Production and Purification of aflatoxin b1 from Local Isolate of *Aspergillus flavus*

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Abstract

Background and Objective: Aflatoxins are cancerogenic compounds produced predominantly by certain strains of the Aspergillus genus. Food and feed contamination by aflatoxin (AF)B_1 has adverse economic and health consequences. Unfortunately, these contaminants can never be completely removed, and on that account, many studies have been carried out to explore an effective process of their detoxification to a threshold level. **Results**: A thermostable enzyme purified from the boiled supernatant was designated as Horseradish aflatoxin-degrading enzyme (HADE). An overall 9.55-fold purification of HADE with a recovery of 39.92% and an activity of 3.85×10^3 U·mg^{-1} was obtained using chromatography on DEAE-Sepharose. peroxidase had an estimated molecular mass of 34 kDa and exhibited the highest activity at 25 °C and pH 7.0. peroxidase is the major protein involved in AFB_1 detoxification. **Conclusion**: PTLC It is reliable as it is a good and reliable method for separating compounds and toxins. It is necessary to study the other compounds that the *Aspergillus fungus* secretes, as they are likely to cause harms more dangerous than aflatoxin B1.

***Key words**: aflatoxin b1, aflatoxins separation, Aspergillus genus , purification by pTLC*

Introduction

Aflatoxins (AFs) are toxic secondary metabolites of *Aspergillus flavus, Aspergillus parasiticus and Aspergillus nomius* [1], and have been found in many crops resulting in serious threat to human and animal health [2]. Although the known AFs have about twenty different forms, four main AFs are commonly found in contaminated food including aflatoxin B1 (AFB1), aflatoxin B2 (AFB2), aflatoxin G1 (AFG1) and aflatoxin G2 (AFG2) [1]. These, AFB1 is considered as the most toxic and many studies indicated that it has genotoxicity, carcinogenicity, embryotoxicity, teratogenicity, immunotoxicity, acute toxicity and chronic toxicity [3]. Therefore, safe and effective strategies are required to remove or degrade AFB1 in food. Many protocols had been conducted for AFB1 removal, mainly including chemical, physical, and biological methods. Using alkaline solution or oxidant to treat contaminated food [4], such as ammonia and ozone, are common chemical methods to degrade AFB1 in food, which have higher degradation efficiency, lower cost and easier operation, but these chemical reagents may cause secondary pollution and have an impact on composition of products. Radiation, mainly including plasma, ultraviolet and electron beam irradiation, is a widely studied physical method for AFB1 degradation, which have been proved capable of AFB1 decomposition and reduced toxicity of its degradation product than native AFB1, but high cost and damage to other components in food limit its wide application [5]. Some cationic clays and microbial cells [6], such as smectite, probiotic bacteria and Saccharomyces cerevisiae, can effectively remove AFB1 through physical adsorption, but

longer treating period and reverse recovery of toxicity result in lower efficiency of physical adsorption [7]. Biological degradation, normally including bacterial fermentation or enzyme catalysis, is another promising method to remove AFB1, which have advantages of mild operation condition, high degradation efficiency and much less toxic products than AFB1. However, for all mentioned methods except radiation, extra substances need to be added into the sample for AFB1 removal, which might result in secondary pollution and extra separation cost, especially for liquid sample. In addition, fast and convenient regeneration of these adsorbents or microorganism also remains significant technical challenges to be addressed. Aflatoxins are resistant to high temperature, strong acid and extremely difficult to be decomposed. At present, there are physical, chemical and biological methods for the elimination of aflatoxins. However, physical and chemical methods are usually limited in practical applications, such as destroying the nutrient substance, incomplete detoxification and high cost of the method[8,9] . The biological method has the advantages of high detoxification efficiency, no damage to food quality and mild reaction conditions [10]. Aims of this study :- production aflatoxins from secondary metabolic of *aspergillus flaves* and Purification aflatoxin b1 and measure by HPLC .

