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وحماية المستهلك

EVALUATION OF THE EFFICIENCY OF CHITOSAN PRODUCED FROM THE STALKS OF *AGARICUS BISPORUS* BROWN AS AN ANTIFUNGAL AGAINST *ASPERGILLUS FLAVUS* AND REDUCING AFLATOXIN B1

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ABSTRACT

The aim of this study was to benefit from the remnants of edible mushrooms production farms in the production of bioactive compounds such as chitosan and characterization it, and then evaluating the efficiency of chitosan in inhibiting growth of *Aspergillus flavus* (*A. flavus*) and preventing it from producing aflatoxin B1 toxins. The prepared chitosan diagnosed via Fourier Transform Infrared (FTIR). Chitosan have been tested in inhibiting *A. flavus* in the concentrations 0.5, 1, 1.5 and 2%. The chitosan inhibition rate of the fungus *A. flavus* has reached 16, 36, 53 and 100% respectively. The effectiveness of chitosan tested in preventing *A. flavus* from producing Aflatoxin (AFB1) in the concentrations 0.5, 1 and 1.5 %. The rate of Aflatoxin B1 production inhibition by chitosan has reached 72.7 ،86.7 and 100% respectively after 21 days of incubation at 25°C after estimating Aflatoxin B1 via High-Performance Liquid Chromatography (HPLC).

Keywords: *Agaricus bisporus*, Chitosan, Aflatoxin B1.

تقييم كفاءة الكايتوسان المنتج من سيقان الفطر *Agaricus bisporus* Brown البني كمضاد فطري تجاه *Aspergillus flavus* واختزال سم الأفلاتوكسين B1

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الخلاصة

هدفت الدراسة إلى الاستفادة من مخلفات مزارع إنتاج الفطر *Agaricus bisporus* Brown (*A.bisporus*) الصالح للأكل في إنتاج المركبات النشطة حيوياً مثل الكايتوسان ومن ثم تقييم كفاءته في تثبيط نمو فطر *Aspergillus flavus* واختزال سم الأفلاتوكسين B1. وجرى تشخيص الكايتوسان بتقانة طيف الاشعة تحت الحمراء (FTIR) Fourier Transform InfraRed ٪. وتم اختبار الكايتوسان في تثبيط فطر *Aspergillus flavus* بتركيز 0.5 و 1 و 1.5 و 2٪. بنسبة تثبيط الكايتوسان للفطر *A. flavus* 16 و 36 و 53 و % على الترتيب.كما اختبرت فاعلية الكايتوسان في منع هذا الفطر من إنتاج سم الافلاتوكسين B1 بتركيز 0.5 و 1 و 1.5٪. اذ بلغت نسبة التثبيط 72.7 و 86.7 و 100 % على التوالي بعد 21 يوم من الحضن في درجة حرارة 25 مْ بعد تقدير سم الافلاتوكسين B1 بأستعمال تقنية الكروموتكرافي السائل عالي الأداء High-Performance Liquid Chromatography (HPLC).

الكلمات المفتاحية : *Agaricus bisporus*، شيتوسان، الأفلاتوكسين B1.

[*]**The research is extracted from the doctoral thesis of the first researcher.**

INTRODUCTION

The chitosan produced from Mushrooms is characterized by many features such as it is hygienically safe as well as the availability of the Mushrooms resources all over the year with the possibility of harvesting the biomass at a low cost via simple fermentation, and producing chitosan from Mushrooms resources does not require demineralization with the possibility of producing high-quality chitosan via treating with acids and bases which makes the process of producing chitosan from Mushrooms resources economically friendly **(Dhillon *et al.*, 2013) .** The demand for chitin and chitosan produced from Mushrooms resources has increased recently according to its distinctive physiochemical characteristics preferred to those produced by traditional crustacean resources and because of the seasonal and limited availability of the crustacean resources as well as the heterogeneous physiochemical characteristics and inconsistent levels to the extent of removing acetyl groups as well as high molecular weight as the traditional method of producing chitin and chitosan reduced the possibility of using it industrially, so fungal chitosan has become a topic of discussion for many modern studies **(Gapsari *et al.*, 2020)**. Many studies which were conducted in Iraq succeeded in extracting chitosan from Mushrooms resources especially the edible Mushrooms such as *Agaricus bisporus* and using chitosan healthily or nutritionally **(Aldulimy *et al.*, 2021; Al Fatima *et al.*, 2021; Fadhil and Mous, 2020)**. Chitosan and its products have got big attention academically and industrially as chitosan has an antifungal activity against plant pathogens and it is used in food industry **(Al-Aubadi, 2021; Al- Aubadi *et al.*, 2020; Salman & Al-aubadi, 2010)**. and chitosan is used medically and pharmaceutically, and in preparing anti-microbial casings as well as manufacturing plasters and removing heavy minerals from water **(Ismail *et al* ., 2015; Taha *et al.*, 2019; Mohsen & Ali, 2022; Yonis *et al.*, 2019)**. The anti-Aflatoxin biocides are used in different parts of the world, and some countries are about to register biocides domestically as an approach towards developing economically friendly antifungal compounds to control the food pollution by fungi. Many studies referred to the role of chitosan in food manufacturing, fungi inhibition and preventing the production of mycotoxins which have cumulative and carcinogenic effects when having them with food item. So, the study aimed to benefitting from the residues of edible mushrooms production farms in producing bioactive compounds such as chitosan and studying its physicochemical properties by FTIR. then evaluating the efficiency of chitosan in inhibiting the growth of *A. flavus* and preventing it from producing Aflatoxin B1.

