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# Physicochemical characterization and cytotoxicity assessment of sodium dodecyl sulfate (SDS) modified chitosan (SDSCS) before and after removal of aflatoxins (AFs) as a potential mycotoxin Binder

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ABSTRACT

Aflatoxins in food and feed with prominent toxic effects have jeopardized public health for decades. This investigation intends to explore synthesized SDS-modified chitosan as new generation of binder for removal of aflatoxin using a straightforward ionic cross-linking approach. The primary objective of this technique was to enhance affinity and adsorption capability of SDSCS towards aflatoxins. In this context, physicochemical properties of SDSCS characterized with advanced analytical techniques such as scanning electron microscope (SEM) coupled with energy dispersive X-ray spectroscopy (EDS) and Fourier transform infrared spectroscopy (FT-IR) before and after removal of aflatoxin. In this study, effect of the pH on the adsorption of aflatoxins (6ppb) indicated that the increase in SDSCS concentration from low (0.5) to high (2 %) resulted in an increase of about 80 %, 78 % and 81 % in the adsorption percentage of $AFB_1$, $AFG_1$, and $AFB_2$ & $AFG_2$, respectively. FT-IR analysis showed the intramolecular interactions of the amine groups of chitosan and sulfate group of SDS formed a stable complex in the removal of aflatoxin that verified with appearance of three new additional peaks at 1323.50, 984.34 and 603.42 $cm^{-1}$. Notably, SEM images revealed that the porous SDSCS network was filled with aflatoxin molecules supported with EDS findings. Also, in vitro cytotoxicity assessments demonstrated that SDSCS protected $HepG_2$ cells against cytotoxic effect caused by aflatoxin (5 μM) in a concentration-dependent manner compared to the control ($p<0.01$). Collectively, the adsorption mechanism may involve attraction of anionic aflatoxin molecule into the interconnected pores of SDSCS complex with numerous cationic active site via hydrogen bond and van der waals force.

## 1. Introduction

Over the last decades, Chitosan (CS) as one of the most abundant chitin-derived linear cationic polysaccharide biopolymer has been intensively explored for its numerous potential applications in biomedicine, waste and water management, agriculture, cosmetics, nutritional enhancement and food processing [1–6]. CS ($(C_6H_{11}NO_4\times2)$) is composed of two repeated units of D-glucosamine and N-Acetyl-D--glucosamine linked by β (1→4) glycosidic bonds. In contrast to similar biopolymers such as cellulose, CS is characterized by multiple functional groups on carbon skeleton including amino ($NH_2$), hydroxyl (OH) and acetamide ($CH_3CONH_2$) that can form complexes with various adsorbates such as mycotoxins and heavy metals through ionic, hydrogen bonds and electrostatic interactions. Moreover, it has been revealed that CS exhibits the favorable biological properties including biosafety, biodegradability and biocompatibility as well as beneficial physiological functions such as antimicrobial, antioxidant, growth promotor activities. With regard to the global challenges due to unavoidable mycotoxin contamination of animal feed, the use of chitosan and its derivatives as a mycotoxin binder (MTB) have been charmed special attention in livestock feed industry [7–14].

Mycotoxins are biotoxic, low-molecular weight secondary metabolites secreted by several fungal species under distinctive environmental conditions such as moisture and temperature, that contaminate agricultural commodities during pre- and post-harvesting periods. Widespread prevalence of mycotoxin contamination of animal feed has been

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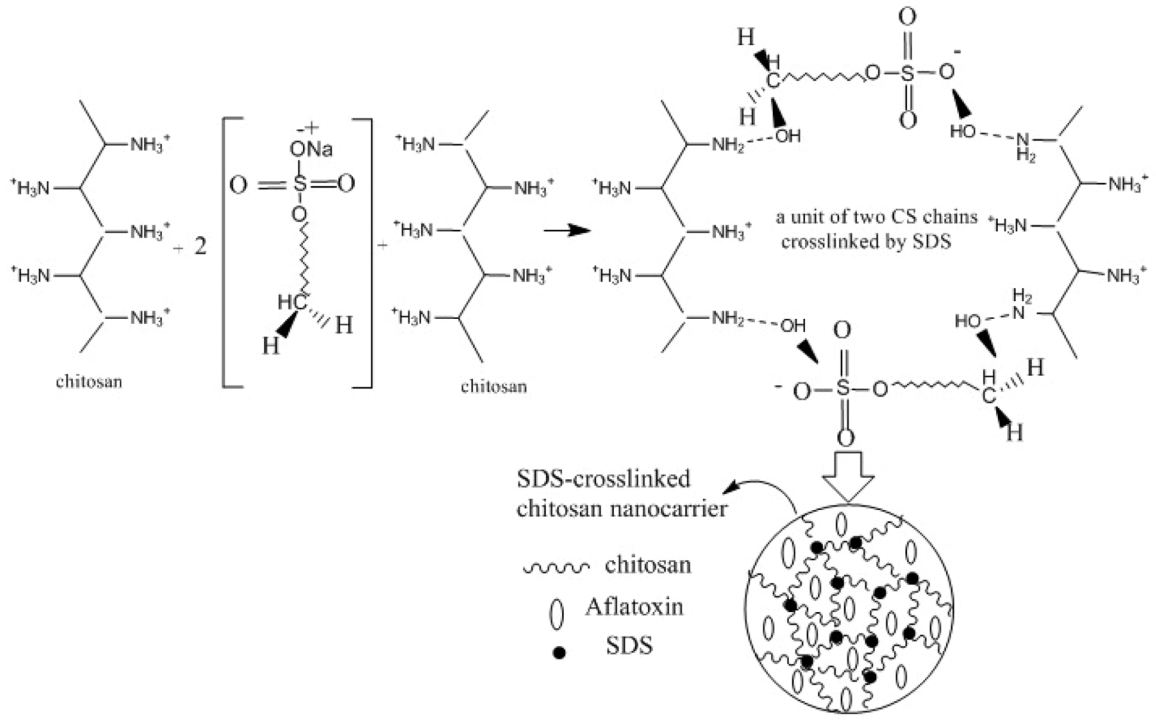



**Fig. 1.** Modeling of entrapped aflatoxin between SDS-modified chitosan.

increasingly predisposed livestock health and production, and potentially consumer health to serious problems. Mycotoxin ingestion leads to a wide range of non-specific symptoms from unthriftiness, anorexia, weight loss, reduced feed efficiency in mild and chronic cases to diarrhea, abortion and death in extreme acute cases on all livestock animals particularly swine and poultry. Among the more than 400 mycotoxins currently identified; such as Deoxynivalenol (DON), Zearolenone (ZEA), Ochratoxin A (OTA), Fumonisin $B_1$ and Trichothecenes (T-2), Aflatoxins are considered to be the most highly toxic due to its potent hepatotoxicity, teratogenicity, mutagenicity, immunotoxicity and carcinogenicity [15–21]. The stable structure of AFs ($C_{17}H_{12}O_6$) is composed of bisfuran ring fused to a coumarin nucleus with a pentanone or six-membered lactone ring. More than 20 types of aflatoxins exist in nature; the main ones are aflatoxin $B_1$ ($AFB_1$), $AFB_2$, $AFG_1$ and $AFG_2$ which $AFB_1$ is the most dangerous one owing to its hepatocarcinogenic property (the rank order of toxicity: $B_1 > G_1 > B_2 > G_2$). In addition, $AFB_1$ concentration generally exceed half of the total aflatoxins in contaminated samples (the rank order of frequency ratio: $B_1 > G_1 > B_2 > G_2$). Consequently, global regulatory limit of aflatoxins has been confirmed on the basis of $AFB_1$ concentration [22–26].

