$ in food is to inhibit their formation. Various biological, physical and chemical agents have been used to decontaminate aflatoxins from feed and food materials (Park, 1993). Interestingly, one of the best promising approaches to minimize the presence of aflatoxin in food is to exploit the binding affinity of aflatoxin to the clay minerals (Phillips *et al.,* 2008). Natural clay minerals are predominantly used in the field of animal nourishment (Phillips *et al.,* 2008) also, it's have been reported to be safe for human consumption as well (Wang *et al.,* 2005).

Natural clay is plentifully available cheap natural source which is nontoxic to ecosystem. Over the last few years, investigation on the modification of the clay to increase their adsorption capacity to remove other contaminants from drinking water in progress (Srinivasan, 2011). Kaolinite Al$_2$Si$_2$O$_5$ (OH)$_4$ is one of the most common clay minerals which is inexpensive and used in many pharmaceutical applications as excipient or dynamic ingredient (Soha *et al.,* 2006). Kaolinite is widely used as it reveals excellent chemical, physical and surface physicochemical properties. Importantly, Kaolinite and its derivatives are used in many pharmaceutical industries as delivery substance on many drugs and protein as it promotes the cellular drug uptake

and has a higher interaction ability with organic molecules (Awad *et al.,* 2017).

Bentonite is considered one of the natural clays and have several applications because of their structural features, low cost, availability and their abundance in nature (Wang *et al.,* 2005). Research recommends that bentonites may be brilliant cell protectors and that they can decrease some of the side effects of drugs like those used for cancer cure (Di Natale *et al.,* 2009). Bentonite particles are effective in promoting growth as adsorbents of many toxins and improving health by decreasing the destructive effects of drugs (Nones *et al.,* 2015). Requirements to limit AFM$_1$ in milk are essential. The introduction of sequestering binders as kaolin and bentonite directly to contaminated milk may be an operational method for the removal of AFM$_1$ residue (Womack, 2015). Therefore, this study aims to detect the incidence AFM$_1$ in raw milk from different dairy shops in Kafr El-Sheikh governorate, Egypt, and to evaluate the efficacy of detoxification methods of AFM$_1$ in milk by using Kaolin and Ca-bentonite.

MATERIALS AND METHODS

Samples collection: Fifty samples of raw milk were randomly collected from different supermarkets and dairy shops in Kafr El-Sheikh governorate. The collected samples were directly transferred to the laboratory on ice and stored in a cool dark place and kept refrigerated until further analysis.

Quantitative detection of AFM$_1$: The fifty randomly collected raw milk samples were subjected to AFM$_1$ detection, where the amount of AFM$_1$ was detected by using Glory Science Aflatoxin M$_1$ kits (A1003), and measure AFM$_1$ concentration by Enzyme-Linked Immuno Sorbent assay. This kit with lower detection limit 0.1 ppb which is a competitive enzyme immuno-assay based on antigen-antibody reaction (Karimi *et al.,* 2007). Samples were prepared and extracted according to the kit's instructions. Briefly, 50 ml milk samples were centrifuged at 3000 rpm/20 min, then the fat layer was discarded. Then we took 10 ml of whey samples (second layer) and added 20 ml of 70% methanol solution; and filtered with what man paper (No. 1). Afterthought, we took 100 µl of the treated samples and added 400 µl of samples diluent. Finally, we collected 50µl of the diluted samples for analysis by ELISA AFM1 kits. The absorbance was measured at 450 nm in ELISA plate reader (PPBI 16). Calculation of results as AFM$_1$ concentration values in the examined raw milk samples were obtained from the standard curve.

Milk samples used in experimental part: 5000 ml of raw milk were brought from a high quality farm and transferred to the laboratory (in an ice box with minimum of delay) where subjected to the following examinations: AFM$_1$ detection as mentioned before and chemical composition analysis (lactose, protein and fat %) was determined by using infra-red spectroscopy (Milko-Scan 133 BN Foss Electric, Denmark) according to manufacturer's instruction.