MATERIALS AND METHODS

Chitosan resource

The residues of *A. bisporus* brown from farms in Baghdad where chitosan was extracted and some of its characteristics were studied according to the study been conducted by **Shahadha *et al.*, (2023)**.

Characterizing chitosan

Chitosan prepared from the stalks of *A. bisporus* brown are characterized by using Fourier transform infrared spectroscopy (FTIR) by mixing dry chitosan with dry potassium bromide in a rate 1: 5 with a ceramic mortar and pestle for 2 min and pressing the mixture by using a hydraulic compressor belongs to FTIR at a pressure of 8 bars for 60 sec. The disc is put in FTIR for being analyzed by using a frequency range between 400 – 4000 cm^{-1} **(Sivakami *et al.*, 2013)**.

Activation of fungus *A. flavus* isolation

The isolation of *A. flavus* is obtained that is characterized on the genetic level and isolated from the local wheatgrass which go back to the marketing of 2020 from a number of silos in Baghdad, and producing Aflatoxin B1 is confirmed by a study conducted by **Mohamed & Al–Shamary, (2022)** in Department of Food Sciences, College of Agricultural Engineering Sciences, University of Baghdad. Fungi isolation of *A. flavus* is activated by using Potato Dextrose Agar (PDA) media, then the plates are incubated at 25°C for 5 d.

Adding chitosan to PDA media

The media is prepared by adding chitosan in concentrations 0.5, 1, 1.5 and 2% for chitosan individually to the PDA media that is sterilized by autoclave at 15 lb (121ºC)for 15 min after cooling it to 50ºC Note that this quantity is calculated from the total size of the media in one flask, as the final size of one media reached 100 mL. then the medium PDA is poured in petri dishes of 9 cm, and then left to solidify **(Saharan *et al.*, 2013)**.

Evaluation of chitosan efficiency in *Aspergillus flavus* growth inhibition on PDA media

A potency test for the extracted chitosan and chitosan nanoparticles that is prepared from the stalks of *A. bisporus* brown is made according to the method mentioned by **El-Mohamedya *et al.*, (2019)** and the inhibition rate is calculated according to the equation described by **(Dewi & Nur , 2017)**.

$$\% \textbf{ inhibition } = \frac{B-A}{B} \times 100$$

where

A: diameter of the growth colony in the test plates

B: diameter of the growth colony in the control plates

spore suspension preparation

Spore suspension was prepared as described by **Cortés-Higareda *et al.*, (2019)**. The spores were counted using hemocytometer and the amount of spores for was adjusted to 10^7 spore/mL.

The inhibition of producing of aflatoxin B1 from *A. flavus* by using chitosan

The test is made according to the method mentioned by **Meng *et al* ., (2020)**. the media is prepared by adding chitosan in the concentrations 0.5, 1 and 1.5 % to the Potato dextrose broth (PDB) media that is sterilized by autoclave 15 lb (121ºC) for 15 min in flasks of capacity 250 mL, as the concentrations are added to both types of chitosan after cooling the PDB media to 50ºC, in each flask by three replicates for each concentration and three replicates are left without adding chitosan as a control treatment. The flasks containing the modified PDB media are inoculated by adding chitosan with 1 ml of the spore suspension of *A. flavus* as each 1 mL contains (1×10^7 spores / mL) and the flasks are incubated in 25ºC for 7 d for producing Aflatoxin.