Materials and Methods

This study was accomplished in Genetic Engineering and Biotechnology Institute for Postgraduate Studies/University of Baghdad, during the period from 25/ 10 / 2019 to 1/5/ 2020.

Collection of A. flavus

Ten of commercial feed were randomly sampled from farmed of common crops The fungi of aspergillus fleavas was isolate form the corn crops and culture in PDA media according [11].

Culture Medium and Inoculum

Thirty-nine grams of PDA added to one liter of distilled water, the medium was autoclaved at 121 °C and 1.5 kg /cm^2 for 15 minutes, after sterilization period, the medium was cooled out to 45 °C, then tetracycline antibiotic (250mg /l) was added to prevent bacterial growth. The medium was distributed in Petri dishes (diameter 10 cm) and left to be solid, then stored in the refrigerator until use. A loop full of *A. flavus* spores taken and then diluted to 10^{-4} with 10 ml of water, then from which 100 µl was withdrawn and inoculated on the Potato Dextrose Agar (PDA) plate by spread plate method and incubated at 25±2°C for 7 days. The inoculum prepared by inoculation universal tubes (1.5 by 15 cm) of PDA with fungal spores. The slanted test tubes were incubator at 25±2°C for 17 days, spore suspensions were prepared by added 5 ml of normal saline to each slant culture of *A. flavus* and shaking it, adjusted to approximately 10^6 spores /ml by using a hemcytometer [12].

Produce AFB1 from *Aspergillus flavus*

After the step of PCR diagnose the next step Production aflatoxins by culture in broth media yeast extract dextrose (YED) ,The toxigenic strains of fungus *A. flavus* used for production AFB1 was cultured in sterile medium which Yeast Extract sucrose Broth medium with Peptone (YEB +P) under static conditions , was added, and inoculated by 10 ml of spore suspension (10^6 spores/ ml) and incubated at 25± 2°C for 21 days.

Extraction of AFs from Liquid Culture

After the incubation period, the AFB1 was extracted from culture and filtered through normal filter paper and then through Whatman no.1 filter paper to separate biomass. To the supernatant was added 100 ml of methanol and acetone (70:30) and diluted in water to each Erlenmeyer flasks and shaken for 15 minutes in the orbital shaker. The mixture extracts and then concentrated by the rotary evaporator near dryness. High-performance liquid chromatography (HPLC) was used for quantitative estimation of AFB1 [13, 14] (AOAC, 2005; Yousefi *et al.,* 2016). AFB1 extract from yeast extract broth medium was used as the stock solution of aflatoxins .

Separated aflatoxins (AF_S) in PTLC :

Aflatoxins it is important to be able to separate a mixture into its chemical components in order to isolate one compound or to assess the purity of the mixture. Thin layer chromatography (TLC) is one of the easiest and most versatile methods of doing this because of its low cost, simplicity, quick development time, high sensitivity, and good reproducibility. TLC is used by many industries and fields of research, including pharmaceutical production, clinical analysis, industrial chemistry, environmental toxicology, food chemistry, water, inorganic, and pesticide analysis, dye purity, cosmetics, plant materials, and herbal analysis. In its simplest form, glass plates are coated with a uniform layer of silica gel (SiO2).steps separated AFs The dissolved the mixture of AFs. is placed on the plate, and the plate is inserted into a screw-top jar containing the developing solvent and a piece of filter paper. When the solvent has risen to near the top of the plate, the plate is removed, dried, and visualized using UV ligh (Santiago & Strobel, 2013).depended in The chromatography shows a characteristic profile with no interference in the retention factor (Rf) of the aflatoxins (AFB_1:0.7, AFB_2:0.6, AFG_1:0.5, AFG_2:0.35) and good separation indicating the good specificity and selectivity of the method. We cut solid phase in cleaning extracts for the aflatoxin analysis [15].