Detoxification of AFM₁ by using natural clay:
Standard stock solution of AFM$_1$ (0.5 µg/ml) was bought from Sigma Aldrich (product No: 34031). The standard was dissolved in acetonitrile and stored at -20ºC. The previously tested raw milk was artificially contaminated with AFM$_1$ standard which resulting in AFM$_1$ concentration (116.2 ng/L). Then we divided the 1600 ml of the artificial contaminated milk sample into two main groups (each 800 ml) one group to detoxify the aflatoxin M$_1$ by kaolin clay (extra pure, high quality kaolin, LOBA CHEMIE PTV. LTD) using different concentrations (5, 10 and 20 gm). The other group to detoxify the aflatoxin M$_1$ by natural calcium bentonite clay (detox powder; X00 17IS9CV; earth living calcium bentonite powder, pharmaceutical, food grade for internal and external use purchased from U.S.A) using different concentrations (5, 10 and 20 gm) as we used for Kaolin. All samples were mixed well with the clay materials as described by Carraro *et al.* (2014) after that all dispersions were shaken for 24 h on the shaker. Sedimentation occurred to all samples for 12h at 20°C then milk samples were separated, and divided to two sub samples, the first for chemical composition analysis (lactose, protein and fat %) by using (Milko–Scan 133B N) as mentioned before; and the second for detection of the AFM$_1$ concentrations which measured by ELISA technique as stated before to study the effect of both clay (kaolin and bentonite) on them.

Statistical analysis: The data were analyzed using the statistical software package SPSS. Multiple comparisons between normally distributed continuous experimental groups were analyzed by the one-way analysis of variance (ANOVA) as a parametric test followed by the Dunnett (two-sided) post hoc test. The data are expressed as mean ± SEM. Statistical significance was assigned as $P \leq 0.05$ and marked as (*).

RESULTS

Determination of AFM₁ in raw milk samples: The fifty randomly collected raw milk samples were subjected to AFM$_1$ detection, where the amount of AFM$_1$ was detected by using Aflatoxin M$_1$ kits. All the examined raw milk samples were positive and contaminated with AFM$_1$ (Table 1). The AFM$_1$ concentration in the examined samples was ranged between 1.4 to 16.2 ppb with the mean value of 10.7±0.89 ppb. By comparing our results to the Egyptian and European AFM$_1$ permissible limits (Table 2), revealed that all the examined raw milk samples exceeded the permissible limit of AFM$_1$ that established by Egyptian standards (2010/7136) and European regulations 466/2001 which stated that the limit is fixed for AFM$_1$ at 50 ng/L for raw and liquid milk. Also, these examined raw milk samples exceed the limit recognized by Food and Drug Administration regulations (2011) which indicated that AFM$_1$ limit in raw milk is 500 ppt.

Table 1: Detection of AFM₁ in the examined raw milk samples

No. of the examined samples	Positive sample		Aflatoxin M₁ concentration(ppb)		
	No.	%	Min.	Max.	Mean ±SE
50	50	100	1.4	16.2	10.7±0.89

Table 2: Number of positive milk samples exceeding the Egyptian, EU and US limits

No. of the positive samples	Egyptian regulations*		European. Regulations**		FDAUS regulations***	
	No.	%	No.	%	No.	%
50	50	100	50	100	50	100

*Egyptian regulation, 2010/7136 (50 ng/L of AFM₁). **European regulations 466/2001 (the limit is fixed for AFM₁ at 50 ng/L for raw and liquid milk) .***FDAUS regulations limit, 500ppt of AFM₁.

Detoxification of AFM₁ in raw milk by kaolin: Kaolin was used to detoxify AFM$_1$ contamination in raw milk samples by using different concentration 5, 10 and 20 gm/L of kaolin (Fig. 1). Our results revealed a significant reduction of AFM$_1$ concentration compared with the control sample. These reductions of AFM$_1$ were reported on all groups using different concentration of kaolin. Briefly, AFM$_1$ concentration after using 5 gm of kaolin was 12.73 ng/L (Fig. 1A) with reduction 89.04% (Fig. 1B). AFM$_1$ concentration after using 10 gm of kaolin was 7.8 ng/L with reduction 93.28%. While AFM$_1$ concentration after using 20 gm of kaolin was 16.13 ng/L with reduction 86.12%.

