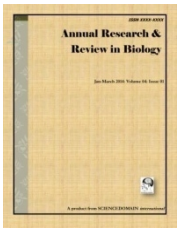

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Effect of *Aloe vera* Extracts on Production of Aflatoxin B1 and Extracellular Proteins by *Aspergillus flavus*

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Authors' contributions

This work was collaboration between the both authors.

Original Research Article

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ABSTRACT

Aims: The purpose of this study is to evaluate the production of aflatoxin B_1 and extracellular protein patterns produced by *A .flavus*.
Study Design: *Aspergillus flavus* is among of fungi that wide dispersal and its massive contamination on feed thus, inevitable to inhibit the growth of this fungus and subsequently production of aflatoxin.
Place and Duration of Study: Department of Animal Science, Faculty of Agricultural Science, Malayer University, between August 2012 and March 2013.
Methodology: The using by acetonic, ethanolic, water, methanolic, chloroform and ethyl ether extracts of *Aloe Vera* fresh leaves, Antitoxin activity of the extracts was evaluated by HPLC. Also, effect of extract on extracellular proteins of *Aspergillus flavus*, analysed by SDS-PAGE technique has been investigated.
Results: The acetone extract of *Aloe Vera* were used to evaluate and study on results obtained from HPLC analysis revealed the inhibition of aflatoxin production in 2000µL in 50mL group for 40.94% and in 2µL in 50mL group for 18.14%. The SDS-PAGE results showed that with decrease in fungal mycelium growth, the proteins production rate was also decreased.
Conclusion: From this study it can be concluded that acetone extracts of *Aloe Vera* can be effective in reducing aflatoxin B_1 and extracellular protein production rates by the fungus *A. flavus*.

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Keywords: Aloe vera; Aspergillus flavus; Aflatoxin B1; HPLC and SDS-PAGE.

1. INTRODUCTION

Medicinal plants are a rich source of antimicrobial agents and normally produce secondary metabolites which can be used in antimicrobial drugs [1]. *Aloe vera* is a succulent plant species originated in northern Africa. The species is frequently cited as being used in herbal medicine since the beginning of the first century AD. Extracts from *A. vera* are widely used in the cosmetics and alternative medicine industries, being marketed as variously having rejuvenating, healing, or soothing properties [2]. There is, however, little scientific evidence of the effectiveness or safety of *A. vera* extracts for either cosmetic or medicinal purposes, and what positive evidence is available is frequently contradicted by other studies. *Aloe Vera* is among of medicinal plants that contains various components including phenol, saponin, anthraquinones, which posses anti-bacteria, antivirus and antifungal activities. One of the microorganisms engaged in oxidation and spoilage of feed are fungi, which decrease the value of feedstuff. Among all, the dominant form of fungus that could normally grow on stored products in favored conditions are *Aspergillus fusarium* and *penicillium* species [3]. These fungi are toxic and carcinogenic and can produce secondary metabolites, which are called mycotoxins. Among all 18 species of my cotoxins, aflatoxin is considered as the most dangerous species that can have several adverse effects which needs a tight control on growth the pathogenic strains of *A. flavus* and *A. parasiticus* fungi and subsequently aflatoxin production [4]. Consumption of feed and foods tuff contaminated with aflatoxin causes acute or chronic diseases such as liver cancer or can be fatal, if consumed in high quantities [5]. One of the strategies to reduce the adverse effects of aflatoxin problems is preventing mold growth on the substrate [6]. In several studies it has been reported that extracts and powders extracted from various herbs and oils have antifungal activity and some of the mare even inhibit the production of aflatoxin [7]. Pitt et al. [8] investigated the mycelium growth of Botrytis *gladiolorum*, *Heterosporium pruneti*, *Fusarium oxysporium* and *Penicillium gladioli* and found that hydro alcoholic extracts of fresh *A. Vera* leaves strongly controlled aflatoxin production. Various herbs such as Centella asiatica, Areca catechu, Piper betle, Momordica charantia, Cirtrus reticulate and Cassia bakeriana have been studied to growth inhibition of *A. flavus* and results showed that raw ethanol extracts of some medicinal plants can inhibit the fungi growth [9]. Aqueous extracts of plants such as *Lupinus* albus, Ammi visnaga and Xanthium pungens could cause the growth of mycelia and aflatoxin production by *A. flavus*fungus. It has found that extracts of Argemone Mexicana and Cyperus rotundus could control the aflatoxin production through inhibition of *A. flavus* growth [10]. Aflatoxin analysis is based on 3 different methods i.e. TLC (thin layer chromatography), HPLC (high performance liquid chromatography) and ELISA (Enzyme-linked immunosorbent assay) (Bullerman et al. [11]). TLC is an old method which show sless accuracy. HPLC is a chemical method which is able to detect and analyze thelevel of chemical compounds with in a mixture of chemicals. This method is highly accurate and rapid method for the detection and quantification of the samples at the same time). The ELISA is a recent method to determine the aflatoxin content in feeds. It is a rapid and simple with high sensitivity method, though it may give false results, therefore, it is better to compare the results with other methods [12]. Inthisstudy, *Aloe Vera* plant extracts treated with organic solvents of acetone, ethanol, methanol, chloroform, ethyl ether and water and the impact of each extracts on aflatoxin production and extracellular proteins pattern of *A. flavus* fungus were studied employing HPLC and SDS-PAGE methods.