Extraction of Aflatoxin B1

Extraction of Aflatoxin B1 was carried out according to method of **Kollu *et al.*, (2009)**. the flasks content is filtered by filter papers Whatman No.1 to get rid of fungal biomass. 25 mL of filtrate was transferred to a 250 mL separation funnel, then 100 mL of chloroform was added, the mixture was shacked, expelling gases accumulated in separating funnel as needed, and leaving the separation funnel for 15 min. The lower layer chloroform is collected and passed through filtering papers that having 10 g of anhydrous sodium sulfate Na_2SO_4 which spread by a sterilized spreader to form a homogenous layer above filtering papers. 10 mL of chloroform are added to the upper layer in the separation funnel and the funnel is shacked to

expel the collected gases then the funnel is left on the holder until the two layers separate, the lower layer is collected and passed through filtering papers that contain anhydrous sodium sulfate then the obtained layer of chloroform is collected and evaporated in the rotary evaporator until it gets dry, then it is kept in small pipes and covered with aluminum foil to prevent its exposing to light, and it is kept in freezer in -18^{o}C.

Identification of Aflatoxin B1 using HPLC

Aflatoxin B1 is characterized according to the method mentioned by **Cota-Arriola *et al.*, (2011)**. using a high-performance liquid chromatography HPLC, mobile phase uses acetonitrile : distilled water (60 : 40) by injecting 50 µL from the sample and the flow rate of mobile phase is 1.2mL/min , and a florescence detector is used to detect fungal toxin according to the wavelengths (Ex=365 nm , Em = 455 nm).

Diagnosis was made based on a match between Retention time (RT) between extracted AflB1 and AflB1 standard. Concentration of AflB1 was calculated according to the following equation:

$$\textbf{concentration}\ (\mu\textbf{g/mL}) = \frac{\textbf{standard concentration x sample's curve area}}{\textbf{standard poison's curve area}} \times \textbf{Dilution factor}$$

RESULTS AND DISCUSSION

characterization of chitosan

The result showed that the numbers of the functional groups of the chitosan extracted from *Agaricus bisporus* brown, (Figure 1) comparing with the spectra of FTIR for a commercial chitosan sample as a standard sample, (Figure 2). the samples of chitosan under consideration give a similar shape of commercial chitosan.

The active group which represents stretching band Hydroxyl appeared for extracted chitosan and commercial chitosan at the wave numbers 3353.95 and 3350.52 cm^{-1} respectively. While the stretching frequency of the group N-H at frequencies 3291.79 and 3289.20 cm^{-1} for extracted and commercial chitosan respectively. This result agrees with what **Poverenov *et al.*, (2018)** mentioned when testing the chitosan extracted from the stalks and fruiting body of champignon. The amide band of the produced chitosan appeared from the stalks of *A. bisporus* brown, commercial chitosan at frequencies 1643.93 and 1638.77 cm^{-1} respectively which represent carbonyl group (C=O) the (AmideI) whose absorbance value on the wave number 1640 – 1700 cm^{1}.

The bands at the wave number 1586.90 and 1587.75 for the extracted chitosan and commercial chitosan respectively refer to the group (N-H) in the second amide bond (Amide II). While the bands whose absorbance value appeared at the wave number 1373.39 and 1372.06 for the extracted chitosan and commercial chitosan respectively represent the bending vibration for the group C-N (Amide III). The band whose absorbance value appeared at the wave number 829.43 and 893.14 cm^{-1} represent glycosidic bond β- (1,4) in the extracted chitosan and commercial chitosan respectively **(Wu *et al.*, 2019)**.