To minimize the negative effects of these mycotoxins in farm animals, inclusion of binders to livestock feed as additive appears to be the most prevalent strategy currently employed, in spite of the fact that different physical, chemical and biological methods have been developed. MTB inhibits the absorption of mycotoxins from gastrointestinal tract (GIT) of animal by adsorbing the toxins to their surface to form a mycotoxin-binder complexes and reduce their bioavailability in GIT and distribution to blood and target organs via elimination in feces. In this manner, adsorption capacity of various organic molecules (yeast cell wall, glucomannan, cellulose, activated charcoal and chitosan) and mineral matrix (bentonite, aluminosillicate and zeolite) have been investigated. Organic binders have been shown to have a high binding activity across a wide spectrum of mycotoxins and do not often interfere with nutrients and pharmaceutical substances while mineral binders non-selectively interact with vitamins and essential elements and their safety remain a concern. Despite of many excellent properties of chitosan, structural instability and desorptive behavior of chitosan on different regions of GIT under various pH levels have been restricted its commercialization as a MTB. However, chemical modification techniques such as surfactant derivatization may improve chemical stability of neat chitosan and improve adsorption and selectivity characteristics towards the removal of mycotoxins [27–32].

Sodium Dodecyl Sulfate (SDS) is a surfactant comprising a hydrophobic carbon chain and a polar sulfate head ($CH_3(CH_2)_{11}OSO_3Na$) could strongly bind to CS through the interactions between $–SO_4^-$ and $–NH_3^+$, respectively and formed an ionically cross-linked chitosan. The proposed mechanism suggested the side chain attachment the amine groups of CS and the sulfate groups of SDS could probably formed three-dimensional porous structures that capable of entrapping adsorbates such as mycotoxin more efficiently through chelation and electrostatic interactions (Fig. 1).

Many studies have focused on evaluating the adsorption property of chitosan and its modified forms towards their application for the removal of $AFB_1$ due to high toxicity and frequency relative to other aflatoxins however, uncommon occurrence ratios of the four aflatoxins have been documented with $AFG_1$ found at equal or even higher levels than $AFB_1$. Therefore, $AFB_1$ cannot just be used to effectively representing the total aflatoxin [33–40].

To the best of our knowledge, no studies have been reported on the evaluation of adsorption efficacy of various concentrations of SDS-modified chitosan as a mycotoxin binder to all aflatoxins. Furthermore, the knowledge on the protective effects of SDS-modified chitosan against all aflatoxins on cell cultures is scarce. Therefore, the present investigation was undertaken to determine the most effective SDS-modified chitosan concentration ranging from 0.5 % to 2 % by weight to simultaneously adsorb $AFB_1$, $AFB_2$, $AFG_1$, $AFG_2$ at different pH in an in vitro model. In addition, physicochemical properties of optimal SDS-modified chitosan concentration before and after removal of aflatoxins were characterized by DLS, SEM-EDS and FTIR to clarify size, surface and shape as well as interactive functional groups, respectively. The other purpose of this study was to compare the ability of different concentrations of synthesized SDS-modified chitosan to protect against aflatoxin-induced cytotoxicity in the hepatocellular carcinoma ($HepG_2$) cell line.

## 2. Materials and methods

### 2.1. Chemicals and cell culture requirements

Chitosan (MW,310 kDa; deacetylation degree 75 %), SDS, acetic acid glacial (98 %), aflatoxin mix ($B_1$ & $G_1$ 5x $B_2$ &$G_2$), acetonitrile, methanol and water were purchased from Sigma Aldrich (St. Louis, MO, USA). All chemicals utilized were of analytical quality and did not necessitate additional purification. Prior to use, all glassware underwent cleaning with deionized water and subsequent drying. To cell cytotoxicity test, hepatocellular carcinoma ($HepG_2$) cell line were procured from the Pasteur Institute of Iran. Dullbecco's modified Eagle's medium, fetal calf serum (5 %), penicillin-streptomycin-amphotericin B, dimethyl sulfoxide (DMSO), thiobarbituric acid (TBA) and MTT (3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide) were purchased from Sigma Aldrich (St. Louis, MO, USA). Lactate dehydrogenase (LDH) assay kits were provided by Biovision Technologies (Pennsylvania, USA).

### 2.2. Synthesis of different SDS-modified chitosan concentrations

First, different weights of chitosan powder (0.5.1,1.5 and 2 gr) were separately dissolved in 1 % acetic acid (100 ml) followed by adding NaOH (2 M) and stirring for 20 min at 300 rpm. Then, obtained gels mixed with a SDS solution (0.6 % w/v) in distilled water and stirred for 60 min. Subsequently, the formed precipitates were filtered and washed with distilled water and dried at 40°C overnight. Yellow-brown SDSCS insoluble complexes (0.5,1,1.5 and 2 % w/w) were ground and sieved to use as aflatoxin adsorbent in order to clarify effective adsorption dose of synthesized SDSCS among various concentrations ranging from 0.5 % to 2 % w/w.

### 2.3. pH – dependent adsorption of aflatoxins to different SDS-modified chitosan concentrations

The adsorption percentage of total aflatoxin ($B_1$,$B_2$,$G_1$,$G_2$) was determined according to the national standard of Iran (INSO 6872). A stock solution of total AF mixture (1000 ng/ml) was prepared in HPLC grade methanol and further diluted to a concentration of 6 ng/ml (20 % higher than the highest tolerance limit authorized by IR/INSO 5925). For the adsorption experiment, 0.04 gr of the binder (SDSCS: 0.5,1,1.5 & 2 % w/w) was weighed and spiked with AF work solution (100 ng/ml, 240 μL) and deionized water (3760 μL) to reach a final AF concentration of 6 ppb (4 ml), and the pH adjusted to 3 and 6.5 with HCL (1 M) and NaOH (1 M), respectively and then incubated at 37ºC for 1 h under constant agitation to simulate the pH conditions in GIT of mono- and polygastric livestock animals. Finally, the samples were centrifuged at 7000 g for 15 min. AF samples (with and without binder) were prepared as independent triplicate for each experiment. An aliquot of supernatant (20 μl) was then injected into the Waters HPLC system (series 1525, Binary pump). The separation was performed at 30 ºC with a InertSustainSwift C18 column (5.0 μm, 150 × 4.6 mm). The fluorescence detection was set at Em:440 and Ex:365 nm. The mobile phase contained H2O (1500 ml), KBr (305 mg), HNO3 (250 μl), MeOH (750 ml) and ACN (250 ml) at a flow rate of 1 ml/min. Recoveries for aflatoxins $B_1$, $B_2$, $G_1$ and $G_2$ were 98±0.8 %, 95±0.7 %, 93±4.4 % and 97±1.5 %, respectively. The limits of detection (LOD) and limits of quantification (LOQ) of 0.15 and 0.45 ng/ml for AFB1, 0.06 and 0.18 ng/ml for $AFB_2$, 0.28 and 0.85 ng/ml for $AFG_1$, and 0.03 and 0.09 ng/ml for $AFG_2$ were obtained, respectively. Each replicate was injected twice. The adsorption percentage of mycotoxins by SDSCS adsorbent was calculated as follows:

$$\text{Adsorption (\%)} = (C_b - C_t) / C_t \times 100$$

where $C_b$ is the AF concentration in blank spiked solution (ng/ml) and $C_t$, the amount of AF in the supernatant of samples (ng/ml) with 0.5 %, 1 %. 1.5 % & 2 % SDSCS