To check the nutritional parameters of the milk before and after kaolin treatment, the amounts of lactose, protein and fat were measured by infrared spectroscopy. No significant changes were reported on any of the lactose, protein and fat content on milk samples after Kaolin treatment (Fig. 2). The protein content was slightly decreased after adding kaolin, ranging from 3.51 to 3.61% comparing to the initial protein concentration 3.95%. While the fat percentage was 3.5% initially and decreased after this treatment to range from 2.69 to 2.9%.
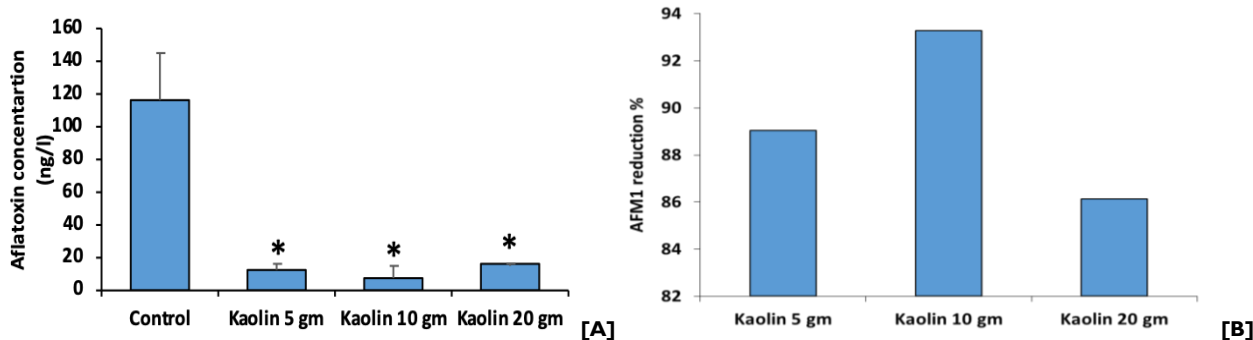

Fig. 1: Detoxification of AFM₁ in the tested raw milk samples after adding kaolin. [A] Effect of adding kaolin at different concentrations on AFM1 content in artificially contaminated raw milk samples. [B] Percentage of AFM1 reduction in raw milk by kaolin treatment. Graphs show mean ± SEM, asterisks indicate P≤0.05 when compared with control group.
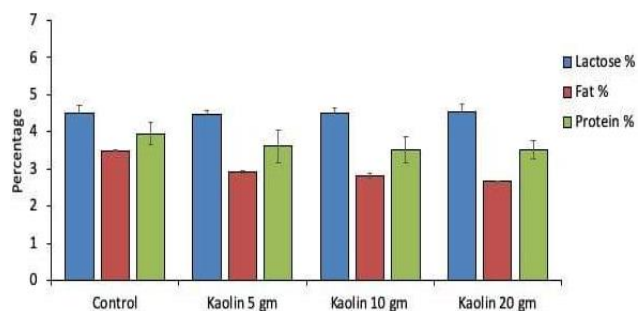
Fig. 2: Chemical composition analysis of milk samples after adding kaolin. Nutritional composition analysis of lactose, protein and fat % in milk samples after adding kaolin at three different concentrations (5, 10, 20 gm), graphs show mean ± SEM.
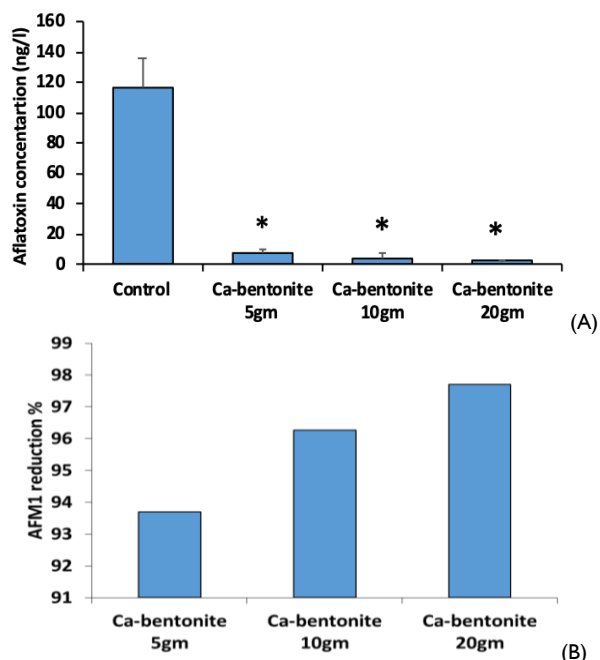