Quantitation of AFB1 using HPLC

The High-Performance Liquid Chromatography (HPLC) analysis of AFB1 carried out in the Department of Chemistry / Ministry of Science and Technology, Iraq. The AFB1 standard obtained from Sigma-Aldrich Chemical Co. (St. Louis, MO, USA). The AFB1 was concentrated and loaded on the matrix; elution and carried out with chloroform: methanol (11.76:0.24), at a flow rate of 5 ml/min. The extract was concentrated to the final volume of 5 ml and the amount of AFB1 in the samples was determined using UV spectrophotometer at 365 nm. The purity of AFB1 in the fraction confirmed by High performance liquid chromatography (HPLC– Shimadzu- LC-2010A) with UV detector at 365 nm as per the instructions given in Supelco instruction manual. The stationary phase was C18 Polaris column. A sample of 20 µl was injected, deionized water: acetonitrile: methanol (50:40:10) was used as mobile phase at a flow rate of 1 ml/min.

Results and Discussion

Morphological in PDA petri dish

Ten samples of corn from local market contaminated with fungi colonies Plates were then incubated at 28 C for 3 days and the fungal colonies were isolated on PDA plates the colonies of this isolate on the surface of PDA medium were contained yellow-green spores on the upper surface and reddish-gold. A. flavus cultures were identified by observing the color of mycelia on culture plate having typical lime green or yellow green color of mycelia as described by Fakruddin *et al*., [16]

Microscopic Characteristics

The *A. flavus* Isolates To ascertain their precise identification, the microscopic characteristics (conidiophores, vesicles, mentula, phialides, and conidia) of these isolates were also examined show in Figure 1 Microscopic examination showed that, hyphae are hyaline and septate, and the conidia produce thick mycelial mats while the conidiophores are rough and colorless, phialides are arranged in one and two rows. The conidiophores were uncolored, thick walled, and coarsely roughened or pitted and were vesicle bearing. Their diameter ranged between 800 and 1200 µm. The vesicles were sub globose in some isolates and globose in others and were also variable in diameter, ranging between 1800 and 2000 µm. The cells were either uniseriate or biseriate or both. For biseriate cells, the phialides were borne on the metals, and, in uniseriate cells, they were attached directly to the vesicles. The metulae covered nearly the entire surface of the vesicles and radiated from the vesicles in all directions. The conidia were globose with thin walls, which were slightly roughened.

All previous morphological features are related to Aspergillus flavus and agree with Rodrigus et al.[17]. microscopy and culture remain commonly used and essential tools [18]. Some changes of Diagnose Procedure can enhance the value of these traditional tools, and give first diagnose by microscopic examinations; by recognizing atypical variants of common aspergilli can improve the laboratory's contribution to rapid diagnosis.

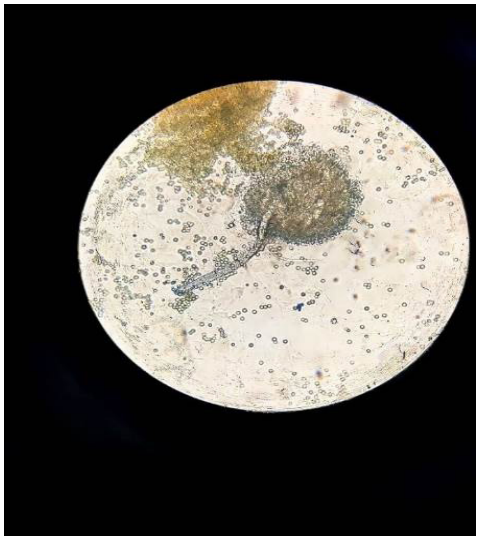

Figure (1) : Microscopic characteristics of A. flavus isolates 40× objective of the Biological Light Microscope.

Plate the fungi *Aspergillus flavus* in media (YES+P)

The result show *A. flavus* in media yeast extract with peptone ,high media Consumed over the days matched by an increase in fungal size ,that mean increase in product of metabolic that contain AFs . that agree with Payne and Hagler, [19], increased mycelial growth is generally associated with increased toxin production .Require an efficient extraction step.

