Evaluation of the Effect of Neem Extract Formulation on Reducing Aflatoxin- Producing Fungi in Pistachio

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Abstract

Introduction: There is not sufficient information about the effect of neem extract on the growth, sporulation level, and production of aflatoxins by *A. flavus*. Therefore, the aim of this study was to further investigate the effect of neem seed extract on the production of aflatoxin AFB_1 by *A. flavus* in culture medium and on pistachios infected with the fungus.

Materials and Methods : Neem seeds were collected from Bandar Abbas in Hormozgan province from August to October 2016. Consecutive ethyl acetate, water, and hexane methods were used to extract Azadirachtin from neem seeds. After drying, the powdered extract was formulated with Tween 20 and sunflower oil. To evaluate aflatoxin production, Potato dextrose broth (PDB) culture medium containing *A. flavus* (6 log CFU / ml) and 0.3 ml extract were prepared and placed in an incubator for 9 days.

Results : The results showed that the amount of fungal mycelium increased in the presence of ethyl acetate extract from neem seed, but the amount of aflatoxin B1 of this fungus decreased around 17% compared to the control sample (free of the formulated extract using the formulation of 1% of this extract).

Discussion: The effectiveness of the formulated extract is attributed to tetranortriterpenoid such as Azadirachtin A. The use of the obtained formulation showed that in addition to the previous properties reported about neem seed extract, which prevents the plants from Insect damage, it can reduce the damage caused by aflatoxin contamination in pistachio. The 1%formulated of this neem extract had no effect on fungal growth, but the most important difference between studies on neem extract and Azadirachtin extracted in this study is the reduction of aflatoxin production by fungi. In other studies, aqueous extract of neem producing aflatoxin was reduced significantly, while Azadirachtin purified in this study had little effect on reducing aflatoxins**.**

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1. Introduction

Pistachio is native to the Middle East and Central Asia and has grown in countries such as Iran, Syria, Turkmenistan, and western Afghanistan. The rate of pistachio production in Iran was 574,987 tons in 2017 and the United States ranked the second with 272,291 tons. The value of global pistachio exports was 1.81 billion dollars, with US share being equal to 842.75 $ and Iran share equal to 199.36 million $ (11%) [1].

Mycotoxins are secondary metabolites resulting from the activity of various fungi that are produced on agricultural products. By contaminating food, mycotoxins reduce nutritional value and cause economic damages on the one hand, and on the other hand, cause health damages such as chronic and acute primary diseases or have secondary effects such as carcinogenesis, genetic mutation, teratogenicity, and immunosuppression. The term mycotoxin first became prevalent in 1962 when 100000 turkeys died in the UK from an unknown disease. The disease was caused by a toxin produced by *Aspergillus flavus* on birds' food. Following this crisis, scientists suggested that other fungal metabolites may be lethal [2]. Mycotoxin contamination is a global problem, and according to the Food and Agriculture Organization of the United Nations, approximately 25% of the world's seeds are contaminated with mycotoxins, and according to the World Health Organization, mycotoxins are one of the leading causes of foodborne illness [3]. Currently, more than 10000 species of fungi have been identified, most of them are fortunately beneficial to humans and are used in the production of bread, cheese, antibiotics, etc. About 50 species of these fungi are harmful to humans, livestock, and poultry, and if they grow on a food, they produce toxins under certain conditions. Mycotoxins are known as secondary metabolic products, meaning that mycotoxins have no specific roles in the normal metabolism and growth of fungi and are usually produced, but not exclusively, by growing fungi [4]. It is now well established that mycotoxins have been responsible for many chronic diseases in human and animal communities, especially in recent years.

Aflatoxins are derivatives of di furanocoumarin which are produced by the polyketide pathway by many species of *A. flavus* and *A. parasiticus*, which grow in many warm and humid regions of the world, and are produced in many food products such as nuts, cereals, dried fruits, and cottonseed. More than 20 different types of aflatoxins are known, with four types of aflatoxins B1, B2, G1 and G2 having the highest abundance in agricultural products. Aflatoxins, especially aflatoxin B1, are associated with carcinogenicity in humans and animals. Acute aflatoxin toxicity causes death, and chronic aflatoxin toxicity can cause immunosuppression, genetic mutations, and effects the nervous system. In addition, acute hepatitis, Reye's syndrome in children have been associated with poor nutrition, and Kwashiorkor [5] [6].