Also, the efficiency of adsorbent was calculated as follows:

$$\text{Efficiency (\%)} = (\text{adsorption pH 3} - \text{desorption pH 6.5}) \times 100$$

### 2.4. Physicochemical characterization of SDS-modified chitosan before and after removal of aflatoxin

#### 2.4.1. Determination of functional groups in SDSCS before and after removal of aflatoxin

In order to identify functional groups of SDS-modified chitosan and their interactive linkages with aflatoxin Fourier Transform Infrared Spectroscopy (FTIR) was utilized. The slight amount of grinded samples (chitosan, SDS-modified chitosan and SDS-chitosan/aflatoxin) mixed with potassium bromide (KBr) and then spectra were recorded in the range of 280–4000 $cm^{-1}$ at a resolution of 4 $cm^{-1}$ with Thermo Nicolet spectrophotometer (AVATAR FT-IR, USA).

#### 2.4.2. Determination of particle size and size distribution of SDSCS binder with aflatoxin

Dynamic Light Scattering (DLS) was subsequently utilized to assess the particle size and size distribution of the SDS-modified chitosan/aflatoxin complex. An adequate amount of dried complex suspended in water was subjected to ultrasound treatment for a predetermined period. Then, prepared sample was analyzed by using HORIBA SZ-100 (Japan) to determine the average diameter, size distribution, and polydispersity index of the suspension.

#### 2.4.3. Morpho-structrural characterization and elemental analysis of SDS-modified chitosan before and after removal of aflatoxin

The surface morphology and structure of SDS-modified chitosan before and after removal of aflatoxin were demonstrated using a SEM (TESCAN MIRA). Prior to SEM imaging, the samples were coated with 15 nm gold layer and the images were taken at a 3KV voltage. Also, the elemental analysis conducted by EDS provide insight into the weight percentage of the constituent elements present within SDSCS and SDSCS/AF.

### 2.5. Cell culture

In this study, human hepatocarcinoma $HepG_2$ cells, which retain normal hepatocyte function and have been used in a number of toxicological assessments, were cultured in Dullbecco's modified Eagle's medium (DMEM) supplemented with 5 % fetal bovine serum, and 60 mg/ml penicillin-streptomycin-amphotericin B at 37°C and 5 % $CO_2$ for 3 days to achieve 80 % cell confluence. Then, cells were trypsinized and resuspended in DMEM medium.

### 2.6. Cell viability assay

Cell viability was measured using the 3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide (MTT) dye reduction assay, to determine the cytoprotective effect of various SDS-modified chitosan concentrations (0.5,1,1.5 & 2 %w/w) against aflatoxins. Cells were seeded in 96-well plate at a density of $5 \times 10^6$ cells per well in DMEM medium and incubated in a humidified 5 % CO2 atmosphere for 24 h. Then, cells were treated with AF (5 μM: $IC_{50}$), alone and combined with different SDSCS concentrations for 24 h. After incubation, 150 μl MTT reagent (75 mg/ml in PBS) was added to each well and the plate was incubated for further 4 h at 37°C. Next, the solution was discarded and cells were lysed with 100 μl of dimethylsulfoxide (DMSO) to obtain purple solution. Finally, the purple solution was removed to empty the wells for absorbance measurement at 570 nm using a microplate reader. Care was taken to ensure that the MTT-containing plates and solutions were

**Table 1**
Percentage of adsorption, desorption and efficiency of different doses of SDS-modified chitosan towards $AFB_1$, $AFG_1$, $AFB_2$ and $AFG_2$ (6 pp) at pH 3 and 6.5.

| SDSCS (%w/w) | Aflatoxins | | | |
|---|---|---|---|---|
| | $B_1$ | $G_1$ | $B_2$ | $G_2$ |
| 0.5 % adsorption | 20.16±1.05 | 22.12±2.2 | 19.91±0.78 | 19.15±0.88 |
| % desorption | 3.08±0.82 | 2.98±1.01 | 1.88±0.78 | 1.76±0.08 |
| % efficiency | 17.08±0.23 | 19.14±1.19 | 18.03±0.75 | 17.39±0.80 |
| 1 % adsorption | 46.14±2.33 | 48.89±3.21 | 44.27±2.89 | 49.65±2.12 |
| % desorption | 2.38±0.92 | 2.05±0.83 | 1.03±0.53 | 1.68±0.14 |
| % efficiency | 43.76±1.41 | 46.84±2.38 | 42.24±2.36 | 46.97±1.98 |
| 1.5 % adsorption | 86.66±3.45 | 88.56±2.35 | 89.80±1.93 | 88.62±1.88 |
| % desorption | 1.04±0.32 | 1.02±0.24 | 0.89±0.03 | 0.93±0.05 |
| % efficiency | 85.62±3.13 | 87.48±2.11 | 88.91±1.9 | 87.69±1.83 |
| 2 % adsorption | 100 | 100 | 100 | 100 |
| % desorption | 0 | 0 | 0 | 0 |
| % efficiency | 100 | 100 | 100 | 100 |

completely protected from light throughout the experimental procedure. Also, all experiments were performed in triplicate. The cell viability percentage of each experiment was calculated using the following formula:

Cell viability (%) = (O.D of treatment–O.D of blank/ O.D of control–O.D of blank) × 100

All absorbance values were corrected background with blank wells which contained growth media alone.

### 2.7. Determination of the intracellular malondialdehyde (MDA) concentration

Membrane lipid peroxidation was determined with malondialdehyde (MDA) concentration in the $HepG_2$ cells using thiobarbituric acid (TBA) reaction. The cells were cultured with the density of $5 \times 10^6$ cells per well at a volume of 1 ml. After as described earlier, the samples were collected, washed twice with PBS and lysed in ice-cold KCl (1.15 %) with Triton X-100 (1 %) by sonication for 5 min. Aliquots (100 µl) of the cell lysates were mixed with 0.2 ml of 8.1 % SDS (8.1 %,) 1.5 ml of acetic acid (20 %) and reached to 4 ml with distilled water. The suspensions were incubated for 2 hr at 100ºC until the color developed, and then cooled. The contents were centrifuged at 5000 × g for 5 min. Finally, the absorbance of the supernatants was measured at 532 nm. The MDA concentrations were calculated by a molar extinction coefficient of $1.56 \times 10^5\ M^{-1}\ cm^{-1}$.