The detection and quantification of aflatoxins .

Require an efficient extraction step. Aflatoxins are generally soluble in polar protic solvents such as chloroform. Thus, the extraction of aflatoxins [20]. This study results shown many of secondary metabolic produce from *A. flavus* when emigrations in Preparative thin layer chromatography (PTLC) (figure. 2),

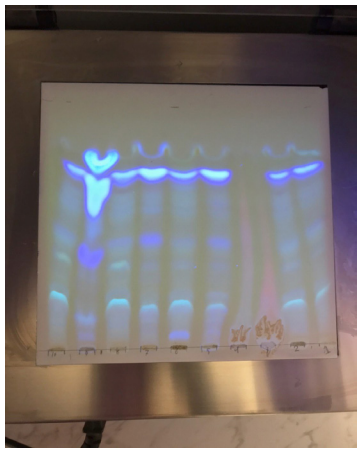

Figure (2): Retention factor (Rf) of the aflatoxins in PTLC with different aflatoxins

(AFB1,AFB2 and another secondary metabolic of fungi).

Depended on the chromatography shows a characteristic profile with no interference in the retention factor (Rf) was of the aflatoxins . Develop plate 50 minutes or until aflatoxins reach Rf 0.4-0.7. remove from tank, evaporate solvent at room temperature and view under long wave UV lamp in a viewing chamber. Observe pattern of 9 florescent spots (Figure.2). All strain of *A. flavus* product AFB1 except 3,4 figure (2): . The results of retention factor (Rf) were of the aflatoxins (AFB_1:0.7, AFB_2:0.6, AFG_1:0.5, AFG_2:0.35) and good separation indicating the good specificity and selectivity of the method. The results agree with Castro and Vargas, [21] who found chromatogram shows retention factor (Rf) of the aflatoxins (AFB1:0.7, AFB2:0.6, AFG1:0.5, AFG2:0.35).

$$RF = \frac{spot\ distance}{SOLVENDISTANCE}$$

Table (1): The retention factor (RF) volume of the aflatoxin in PTLC.

No.of spot	Distance spot	Distance solvent	Rf	Type of spot depended on RF
spot1	12 cm	17 cm	0.7	AFB1
Spot2	11 cm	17 cm	0.6	AFB2
Spot3	8.5 cm	17 cm	0.5	AFG1
Spot4	6 cm	17 cm	0.35	AFG2

Cut spot of the aflatoxins (AFB1) depended on RF factor in PTLC after that dissolved measurer in UV spectrophotometer for chose strain that product high concentration of aflatoxin B1. AFB1 have absorption maxima at 362 nm [23]. AFB1 measured by a UV-detector at 360 ml [22]. A spot that cut from PTLC dissolved in chloroform solvent and measurer UV spectrophotometer AFB1 have absorption maxima at 362 nm [24]. The results shown the outweigh strain 9 to produce AFB1 comparing with another isolated strains (table 2) , this strain (strain 9) which used to produce AFB_1 for test of cytogenetic in this study.

Table 2: Results measurer UV spectrophotometer of AFB1 362 nm .

Strain no.	1	2	3	4	5	6	7	8	9	10
O.D 360	0.065	0.008	0.0	0.0	0.029	0.045	0.006	0.055	0.17	0.018

The study find the strain 9 have high production of aflatoxin b1 and mycelial wight.that agree with [20] .because increased mycelial growth is generally associated with increased toxin production, mycelial growth may increase the AFB_1 production capacity. That result associated aflatoxin b1 production as secondary metabolic so when increase mycelial of fungi that mean increase the product secondary metabolic and aflatoxin b1 just secondary metabolic from *A. flavus* .

- Production AFB1 from strain 9 .