Fig. 3: Detoxification of AFM_1 in the tested raw milk samples after adding calcium bentonite. [A] Showed the reduction of AFM_1 content after adding Ca-bentonite at three concentrations (5, 10, 20 gm). [B] Percentage of AFM1 reduction in raw milk by Ca-bentonite treatment. Graphs show mean ± SEM, asterisks indicate P≤0.05 when compared with control group.

Detoxification of AFM_1 in raw milk by bentonite: Addition of calcium bentonite at different concentrations 5, 10 and 20 gm/L to the artificially contaminated raw milk samples (Fig. 3) with AFM_1 exhibited a significant difference in the reduction of AFM_1 level compared with the control samples (AFM_1=116.2 ng/L) where AFM_1 reductions were 7.33 (93.69%), 4.33 (96.27%) and 2.66 (97.71%) ng/L with addition of three different amounts of bentonite, respectively (Fig. 3A, B). Furthermore, when examined chemical composition (lactose, protein and fat

%) of tested milk samples to determine the effect of bentonite on milk nutritional parameters. No significant changes were reported on any of the lactose, protein and fat content on milk samples after bentonite treatment (Fig. 4). We noticed slightly changed on some parameters as, fat % was decreased from 3.5 to 2.66%, that still statistically not significant comparing to control samples.

Interestingly, by comparing the detoxification effect of kaolin and calcium bentonite on AFM_1 concentration in tested raw milk (Fig. 5), we observed that Ca-bentonite had a greater significant reduction of aflatoxin content using 5 gm and 10 gm compared to the same concentration of kaolin (P=0.04, 0.01; respectively).

DISCUSSION

Milk has been well-defined as a complete food as it contains energetic nutrients including proteins, lactose, essential fatty acids, vitamins and minerals in well-adjusted quantities, but it can also contain chemical hazards and contaminants, which institute a technological risk factor for milk products, consumer health and the associated commercial image (Licata et al., 2004). As AFM_1 is a worldwide problem, numerous studies have been directed for determining the incidence and concentration of AFM_1 in milk using different methods worldwide. Therefore, this study aims to detect the incidence AFM_1 in raw milk from different dairy shops in Kafr El-Sheikh governorate in Egypt, and to evaluate the efficacy of detoxification methods of AFM_1 in milk by using Kaolin and Ca-bentonite.

In this study, AFM_1 has been detected in all the examined raw milk samples and it is concentration was ranged between 1.4 to 16.2 ppb which exceeded the permissible limit of AFM1 that established by Egyptian and European standards. Maximum AFM_1 level in liquid milk and processed or dried milk products anticipated for adults has been established 50 ng/L and 25 ng/L for milk proposed for infants (European Commission Regulation, 2004).

Conversely, the US Food and Drug Administration stated the maximum allowable level of 0.5 ug/kg in milk. While, comparing our estimations with the permissible limits, our results (Table 2) revealed that all the examined raw milk samples exceeded the permissible limit of AFM_1 that established by Egyptian standards (2010/7136) and European regulations 466/2001(50 ng/L). Also, these findings surpassed FDAUS regulations (2011) limit (500ppt) for raw milk. There are consequently differences in the maximum tolerance limit for AFM_1 in different countries. Previous studies by Shaker and Elsharkawy (2014) estimated 93% of examined milk samples were above the European Commission (EC) standard of aflatoxin (50ng/L).
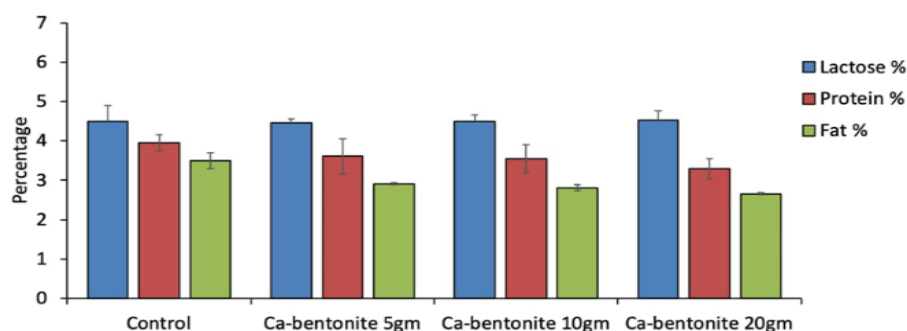