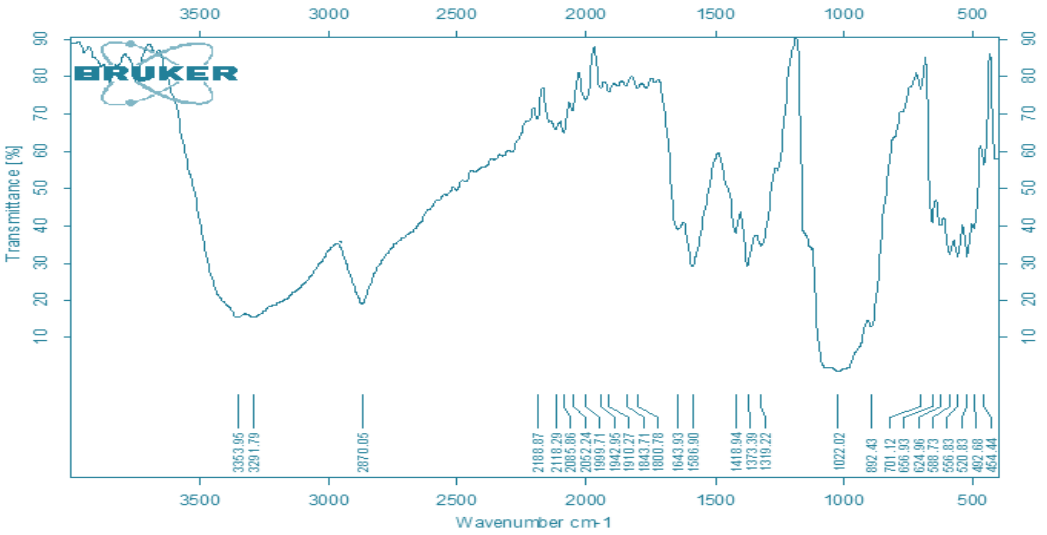

Figure (1): Infrared spectra of the chitosan extracted from the stalks of *A.bisporus* brown.

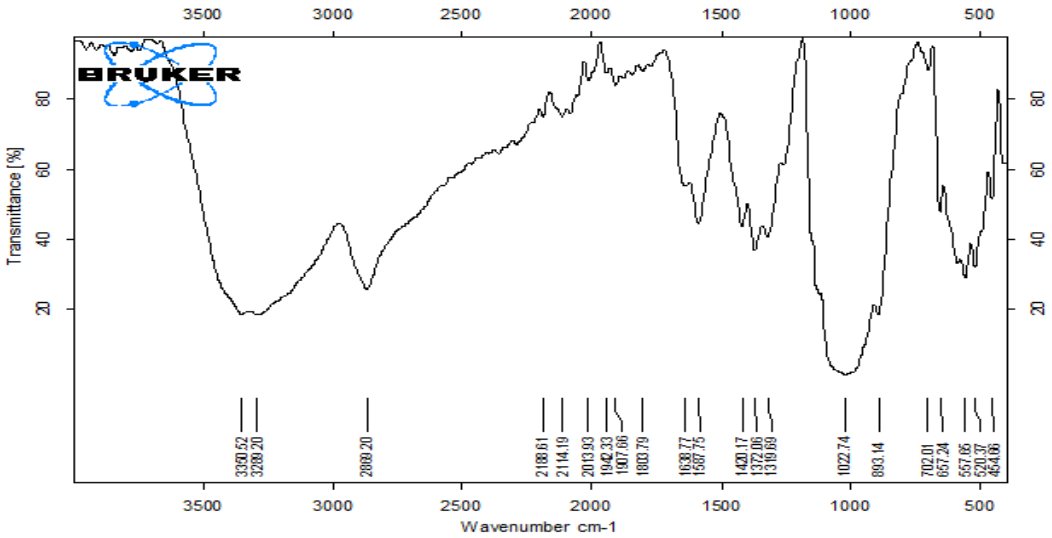

Figure (2): Infrared spectra of commercial chitosan.

Evaluation of chitosan efficiency in *Aspergillus flavus* growth inhibition on PDA media

It is clear from (Figure 3) that the inhibiting potency of chitosan increases directly with increasing concentration. The results show that adding chitosan to culture medium in concentrations 0.5, 1, 1.5 and 2% leads to inhibiting the growth of *A. flavus* 16, 36, 53 and 100 % respectively. these results correspond with what **Dewi & Nur, (2017**) found while he was studying the most potent concentration of chitosan towards some types of *Aspergillus*.