### 2.8. Lactate dehydrogenase (LDH) release assay

LDH release is a method to measure the membrane disintegrity as a function of the amount of intracellular LDH released from the disrupted

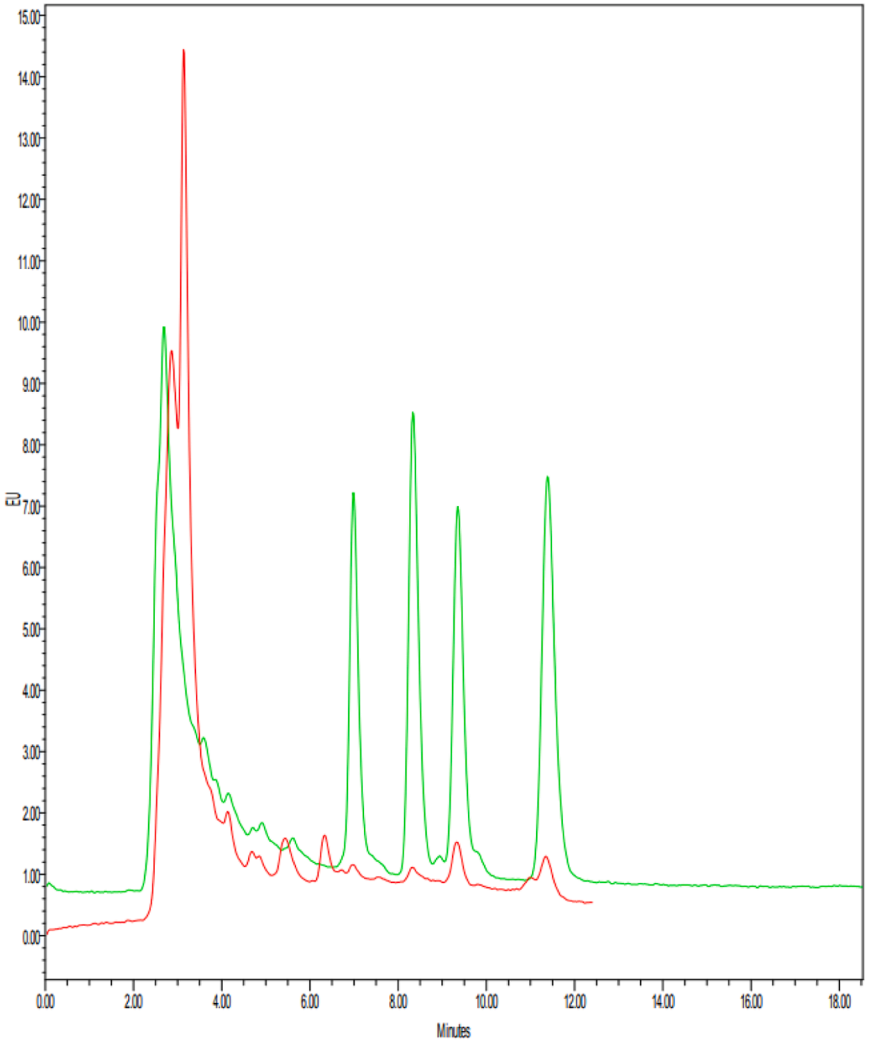



**Fig. 2.** HPLC chromatograms of aflatoxins spike (6ppb) which peaks related to solvent, $AFG_2$, $AFG_1$, $AFB_2$ and $AFB_1$ from the left to right, respectively (top) and removal of aflatoxins after adsorption to SDS-modified chitosan (2 %) (below).

cell membrane to the cell culture medium. The LDH assay was carried out using a commercially available in vitro cytotoxicity test (LDH Assay Kit, Biovision, California, USA). In brief, at the end of the experiment, 50 µl of culture supernatant from each well was collected and carefully transferred to new microtiter plate. Then, 50 µl of the buffer reagent was added and incubated at 25°C for 30 min and finally added 50 µl of kit stop solution and read absorbance at 490–520 nm.

### 2.9. Statistical analysis

All data obtained from at least three independent experiments subjected to a one-way analysis of variance and then, the means were compared using Tukey-Kramer and Dunnett's post hoc analysis. The result expressed as mean ± standard deviation. A probability of $p < 0.01$ was considered significant. Statistical package SPSS Version 12 was used for the statistical analysis.

## 3. Results and discussion

### 3.1. Effect of SDSCS dose on the adsorption behavior towards aflatoxins

To determine the effect of the adsorbent dose on the adsorption (removal) and desorption (release) of aflatoxins during adsorption process at pH 3 and 6.5 respectively, the experiments were carried out by varying the dosage of SDSCS (from 0.5 % to 2 %w/w). As shown in Table 1, the increase in SDSCS concentration from 0.5 % to 2 % resulted in an increase of about 80 %, 78 % and 81 % in the adsorption percentage of $AFB_1$, $AFG_1$, and $AFB_2$ & $AFG_2$, respectively. Also, the obtained results demonstrated that the amount of different aflatoxins desorbed decreased with the increase in the SDSCS dose. Thus, with regard to percent efficiency, 2 % SDS-modified chitosan was considered as the optimum dose for the removal of all aflatoxins in this study. This observation of the effect of the adsorbent dose has also been documented by other bioadsorbent in metal ions sorption [41]. This trend in removal is attributed to the increasing number of active sites and surface area for aflatoxins binding on SDS-modified chitosan as the weight of the binder increases from 0.5 % to 2 % due to the more adsorption sites and the availability of larger surface area.

### 3.2. Effect of pH on the adsorption behavior of SDSCS towards aflatoxins

The solution pH is one the most crucial parameters in the adsorption behavior of binder as it influences uptake (absorption) and release (desorption) of the adsorbate onto and from the adsorbent due to the nature of functional groups and surface charge of both the adsorbent and adsorbate. The protonation and deprotonation of both functional groups in the SDS-modified chitosan and aflatoxins will produce different surface charges. As under acidic condition (pH=3), active site on the SDSCS such as amine groups become protonated and then the surface of the binder will be positively charged and attract anionic aflatoxin molecules due to negative charges of the oxygen atom of aflatoxins, indicating that the mechanism is dominated by electrostatic forces such as hydrogen bond and *Van der Waals* interactions. Therefore, the polycationic nature of SDSCS allows the adsorption of polar molecules such as aflatoxins in acid aqueous media of upper region of GIT. Under neutral condition (pH=6.5), SDS-modified chitosan in contrast to chitosan and chitosan soluble complex (treated with NaOH) can retain aflatoxins inside the highly stable physical network which is formed by the complexation of chitosan and SDS. As shown in Fig. 2, the optimum dose of SDSCS was 2 %, which completely adsorbed all aflatoxins at different pH (3 and 6.5). This result is in agreement with previous studies, in which glutaraldehyde- modified chitosan and nonionic surfactant modified montmorillonite have the highest adsorption capacity for $AFB_1$ [42–44]. It can be noted from the data depicted in Table 1, that the adsorption capacity of SDSCS towards $AFB_1$, $AFG_1$, AFB2 and $AFG_2$ are the same irrespective of the pH value of the solution. Therefore, it seems that SDSCS binder leads to the highest adsorption capacity for aflatoxins under different pH circumstances such as upper and lower regions of GIT [45–51].