Fig. 4: Chemical composition analysis of milk samples after adding calcium bentonite. Nutritional composition analysis of lactose, protein and fat % in milk samples after adding calcium bentonite at three different concentrations (5, 10, 20 gm), graphs show mean ± SEM.
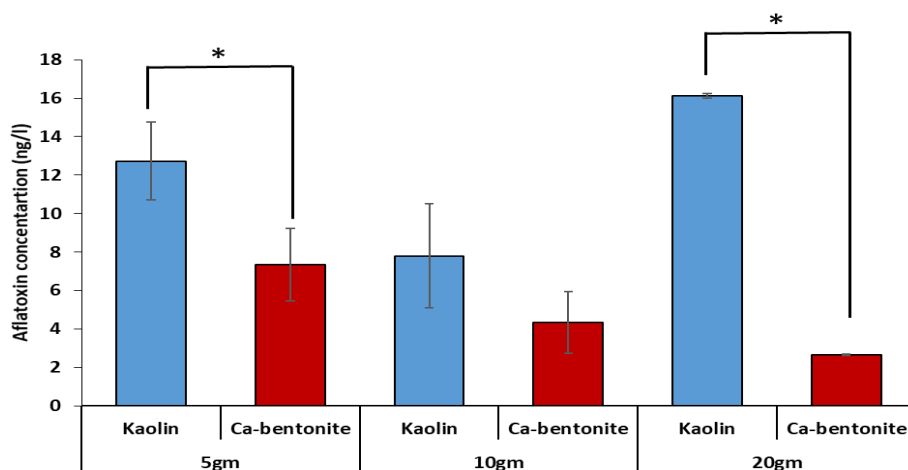
Fig. 5: Comparison between Kaolin and Ca calcium bentonite detoxification effect and the recovery of AFM_1 in the tested raw milk. Comparing between the effect after adding the same concentration of kaolin and Ca-bentonite on AFM1 reduction. Graphs show mean ± SEM, asterisks indicate $P \leq 0.05$ when compared with control group.

Previous studies by Shaker and Elsharkawy (2014), and Deeb et al. (2016) detected a higher incidence of AFM_1 in the examined raw milk samples in Egypt, the reported AFM_1 concentration was 97.92 and 95.6%, respectively. In our study, AFM_1 levels in the examined raw milk samples appeared to be different from some other studies. However, comparison among the countries is difficult due to different analytical techniques applied, climatic period, storage conditions and the period of sampling, etc. Knowing that the incidence of AFM_1 in milk initiates from the occurrence of AFB_1 in feed (Tsakiris et al., 2013). Also, difference may be attributable to a diverse origin of feed. Ingestion of contaminated milk with AFM_1 toxin can cause acute and chronic mycotoxicosis. So, it is critical to control and mitigate such toxin (Naeimipour et al., 2018; Bhatti et al., 2019).