The results as shown in figure.(3) production of aflatoxin B1 as a bar line. Show retention factor (Rf) (in strain 9 only) of the aflatoxins in PTLC and show different aflatoxins (AFB1, AFB2 and another secondary metabolic of fungi) after that cut line we cut only (RF 0.7) as shown in (figure 3) and dissolved in solvent and measure concentration by HPLC as shown in (Figure 4).

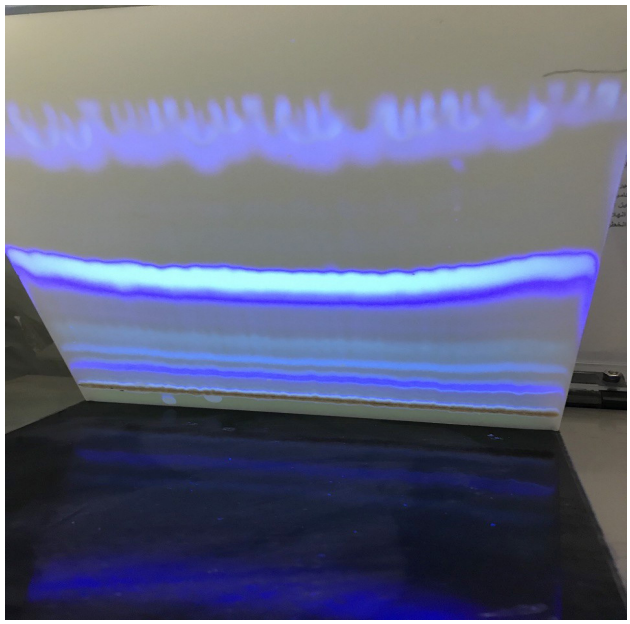

Figure (3) production aflatoxin b1 from strain 9 in PTLC.

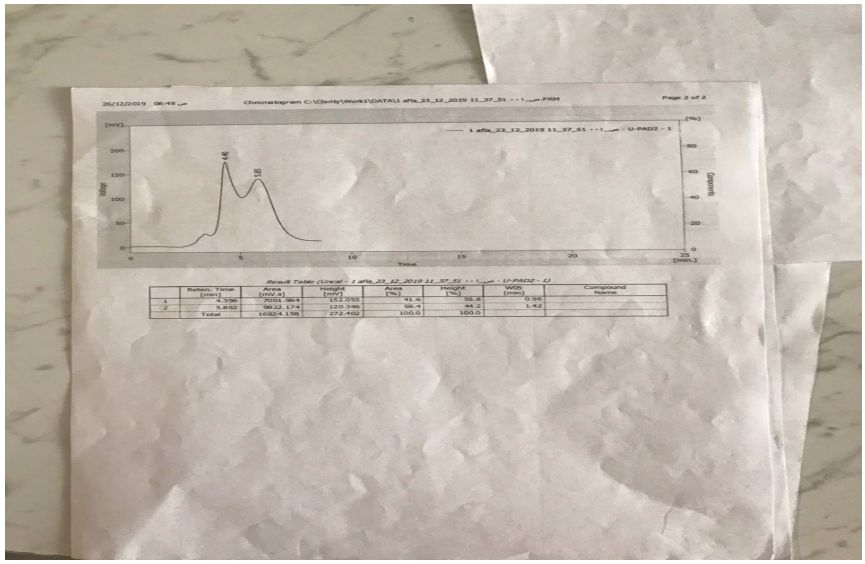

Figure (4) : HPLC measure stander of aflatoxin b1 .

$$con.sample = \frac{C.st \times Are.sam}{Are.st.} \times \frac{D.F}{Vulm}$$

$$con.sample = \frac{0.125 \times 7001}{9733} \times \frac{10}{0.1}$$

$$con.sample = 8.99 \ ppb$$

Conclusion

The tests that detect mycotoxins in the market must be increased and reduced before reaching the final consumer, as they are in such high proportions that they can cause cancer. In cases of necessity, the necessary methods must be followed to reduce or reduce the harmful effect of aflatoxin, especially aflatoxin B1. PTLC It is reliable as it is a good and reliable method for separating compounds and toxins. It is necessary to study the other compounds that the

Aspergillus fungus secretes, as they are likely to cause harms more dangerous than aflatoxin B1.