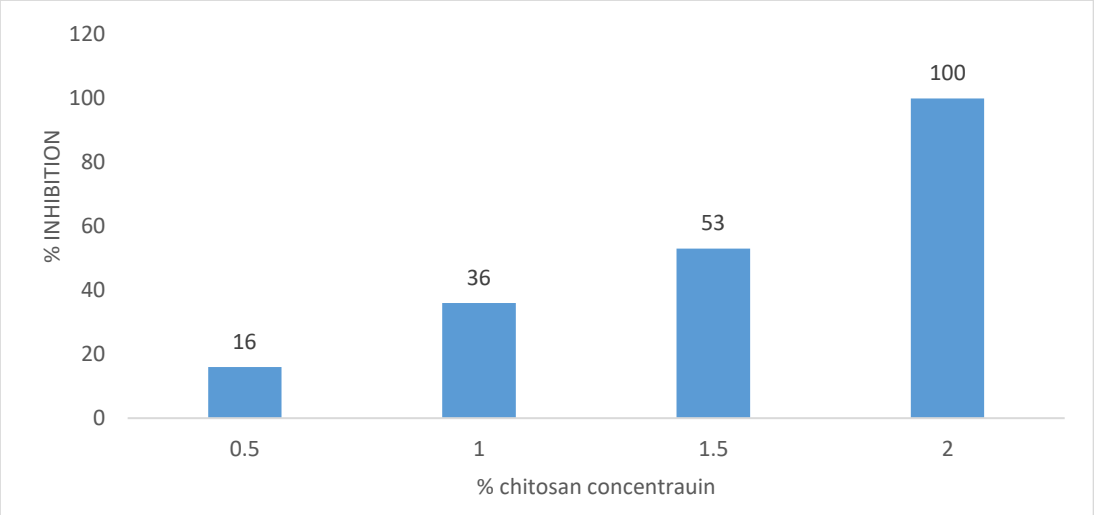

Figure (3): The effect of adding chitosan on the growth of *Aspergillus flavus* by using PDA media

The results contradict the study carried out by **Bukola *et al.*, (2023)**, which included a study of the inhibitory effectiveness of chitosan extracted from shrimp peels against *A.flavus*, which found that the highest inhibition rate was at a concentration of chitosan 0.5 mg / mL, which amounted to 61.7%, in While the higher concentrations 0.25, 0.75 and 1 mg / mL gave a lower inhibition rate, which amounted to 6.80, 20.4 and 12.2 %, respectively, and the reason for this result was explained by the fact that it is possible that chitosan has a stimulating effect on the defensive enzyme as another mechanism for the high activity of the fungus towards chitosan **(Xing *et al.*, 2015)**. This variation in the inhibitory activity of chitosan may be due to several reasons, including those related to the target isolate of the fungus. The varying tolerance of *A. flavus* isolates to different concentrations of chitosan may also be due to the difference in the unsaturated fatty acid composition, which is an essential part of the phospholipids in the layer bilayer lipids in the cell membrane which represent an important factor affecting membrane stability and fluidity, in general, the antifungal activity of chitosan is attributed to the ability of chitosan to easily penetrate fungal cell membranes and then bind to specific enzymes responsible for fungal growth and thus reduce its activities. In addition, increasing the concentration of chitosan increases the density of the cation charge $NH3^{+}$ in the chitosan solution, which can It easily attaches to the fungal membrane and changes its permeability, which in turn leads to the death of microorganisms **(Yien *et al.*, 2012)**.

The inhibition of producing of aflatoxin B1 from *A. flavus* by using chitosan

The rate of inhibiting the production of aflatoxin B1 from *A. flavus* in concentrations 0.5, 1 and 1.5 % for chitosan reaches 72.7, 86.7 and 100 % respectively (Figure 4). These results indicate that in addition to the ability of chitosan to inhibit fungal growth and aflatoxin production, chitosan has the ability to reduce Aflatoxin This result agrees with what was mentioned by **Solís-Cruz *et al.*, (2017)** which indicated that chitosan has the ability to reduce aflatoxin B1 by up to 34%.

The potency of chitosan in reducing Aflatoxin B1 may be due to the capability of these substances to adsorb Aflatoxin by forming bonds between the potent groups of chitosan with

other groups on Aflatoxin that are different in charge, which leads to the conversion of Aflatoxin into less-toxin compounds or toxin reduction, as chitosan contains a group of positive amines while Aflatoxin B1 contains negative oxygen. Aflatoxin, according to its combination, provides 6 positions of oxygen atoms that are available to react with the amino group in chitosan. Mostly, the reaction occurs between chitosan and Aflatoxin B1 at the oxygen atom number 6 according to energy and the negative charge which is higher than the oxygen in that position. Reaction occurs in the other positions depending on energy, so it is possible to say that the possibility of adsorption happens depending on energy, so chitosan can be an electrostatic attracting factor that is responsible for adsorption **(Juarez-Morales *et al.*, 2017)**.