2. MATERIALS AND METHODS

2.1 Initial Preparation of Plant

Aloe vera fresh leaves were treated from the farms of Malayer, (located in the northwestern Iranian province of Hamadan), during September 2012. Later, the fresh leaves washed with distilled water and have disinfected by ethanol70%.Later, they have chopped into the small pieces and were exposed to 60°C for 3 days to get dry. After complete drying, leaf parts were powdered using Electric Grinder.

2.2 Preparation of Extracts

30g of powdered plant material are mixed with 100 mm of various solvents of acetone, ethanol, water, methanol, chloroform and ethyl ether and kept in room temperature for 72h. Later, the plant contents of each solvent were filtered through Whatman No.1 paper filter and separated part evaporated in 65°C in Water Bath till complete dry. Dried extracts were powdered and again dissolved in small portion in quantities equal to the volume of the respected solvent and distilled water (50:50) and kept in 4°C for further study [13].

2.3 Fungus Strain

Inthisstudy, *Aspergillusflavus* (ATCC5004) were obtained from the Department of Mycology, Pasteur Institute of Iran. Fungi were cultured on Potato Dextrose Agarmedium. The fungus was cultured in the laboratory and preserved. It has been sub-cultured on PDA for 7 days in 28°C and slants were kept in 4°C for further study after 7days of growth.

2.4 Aflatoxin B1 Production Assessment

After evaluation of *A. Vera* leaves extracts of different solvents on inhibition of growth, it has been found that acetone extracts is the best and applicable extracts to investigate the aflatoxin content using HPLC method. Initially, 1mm of *A. flavus* fungus suspension in cubated in 50ml of YESB medium in different conical flasks reflecting 0, 2, 20, 200 and 2000µL in 50mL of *Aloe Vera* extracts and kept in 28°C for 7 days having 3 replicates per treatment. After day 7, grown mycelia on the medium are withdrawn and washed with distilled water to weigh after drying for calculation of fungi growth index. The YESB medium after cleansing is filtered through Whatman No.1 paper filter and sent to the standard laboratory to analyze with HPL C and post cleanup process; samples were analyzed through KHA-S001 method [14].

2.5 Measuring the Extracellular Protein Patters Produced by *A. flavus*

To study the extracellular protein patters produced by *A. flavus*, the standard strain is grown in PDA medium. Spores are washed thoroughly from the fungi plate through a mixture of distilled water and Triton X using a microbial loop and 1mm (105 spore/ml-1) of this suspension is incubated in PDB medium. In sterile condition 0, 2, 20, 200 and 2000µL in 50mL of acetone extract is added into the PDB medium and kept in 28°C in shaker incubator for 7-10 days. These samples are also set in 3 replicates. At the end of 7-10 days, the big particles of fungi mycelia are separated by cleansing, in sterile condition and the rest of medium is centrifuged in 4000rpm for 10 minutes and supernatants were collected and kept in -20°C for SDS-PAGE analysis.

2.6 SDS- PAGE

The obtained proteins from the samples were analyzed by SDS-PAGE. Separating gel (12%) and compressing gel (4%) were used. 20ml of sample and 10µL of 2 x SDS gel loading buffer poured in tube and mixed for 7 minutes in 100°C and 30µL of samples was added to gel. Along with samples, standard marker is also undergone electrophoresis which had protein components in different sizes and with strong bands of about 23 and 60 KDa. Staining was done by using Coomassi brilliant blue G-250.