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Conflict of Interest: Nil

The authors declare that they have no financial conflict of interest.

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Ethical Clearance : Taken from institutional ethical committee.

References

1. Zhuang, Z., Huang, Y., Yang, Y. & Wang, S. Identification of AFB1-interacting proteins and interactions between RPSA and AFB1. *Journal of Hazardous Materials*, *2016*, *301*, 297–303. https://doi.org/10.1016/j.jhazmat.2015.08.053.
2. Xu, L., Zhang, Z., Zhang, Q. & Li, P. Mycotoxin Determination in Foods Using Advanced Sensors Based on Antibodies or Aptamers. *Toxins*, *2016, 8*(8), 239. https://doi.org/10.3390/toxins8080239
3. Azziz-Baumgartner, E., Lindblade, K., Gieseker, K., Rogers, H. S., Kieszak, S., Njapau, H., Schleicher, R., McCoy, L. F., Misore, A., DeCock, K., Rubin, C., Slutsker, L., Nyamongo, J., Njuguna, C., Muchiri, E., Njau, J., Maingi, S., Njoroge, J., Mutiso, J.&,Bowen, A. Case-control study of an acute aflatoxicosis outbreak, Kenya, 2004. *Environmental Health Perspectives*, *2005*, 113(12), 1779–1783. https://doi.org/10.1289/ehp.8384
4. Diao, E., Hou, H., & Dong, H. Ozonolysis mechanism and influencing factors of aflatoxin B1: Areview. In *Trends in Food Science and Technology 2013,* 33, 1, 21–26. Elsevier. https://doi.org/10.1016/j.tifs.2013.06.002
5. Wang, B., Mahoney, N. E., Pan, Z., Khir, R., Wu, B., Ma, H., & Zhao, L. Effectiveness of pulsed light treatment for degradation and detoxification of aflatoxin B1 and B2 in rough rice and rice bran. *Food Control*, *2016, 59*, 461–467. https://doi.org/10.1016/j.foodcont.2015.06.030
6. Verheecke, C., Liboz, T., & Mathieu, F. Microbial degradation of aflatoxin B1: Current status and future advances. *International Journal of Food Microbiology*, *2016, 237*, 1–9. https://doi.org/10.1016/j.ijfoodmicro.2016.07.028
7. Hernandez-Mendoza, A., Guzman-de-Peña, D., & Garcia, H. S. Key role of teichoic acids on aflatoxin B_1 binding by probiotic bacteria. *Journal of Applied Microbiology*, *2009, 107*(2), 395–403. https://doi.org/10.1111/j.1365-2672.2009.04217.x
8. Di Stefano, V., Pitonzo, R., Cicero, N., & D'Oca, M. C. Mycotoxin contamination of animal feedingstuff: detoxification by gamma-irradiation and reduction of aflatoxins and ochratoxin A concentrations. *Food Additives and Contaminants - Part A Chemistry, Analysis, Control, Exposure and Risk Assessment*, *2014*, *31*(12), 2034–2039. https://doi.org/10.1080/19440049.2014.968882
9. Di Stefano, V., Pitonzo, R., Bartolotta, A., D'Oca, M. C., & Fuochi, P. Effects of γ-irradiation on the α-tocopherol and fatty acids content of raw unpeeled almond kernels (Prunus dulcis). *LWT - Food Science and Technology*, *2014, 59*(1), 572–576. https://doi.org/10.1016/j.lwt.2014.04.055
10. Wu, Q., Jezkova, A., Yuan, Z., Pavlikova, L., Dohnal, V., & Kuca, K. Biological degradation of aflatoxins. In *Drug Metabolism Reviews* , *2009*, 41, (1), 1–7. https://doi.org/10.1080/03602530802563850
11. Barwant, M., & Lavhate, N. Isolation and Maintenance of fungal pathogens *Aspergilius niger* and *Aspergilius flvaus*. *2020*, www. iaset. us.