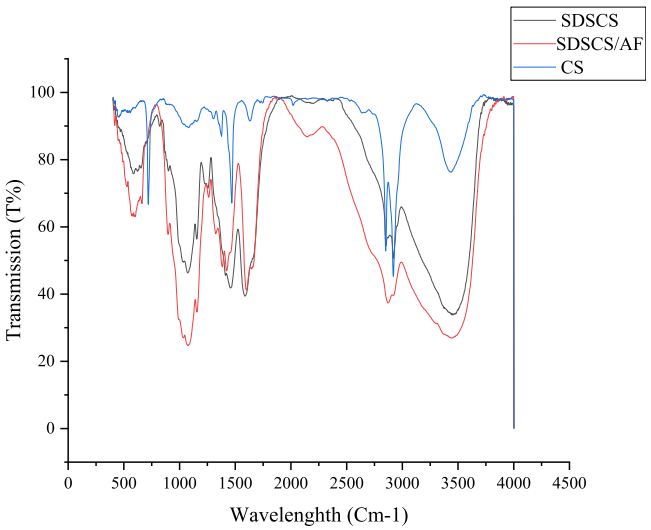



**Fig. 3.** FTIR Spectra of chitosan (CS) and SDS-modified chitosan (SDSCS) before and after removal of aflatoxin (SDSCS/AF).

### 3.3. Physicochemical characterization of SDS-modified chitosan before and after removal of aflatoxin

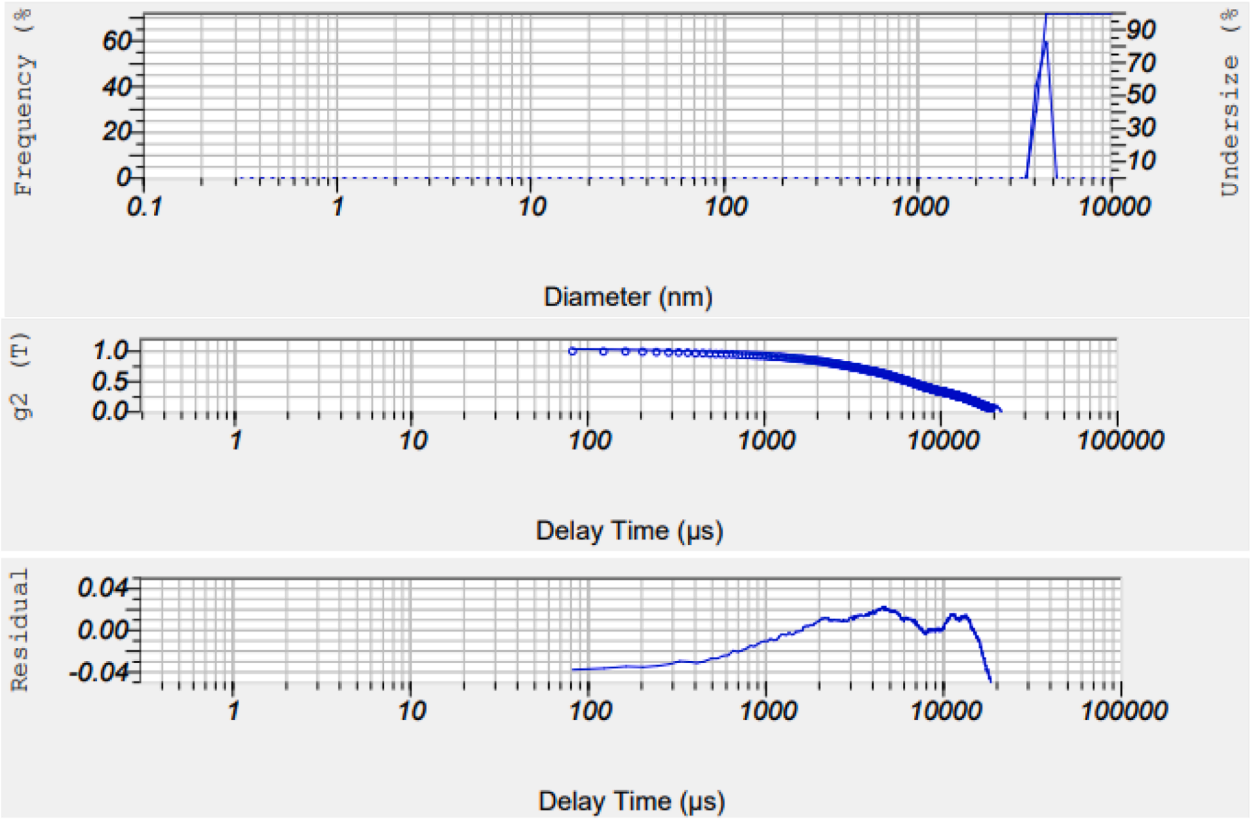
#### 3.3.1. Determination of functional groups in SDS-modified chitosan before and after removal of aflatoxin

FTIR spectra was used to confirm the chemical composition of the synthesized SDS-modified chitosan and to identify functional group interactions with aflatoxin. As depicted in Fig. 3, the FTIR spectrum of chitosan displayed a characteristic broad tongue-like band at 3437.44 $cm^{-1}$ related to OH vibrational stretch, which overlapped with the $NH_2$ stretch due to primary amine; two bands at 2918.65 and 2850.45 $cm^{-1}$ attributed to the stretch of the aliphatic CH in the biopolymer backbone. The two peaks of carbonyl (C═O) at 1747.83 and 1630.43 $cm^{-1}$ characterized the amide I and II groups and at 1469.23 $cm^{-1}$ secondary absorption of the alkenes ($CH_2$) was also found. The spectra were complemented by bands at 1376.65 and 1306.53 $cm^{-1}$ related to C-N stretch, at 1155.25, 1060.24, 719.23 and 452.32 assigned to symmetric and asymmetric bridge stretching vibrations of glycoside bonds (C-O-C). As observed in Fig. 3, SDS-modified chitosan was successfully synthesized by the emerging peaks at 1254.15 (S═O stretch), 1073.96 (C-O-S stretch), 1033.37(C-O-S stretch), 8220.89 (C-O-S stretch) and 661.52 (S-O stretch) $cm^{-1}$ related to the insertion of sulfate group after SDS modification. Also, the characteristic bands of CS were shifted and had lower intensities at 2920.45, 2852.46, 1658.57, 1589.56, 1457.30 and 1413.39 $cm^{-1}$ demonstrating the formation of complex between CS and SDS. The adsorption capacity of SDSCS complex in the removal of aflatoxin was verified with appearance of three new additional peaks at 1323.50, 984.34 and 603.42 $cm^{-1}$ concerning to incorporation of aromatic ring moiety of aflatoxin in SDSCS (Fig. 3). In addition, characteristic bands of the adsorbent shifted and weakened, indicating that there were electrostatic interactions between binder and aflatoxin[52–56].