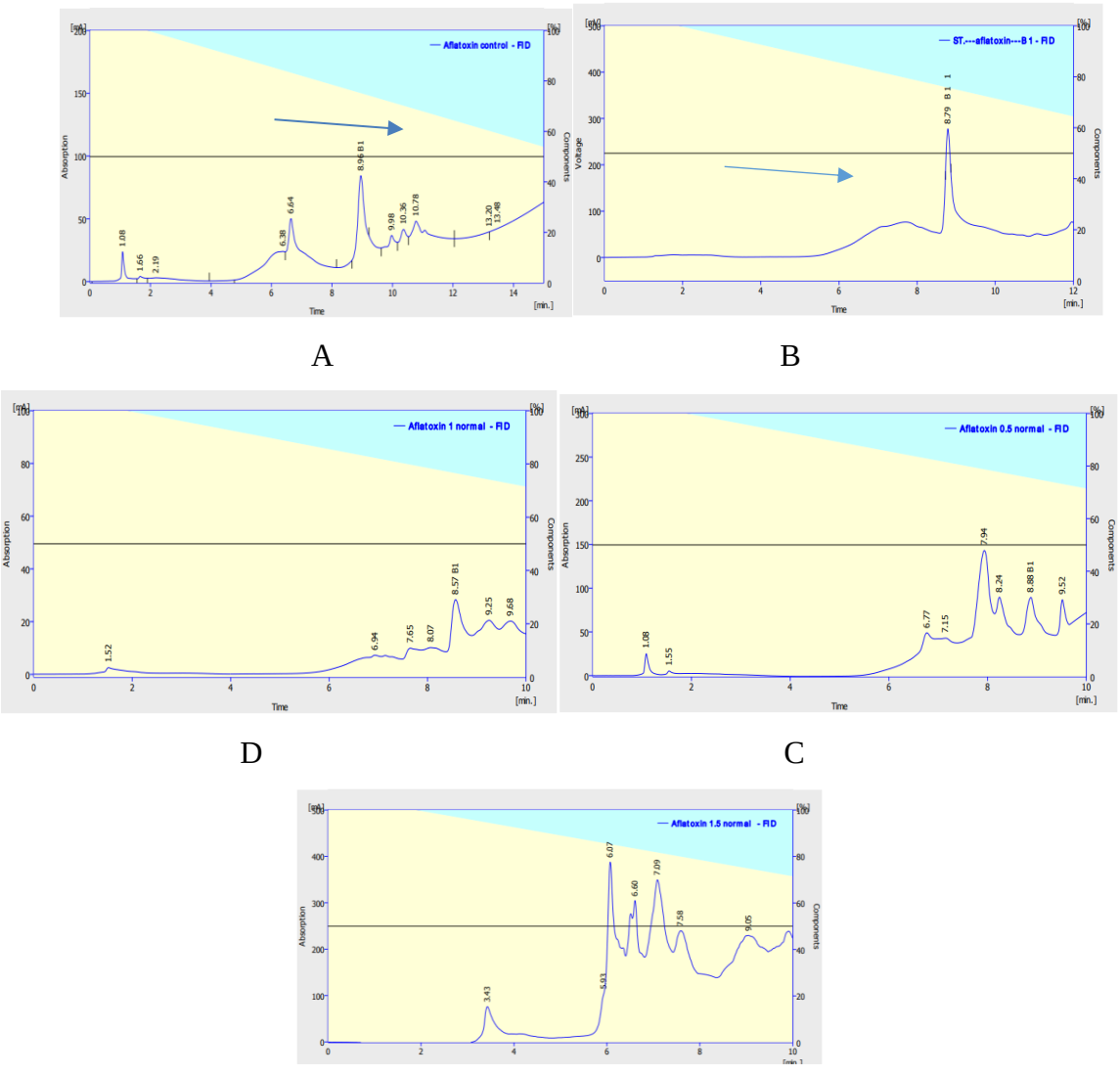

Figure (4): The effect of chitosan in inhibiting the production of Aflatoxin B1 from *A. flavus* by HPLC, (A): Standard Aflatoxin B1 poison, (B) Control coefficient, (C) Chitosan in concentration 0.5%, (D) Chitosan in concentration 1% (E) Chitosan in concentration 1.5%.

CONCLUSIONS

The results of this research showed that chitosan possesses a high inhibitory activity against *Aspergillus* fungus, and it also has effectiveness towards inhibiting the production of aflatoxin B1, as there was a direct relationship between the concentrations of chitosan and inhibiting the growth of the fungus and preventing it from producing the toxin AFB1.

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