The other previous studies have showing that there have been no considerable changes in AFM_1 content after heat treating such as pasteurization and sterilization (Jasutiene et al., 2007), as thermal handling during processing stages is not measured as an outstanding way to decrease AFM_1 in milk. Even though pasteurization and sterilization may reduce many microorganisms from milk, but they are not effective in AFs reduction (Naeimipour et al., 2018). Consequently, different approaches were studied to detoxify AFM1 from dairy products such as oxidation by hydrogen-peroxide besides of heat-treatment, biodegradation, binding of AFM1 to adsorbents such as diverse mineral clays (kaolin, bentonite, potassium sulphite, zeolites, smectite or activated carbons (Soha et al., 2006; Phillips et al., 2008 and Carraro et al., 2014). These absorbents can decrease AFs contents in milk due to their high variability and ability to bind to AFM1 in a stable mode (Naeimipour et al., 2018). Therefore, due to the essential need to healthy non contaminated milk, we tried to detoxify AFM_1 concentration in the contaminated milk by using the natural healthy clay as kaolin and calcium bentonite.

Kaolin was vended as an anti-caking additive for animal feed. It was stated to sorb AFB_1 with high attraction and high ability in aqueous solutions and was shown to save broiler and leghorn chicks from the poisonous effect of 7500ppb aflatoxin in the diet (Phillips et al., 2008). Also, kaolinite used as protectors for gastrointestinal tract. Its therapeutic action depends on its great specific area and sorption ability as they adhere to the gastric and intestinal mucosa and guard them (Carretero, 2002). In this study, Kaolin was used to detoxify AFM_1 contamination in raw milk samples by using different concentrations 5, 10 and 20 gm of kaolin. Our results revealed a significant reduction of AFM_1 concentration in the artificially contaminated raw milk samples. We noticed that the best amount in the adsorption of AFM_1 was 10 gm kaolin, where the reduction % was 93.28%. Interestingly, no significant changes were reported on any of the chemical constituents of the tested milk (lactose, protein and fat) after Kaolin treatment.

Bentonite is absorptive aluminum phyllosilicate clay. It is also called Montmorillonite clay, which originated from the area of France named Montmorillon, where it was first established. It has been eaten and used from earliest time till now as human trusts in its therapeutic profits (Moosavi, 2017). Moreover, by using bentonite at the same different amounts of (5, 10 and 20 gm) to the artificially contaminated raw milk samples, it exhibited a major reduction of AFM_1 level compared with the control sample (AFM_1=116.2 ng/L) by increasing clay amount as AFM_1 reductions reached to 97.71% with 20 gm bentonite added. Also, it resulted in slightly non-significant changes in the tested milk components (lactose, protein and fat %). Previous study found that supplementation of bentonite and activated charcoal by 1% for early lactating goats lead to substantial reduction of AFM_1 concentration of milk and remainder of aflatoxin from feed to milk without causing any change in the composition of milk (Rao and Chopra, 2001). Our findings were nearly comparable with that established by Soha et al. (2006) as they added bentonite and (HSCAS) hydrated sodium calcium alumino silicate at 0.5 and 2% to naturally AFM_1 contaminated milk exceeding 0.05 µg/kg, and the results showed that bentonite decreased AFM_1 levels by 90% at both concentrations 0.5 and 2% levels while HSCAS reduced it by 47.7 and 77.8% at 0.5 and 2%, respectively.

This study revealed that both clays had a respectable effect on AFM1 adsorption from the contaminated raw milk, and calcium bentonite is more effective in AFM1 detoxification than kaolin. Kaolin was less operative in AFM_1 detoxification than the bentonite. So, this trial may be professionally in AFM_1 recovery from milk and this

technique has certain advantages rather than other recovery methods (biochemical or chemical) as it is effortlessly used, mechanical separation of the toxin; and it is able to produce a safe and harmless product for human consumption.

Conclusions: our results exhibited a high contamination frequency AFM1 in raw milk market in Egypt that represents a great hazard to consumers in this area. Our results show that kaolin and bentonite clay exhibited a significant role in AFM1 detoxification from the artificially contaminated milk with no substantial change in nutritional constituents (lactose, protein and fat %) of the treated milk before and after treatment. Future investigations and more studies are needed to explore the mechanism by which clay minerals decrease the aflatoxin contamination in raw milk.

Authors contribution: AM, IA, RB and AS made the concept and design of the project. AM, WA and RB collected the data and wrote the manuscript. RB, AM and WA did the analysis of the data as well as interpretation of the results. IA and AS also critically supervised the entire process and reviewed the manuscript.

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