#### 3.3.2. DLS analysis of SDS-modified chitosan/aflatoxin complex (SDSCS/AF)

When suspended particles interact with monochromatic light, the wavelength of the light is altered upon contact with the particles. Dynamic Light Scattering (DLS) analysis exploits the Brownian motion of these particles in dispersion, with a detector capturing the resulting

**Table 2**
Particle size and size distribution of SDS-modified chitosan complex with aflatoxin.



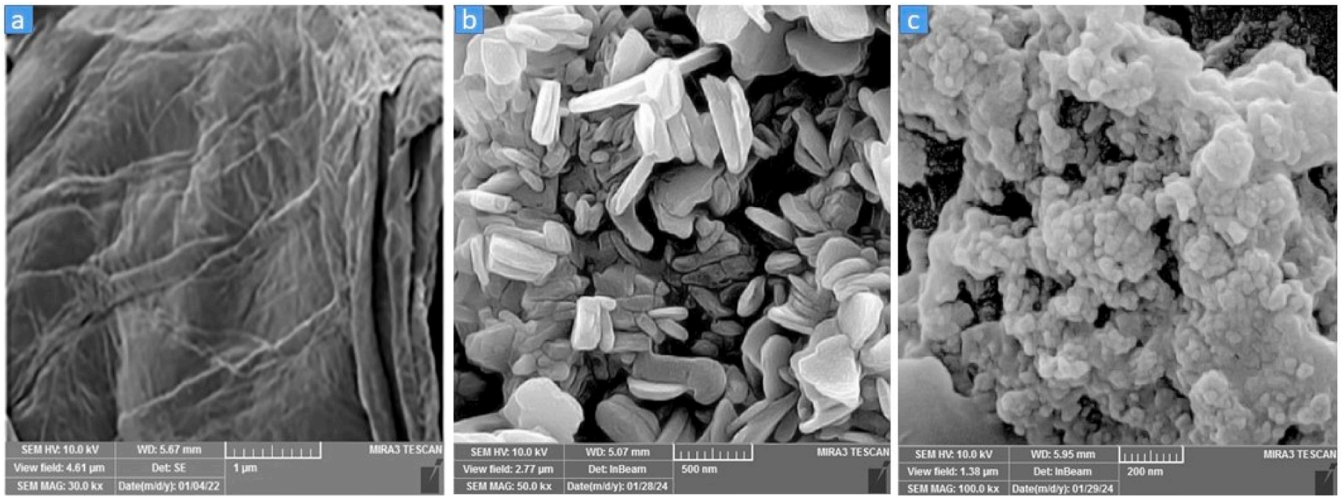




**Fig. 4.** Scanning electron microscope (SEM) images: (a) chitosan, (b) SDS-modified chitosan and (c) SDS-modified chitosan after removal aflatoxin.

signal. As depicted in Table 2, SDSCS/AF particles exhibited a particle size distribution in the range of approximately 2–3 µm. These DLS results suggest that SDS-modified chitosan particles possess an appropriate size, making them suitable for a diverse biomedical and industrial applications [57–63].

*3.3.3. Morpho-structrural alterations of SDS-modified chitosan before and after removal of aflatoxin*

The utilization of SEM facilitated the examination of particles morphology and structural alterations in the neat chitosan crosslinked with SDS (SDSCS), as well as confirmed the incorporation of aflatoxin into the synthesized adsorbent (SDSCS/AF). As shown in Fig. 4a, the morphology of the neat chitosan distinguished by a uniform membrane surface devoid of any discernable pores, which was in accordance with other reports. In the contrary, SDS modification of chitosan (SDSCS) generated strong intermolecular interactions leading to the creation of porous structure with a diverse topological shaped such as spherical, granular, toroidal and globular forms in the network (Fig. 4b); this result

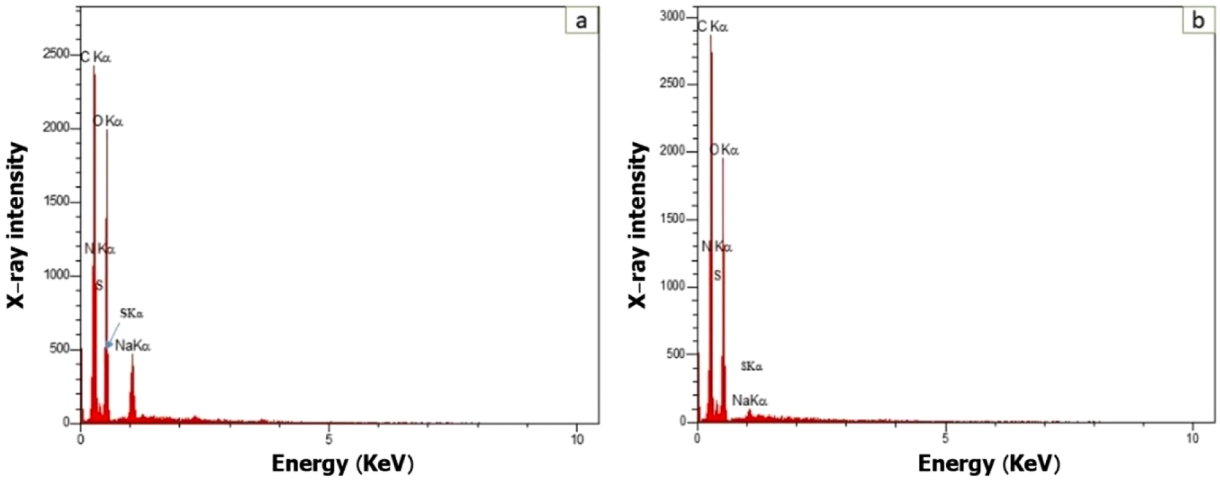



**Fig. 5.** EDS spectrum of (a) SDS-modified chitosan and (b) SDS-modified chitosan after removal of aflatoxin.

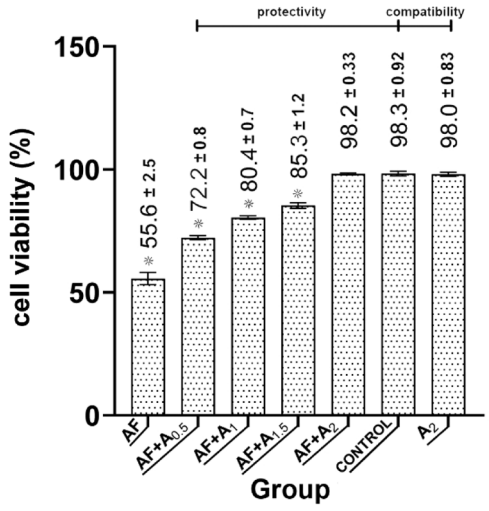



**Fig. 6.** Cytoprotective effect of different doses of SDS-modified chitosan (0.5,1,1.5 and 2 %) against aflatoxin-induced cytotoxicity in $HepG_2$ cells. As shown, the high dose of SDS-modified chitosan ($A_2$) is not toxic to $HepG_2$ cell when compared to the control. The results of triplicate independent experiments are shown as the mean ± SEM; significant difference from the control: * $P<0.01$.