12. Taheri, S., Banaee, M., Nematdoost Haghi, B. & Mohiseni, M. Evaluation of Nephrotoxic Effects of Aflatoxins on Common Carp (Cyprinus carpio). *Iranian Jornal of Toxicology*, *2017*, *11*(2), 51–58. https://doi.org/10.29252/arakmu.11.2.51.

13. Association of Official Analytical Chemist (AOAC) . Official methods analysis. Natural toxins, *2005*, 17th edn. Chapter 49, Gaithersburg, MD.

14. Yousefi S. (2009). Aflatioxin Productions by Asperillus flavus isolates from green-tiger shrimps *(Penaeus semisulcatus)* . Iranian Journal of Microbiology, 2009, 1 (4), . www.SID.ir

15. Paulsch, W. E., Sizoo, E. A., & Van Egmond, H. P. Liquid Chromatographic Determination of Aflatoxins in Feedstuffs Containing Citrus Pulp. *Journal of AOAC International, 1988, 71*(5), 957–961. https://doi.org/10.1093/jaoac/71.5.957

16. Fakruddin, M., Chowdhury, A., Hossain, M. N., & Ahmed, M. M. Characterization of aflatoxin producing *Aspergillus flavus* from food and feed samples. *SpringerPlus*, *2015*, *4*(1), 1–6. https://doi.org/10.1186/s40064-015-0947-1

17. Rodrigues, P., Soares, C., Kozakiewicz, Z., Paterson, R., Lima, N.,& Venâncio, A. *Identification and characterization of Aspergillus flavus and aflatoxins*. *2007*,

18. McClenny, N. Laboratory detection and identification of *Aspergillus* species by microscopic observation and culture: the traditional approach. *Medical Mycology*, *2005, 43*(s1), 125–128. https://doi.org/10.1080/13693780500052222

19. Payne, G. A. & Hagler, W. M. Effect of specific amino acids on growth and aflatoxin production by Aspergillus parasiticus and Aspergillus flavus in defined media. *Applied and Environmental Microbiology*, *1983*, *46*(4).

20. Bertuzzi, T., Rastelli, S., Mulazzi, A., & Pietri, A. Evaluation and Improvement of Extraction Methods for the Analysis of Aflatoxins B 1, B 2, G 1 and G 2 from Naturally Contaminated Maize. *Food Analytical Methods*, *2012, 5*(3), 512–519. https://doi.org/10.1007/s12161-011-9274-5.

21. CASTRO, L. de, & VARGAS, E. A. Determining aflatoxins B1, B2, G1 and G2 in maize using florisil clean up with thin layer chromatography and visual and densitometric quantification. *Ciência e Tecnologia de Alimentos*, *2001*, *21*(1), 115–122. https://doi.org/10.1590/s0101-20612001000100024.

22. Aiko, V., & Mehta, A. Control of Aspergillus flavus and aflatoxin production with spices in culture medium and rice. *World Mycotoxin Journal*, *2013*, 6(1), 43–50. https://doi.org/10.3920/WMJ2012.1437

23. Jubeen, F., Bhatti, I. A., Zargham Khan, M., And, Z.-U.-H. & Shahid, M. Effect of UVC Irradiation on Aflatoxins in Ground Nut (Arachis hypogea) and Tree Nuts (Juglans regia, Prunus duclus and Pistachio vera). In *J.Chem.Soc.Pak* , *2012,* . 34, (6).

24. Santiago, M. & Strobel, S. (2013). Thin layer chromatography. In *Methods in Enzymology 2013*, 533, (303–324). Academic Press Inc. https://doi.org/10.1016/B978-0-12-420067-8.00024-6