3. RESULTS AND DISCUSSION

The impact of acetone extract of *Aloe Vera* on production of aflatoxin B1byA. flavus:

As shown in Table1, the acetone extract of *Aloe vera* had the positive impact in reduction in aflatoxin B1 by *A. flavus*. HPLC results revealed that aflatoxin production inhibition in 2000µL in 50mm was 40.94% and in the minimal concentration (2µL in 50mm) found 18.14%, where as in control group (without extract impact), the aflatoxin production was found 77.2ng/g. In concentrations of 20 and 200µL in 50mm of the extract, the produced aflatoxin rates were 52.6 and 59.8ng/g. The dry weight of the mycelium produced in YES medium in concentrations of 0, 2, 20, 200 and 2000µL in 50mm were 1.93, 2.43, 2.86, 3.02 and 3.58g, respectively. Results showed that in YES medium, the fungus production in concentration of 2000µL was not completely inhibited, whereas in the same concentration of PDA medium, it was 100% inhibited.

Table 1. Evaluation of *Aloe Vera* acetone extract on Mycelia Biomass (g) and aflatoxin produced from *A. flavus*in YES medium

Concentrations µlin 50ml	Mycelium Dry Weight (g)	Aflatoxin B_1 ng/g	Aflatoxin production Inhibition (%)
0	3.58	77.2	0
2	3.02	63.2	18.14
20	2.86	59.8	22.54
200	2.43	52.6	31.87
2000	1.93	45.6	40.94

Impact of Acetone extract of *A. Vera* on extra cellular proteins patterns of *A. flavus*:

Due to the dissolution of some of the active compounds of *Aloe Vera* in different solvents, there would be a significant effect on the production and synthesis of some cellular components and metabolites. Some compounds of *Aloe Vera*, such as different enzymes like aliiase, alkaline phosphatase, amylase, carboxypeptidase, catalase, cellulase, lipase, peroxidase, etc. can affect production of fungal proteins during their biosynthesis pathway. Production profile of fungal extracellular proteins in different concentrations of 0, 2, 20, 200 and 2000µL in 50mL of PDB medium inoculated with *A. flavus* were analyzed by SDS-PAGE method. After incubation for 7 days at 28°C and mycelia isolation and sample preparation, SDS-PAGE was performed (Fig. 1). In this analysis, 24 protein bounds were observed with the molecular weight between 10 and 140 KDa. The results showed that due to the decline in the growth of fungal mycelium, protein production rate has also declined.