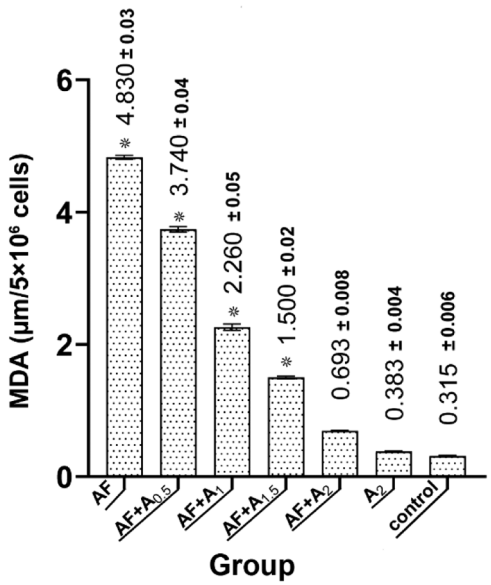



**Fig. 7.** Effects of different doses of SDS-modified chitosan (0.5,1,1.5 and 2 %) against aflatoxin-induced lipid peroxidation in $HepG_2$ cells. The results of triplicate independent experiments are shown as the mean ± SEM; significant difference from the control: * $P<0.01$.

was similar to those reported in other studies. Remarkably, SDS-modified chitosan after removal of aflatoxin exhibited distinct granular shaped particles with different sizes, which was attributable to aflatoxin molecules, became trapped inside the pores of SDSCS network (Fig. 4c). Therefore, the images revealed that the porous SDS-modified chitosan structure was able to adsorb aflatoxin molecules and formed stable SDS-modified chitosan with aflatoxin (SDSCS/AF) complex [64–67].

### *3.3.4. EDS elemental mapping analysis of SDS-modified chitosan before and after removal of aflatoxin*

The chemical composition of the SDS-modified chitosan was also evaluated using EDS analysis which was coupled to the SEM. This technique was also used to measure the chemical species on the adsorbent surface after aflatoxin absorption. The results depicted in Fig. 5a indicates that the carbon (41.80 %), oxygen (44.09 %), nitrogen (11.55 %) and sulfur (2.56 %) were the major constituent of SDSCS adsorbent. As anticipated, additional sulfur peak originating from SDS was identified as the primary contributor to the cross-linking of the chitosan layers. From the Fig. 5b, the EDS analysis of SDS-modified chitosan after removal of aflatoxin indicated that there were increases in the carbon (43.30 %) and nitrogen (13.31 %) and decreases in oxygen (42.87 %) and sulfur (0.52 %). This was associated to uptake of the aflatoxin molecules from aqueous solution onto the SDS-modified chitosan adsorbent [68].

### *3.4. In vitro cytotoxicity assessment*

In order to verify SDS-modified chitosan are not toxic (cytocompatible) to $HepG_2$ cell as well as to evaluate the cytoprotective effect of different dose of the SDS-modified chitosan (0.5, 1, 1.5 and 2 %w/w) against aflatoxin-induced cell death in $HepG_2$ cell, cell viability test (MTT assay) was carried out after 24 h exposure (Fig. 6). Furthermore, MDA and LDH assays were carried out to examine to what extent SDS-modified chitosan could prevent oxidative stress and preserve cell membrane integrity following aflatoxin exposure in $HepG_2$ cell, respectively (Figs. 7 & 8). As observed in Fig. 6, there was not statistical

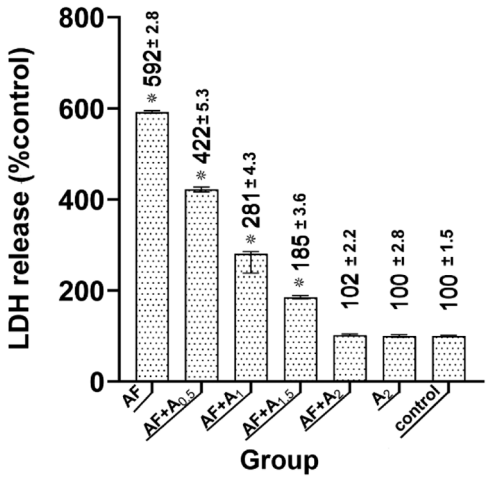



**Fig. 8.** Effects of different doses of SDS-modified chitosan (0.5,1,1.5 and 2 %) against aflatoxin-induced LDH leakage in $HepG_2$ cells. The results of triplicate independent experiments are shown as the mean ± SEM; significant difference from the control: * $P<0.01$.

significant difference of cell viability after 24 h treatment with 2 % SDS-modified chitosan when compared with control group ($p>0.01$). It clearly demonstrated that SDS-modified chitosan was not toxic to $HepG_2$ cell at optimum concentration. Also, the significant decrease in percentage cell viability (56.56 ± 2.5 %) observed after 24 h exposure with 50 % inhibitory concentration ($IC_{50}$) of aflatoxin (5 μM) when compared with control group ($p<0.01$). Furthermore, data analysis indicated a significant percentage of cell viability from low to high doses of SDSCS adsorbent in the cells exposed to 5 μM aflatoxin ($A_{0.5}$: 73.44 ± 0.82 %, $A_1$: 81.79 ± 0.71 %, $A_{1.5}$: 86.77 ± 1.22 % and $A_2$: 99.89 ± 0.01) when compared to the control group ($p<0.01$). Therefore, it can be concluded that SDS-modified chitosan protects $HepG_2$ cells against cytotoxic effect caused by aflatoxin in a dose-dependent manner. Corroborating with physicochemical characterization results of this study, it might indicate aflatoxin participates in a specific binding mechanism with SDS-modified chitosan. The adsorption mechanism may involve attraction of AF into the pores of SDSCS network via dipole-dipole interactions. This adsorption led to reduced availability of AF and, consequently, increased cell viability.

Previous studies have suggested that cell death induced by aflatoxin may involve necrosis and apoptosis. Necrosis is characterized by cell membrane damage, leading to LDH release. So, other clues supporting SDS-modified chitosan can prevent necrosis mediated by aflatoxin is related to examine cell membrane integrity by measuring MDA and LDH leakage from $HepG_2$ cells. It is well known that aflatoxin upon entry into the cell particularly hepatocyte is metabolized by microsomal mixed function oxidase (MFO) to reactive epoxide intermediate. The 8,9 epoxide can bind to vital cellular macromolecules such as phospholipids, DNA, RNA and proteins, causing oxidative stress-induced cell death. As a consequence of oxidative stress, lipid peroxidation (LPO) is initiated by the attack of free radicals to polyunsaturated fatty acids (PUFA) on cellular or organelle membranes. Malondialdehyde (MDA), a toxic aldehyde end product of LPO causes structural changes that mediates cellular and subcellular membrane disintegration. As shown in Fig. 7, the results indicate that aflatoxins induce LPO in aflatoxin-exposed cells while markedly reduction in MDA levels observed in the SDSCS-treated cells from low to high doses (0.5–2 % w/w) concomitantly exposed with aflatoxin (5 μM) in a concentration-dependent manner compared to the control ($p<0.01$). As far as, the high dose of SDSCS-treated cells did not demonstrate statistically significant difference in MDA levels compared to control cells ($p>0.01$).

As shown in Fig. 8, aflatoxin-exposed cells markedly released LDH from inside the $HepG_2$ cells into the culture media due to compromised cell membrane integrity. However, a significant decrease in the LDH release was observed in a dose-dependent manner after treatment with different concentrations of SDS-modified chitosan ($p<0.01$). As far as, the high dose of SDSCS-treated cells did not demonstrate statistically significant difference in LDH release compared to control cells ($p>0.01$).

Collectively, the findings indicate that SDS-modified chitosan serves as an effective protective agent for HepG2 cells against the cytotoxic effects caused by aflatoxin when used at a concentration of 2 % w/w [69–76].

**Table 3**
Comparison of in vitro effectiveness of different bioadsorbent to adsorb and desorb aflatoxin $B_1$ with SDS-modified chitosan.

| Bioadsorbent | Inclusion (% w/w) | $AFB_1$ (μg/ml) | pH | Adsorption (%) | Desorption (%) |
|---|---|---|---|---|---|
| SDSCS | 2 | 6 | 3 and 6.5 | 100 | 0 |
| Grape pomace | 0.5 | 1 | 3 and 8 | 82 | 4 |
| Almond hull | 1 | 1 | 7 | 87 | 6.4 |
| Oven-dried banana peel | 6 | 0.5 | 3 and 9 | 74.9 | 13.6 |
| cellulose | 0.5 | 0.01 | 2 and 6.8 | 31 | NR |
| Lignin (Lettuce) | 0.5 | 0.1 | 2, 5 and 7 | 84 | NR |

## 4. Comparison with other adsorbents

The in vitro effectiveness of SDS-modified chitosan (this study) to adsorb and desorb aflatoxin $B_1$ was compared with those of different bioadsorbents reported in the literature. The comparison is summarized in Table 3. It was regarded the concept of dose (% w/w) was important when comparing the adsorption affinity of different adsorbent [77–81]. Also, adsorption capacity for other aflatoxins ($G_1$, $G_2$ & $B_2$) were not reported to compare with findings of this study.

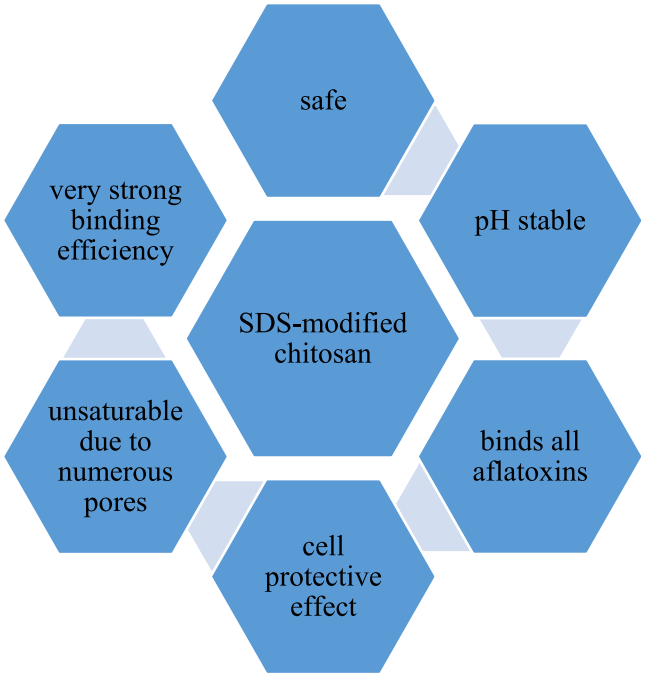



**Fig. 9.** Criteria for selecting the SDS-modified chitosan as a potential mycotoxin binder.

## 5. Conclusion

Chitosan cross-linked with sodium dodecyl sulfate (SDSCS) as a bioadsorbent was successfully synthesized at different concentrations ranging from 0.5 % to 2 % w/w with the aim of improving its adsorption capacity for removal of aflatoxins. Physicochemical characterization of SDS-modified chitosan using advanced analytical techniques such as scanning electron microscope (SEM) coupled with energy dispersive X-ray spectroscopy (EDS) and Fourier transform infrared spectroscopy (FT-IR) were carried out before and after removal of aflatoxin. In vitro pH analysis clarified as the concentration of SDSCS increased, the adsorption efficiency increased. So, SDS-modified chitosan at concentration of 2 % w/w indicated optimum adsorption dose which completely adsorbed all aflatoxins ($B_1$,$B_2$,$G_1$,$G_2$). Also, the findings demonstrated effect of pH on adsorption behavior of SDSCS towards aflatoxins was negligible, which was in accordance with FT-IR analysis showed the intramolecular interactions of the amine groups of chitosan and sulfate group of SDS formed a stable complex at different pH conditions. Notably, SEM images revealed that the porous SDSCS network was filled with aflatoxin molecules supported with EDS findings. Moreover, in vitro cytotoxicity assessment demonstrated that SDS-modified chitosan protected $HepG_2$ cells against cytotoxic effect caused by aflatoxin. Taken together, it seems that SDS derivatization of chitosan generated more active site in the SDS-modified chitosan, increasing the affinity and capacity of adsorption of aflatoxins (100 % at SDSCS concentration of 2 % w/w) via electrostatic interactions such as hydrogen bond and Van der Waals forces. These findings suggest that the SDS-modified chitosan might be an excellent candidate as a mycotoxin binder additive (Fig. 9). Although in vitro results have been promising to test the adsorption capability of mycotoxin binders, further in vivo testing is required in order to determine actual potential of mycotoxin binders because of there is sometimes a discrepancy between the adsorption effects of mycotoxin binders observed in vitro and in vivo. Also, multi-mycotoxin adsorption experiments should be conducted in order to examine competitive biosorption.

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## CRediT authorship contribution statement

**Afsaneh Moghaddam Jafari:** Writing – original draft, Visualization, Validation, Investigation, Formal analysis, Data curation. **Amir Moghaddam Jafari:** Writing – review & editing, Writing – original draft, Visualization, Validation, Supervision, Project administration, Methodology, Investigation, Funding acquisition, Data curation, Conceptualization. **Asma Golmakani:** Visualization, Validation, Methodology, Investigation, Formal analysis, Data curation.

## Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper. Amir Moghaddam Jafari reports financial support was provided by Khorasan Razavi Technology and Science Park, Mashhad, Iran. Amir Moghaddam Jafari reports a relationship with Khorasan Razavi Technology and Science Park, Mashhad, Iran that includes: funding grants. If there are other authors, they declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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## Data availability

Data will be made available on request.

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