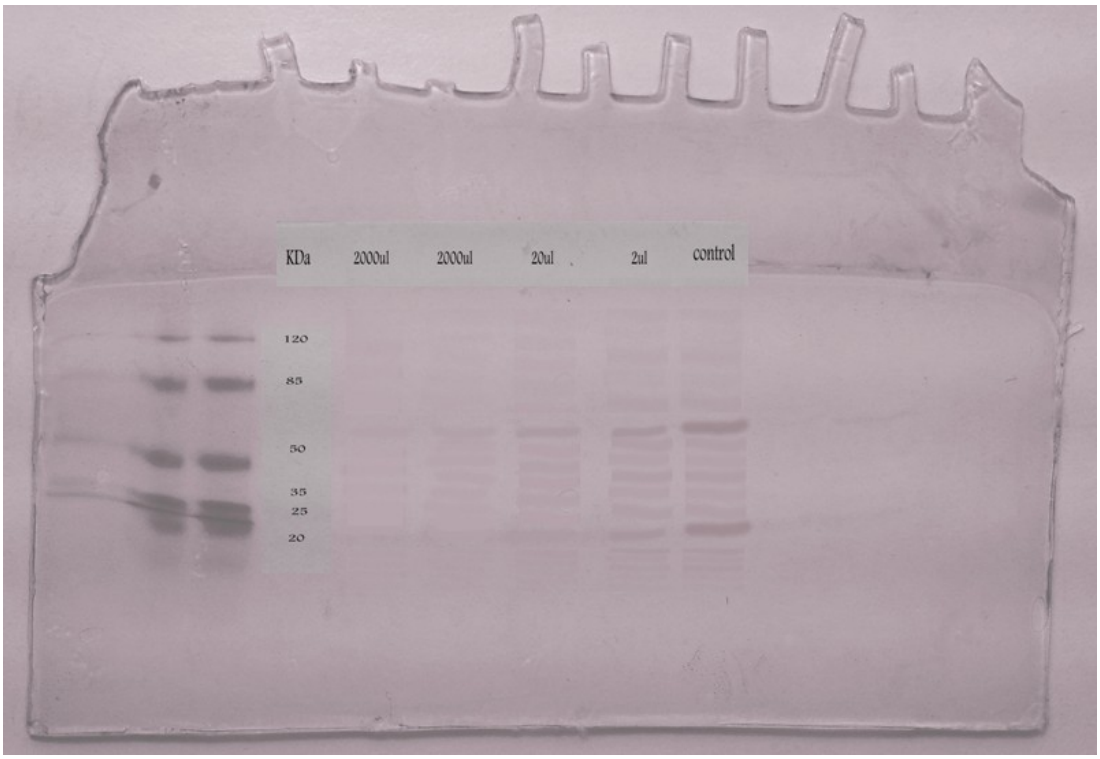

Fig. 1. The fungal extra cellular proteins pattern in different concentrations of 0, 2, 20, 200 and 2000µL in 50mL of PDB medium, inoculated with *A. flavus* by SDS-PAGE.

Considering the ability of rapid growth rate of the fungus *A. flavus* on feed and food stuff, and can damage in the field of food industry, health and economic development, therefore inhibiting fungal growth can greatly help the human community and animal health. Results obtained from this study can provide the basic information about the usefulness and effectiveness of different extracts of *Aloe Vera* plant to reduce the fungal growth and anti fungal activity of this plant. Studies on the antifungal activity of *A. Vera* have been conducted by different researchers across the globe. It has been proved that extracts of *Aloe Vera* have anti-fungal effects on *A. flavus*, *A. glaucus*, *Candida albicans*, *C. tropicalis*, Trichophyton mentagrophytes and *T. rubrum* [15]. The highest antifungal activity of acetone extract of *Aloe Vera* plant was reported on the growth of *A. niger* and *A. flavus*, which are in agreement with the findings of current study [16] .It is found that Hydro Alcoholic extracts from the leaves of *A. Vera*, had the inhibitory effect on mycelia growth of Botrytis gladiolorum, *fusarium oxysporium*, Heterosporium pruneti and *Penicillium gladioli* [15]. Study on the *in vitro* antifungal effect of extracts of *A. Vera* in medium and reported that the plant extract significantly effective on mycelium growth of *Botrytis gladiolorum*, *Fusarium oxysporum*, *Heterosporium pruneti* and *Penicillium gladioli*, compared with the respective control groups. A good number of reports in finding the best suitable methods to control the fungal contamination in foods were performed. In a study of [14], the effect of *A. niger*, as interfering agent in the production of aflatoxin by *A. flavus* was reviewed and concluded that the aflatoxins were not isolated corn which were contaminated with A. niger. They have analyzed the aflatoxin concentration by TLC method and found that in different treatments, the low pH(2.8-3), aflatoxin production completely stops. Study the *in vitro* effect of Neem

Oil on growth, morphology and aflatoxin production by *A. flavus* and found that at concentrations of 0.5 to 4 percent of the oil, aflatoxin production was approximately 95% inhibited, but the fungal growth was not reduced [17]. These treatments completely inhibited fungal growth at 750ppm and both the oils completely in habited the toxin production at concentrations of 750ppm and 500ppm. Some compounds of *A. Vera*, such as different enzymes, including aliiase, alkaline phosphatase, amylase, carboxypeptidase, catalase, cellulase, lipase, peroxidase, etc. can be effective in production of proteins obtained from fungal during their biosynthesis pathway. In this study, the production profile of the fungal extra cellular proteins in concentration of 0, 2, 20,200and2000µLin 50mL of medium by SDS-PAGE were evaluated and results showed that following the reduction in fungal mycelia growth, the produced protein rates are also reduced. It has been studied the effect of UV-C radiation waves on extracellular proteins pattern of *A. parasiticus*, employing the SDS-PAGE technique; found that the 12 proteins produced by this fungus were reduced with UV-Cradiation waves [18].

4. CONCLUSION

According to different solubility of various compounds found in *A. Vera*, in any particular solvent some specific compounds are isolated from this plant, thus, each of the extracts obtained from different solvents, have arrange of antifungal or antimicrobial activities. In this study, among all tested extracts, the highest antitoxin activity of acetone extract on the *A. flavus* production of aflatoxin is found. Although preventing the growth of fungi is the best practices to avoid aflatoxin contamination in food, but other measurements at different stages of its production are also necessary. Therefore, the advantage of the compounds produced by plants as a source of safe, harmless and more effective controlling agents than synthetic antimicrobial agents must be considered.

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

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