Nuts, oilseeds, and cereals with high fat and carbohydrate are the most susceptible products for contamination and the most suitable and natural medium for the growth of aflatoxin-producing fungi. Therefore, the presence of different species of Aspergillus in pistachios should be seriously considered to prevent aflatoxin production. Factors such as early cracking and 78-88% humidity facilitate fungal growth and aflatoxin production. Aflatoxin

contamination in pistachios is undoubtedly a problem in Iran and other pistachio producing countries, because its control and monitoring is a constant concern of consumers in order to maintain their safety and health. Iran National Standards Organization has set the maximum tolerance or limit of aflatoxins in pistachios at 8 and 10 ng/g for aflatoxin B1 and total aflatoxin to protect Iranian health [1].

Neem is a fast-growing, evergreen tree native to the Indian subcontinent, Pakistan, Myanmar, Nepal, Bangladesh, Sri Lanka, and the Maldives, and is widely cultivated in Africa and southern Iran. Its different parts have been used as an anti-inflammatory, analgesic, antipyretic, laxative, blood purifier, antibacterial, antiseptic and wound healing agent in traditional medicine. Neem has more than 35 biologically active substances, and Azadirachtin is the predominant active ingredient in the seeds, leaves, and other parts of neem tree [7].

Although it seems that all parts of the neem tree are naturally resistant to pests and diseases, the seeds seem to be the most resistant. Formulations and seed extracts are effective against many types of pests, including gypsy moths, Japanese beetles, aphids, and more. Neem is an inhibitor of aflatoxin production by productive fungi. Various studies have also shown that the aqueous extract of neem plant prevents the production of aflatoxins by the fungus while it has had no effects on the growth of the fungus [8].

Due to the rapid growth of *A. flavus* on food products and its damages to the food industry, health and economy; inhibiting the growth of this fungus could reduce the aflatoxins contaminated food products and help the economy by increasing the export, and contribute to human and animal health [9]. Every year, large amounts of chemical pesticides are used in pistachio orchards to reduce the damage caused by pests. Due to the strategic nature of food products and the agricultural industry in the food safety of each country, the use of chemical pesticides is one of the most important issues in the world. Consumers of agricultural products have expressed concern about the potential residual effects of these compounds on human health and the environment, and policymakers have reacted by revising the existing policies and developing new methods. For this purpose, the European Commission completed a pre-1993 study of pesticides available in the market in 2009 and removed more than two-thirds of these substances. Plant-based pesticides have already been developed in several important areas of health, and although they are small compared to other biotoxin markets, they are expanding day by day. In most cases, this is due to increased public demand for pest management products with less impact on human health and the environment, both in terms of food production ("organic" and "sustainable production") and in terms of public health and pest management. The continued loss of conventional pesticides through regulatory measures or the loss of efficiency due to increased resistance to pests, as well as the recent EU policies to eliminate harmful pesticides, create opportunities for plant-based pesticides. Neem has been used as a natural insecticide for many years due to its active ingredient such as Azadirachtin, and over time it has been used as an antifungal agent. The use of essential oils and extracts of medicinal plants due to their medicinal, antifungal, antibacterial, and antioxidant properties in the pharmaceutical, food, and animal feed industries is progressing and in many recent studies, finding natural plant- based antifungal compounds has attracted the attention of the

researchers. Volatility, low solubility in water, rapid oxidation, chemical instability of plant essential oils in the presence of light, air, humidity, and high temperature are the disadvantages of essential oils. One of the most important solutions to these disadvantages is to provide a new formulation and make some changes to increase the quality and effectiveness of essential oils. Research has shown that crops damaged by insects are more likely to be contaminated with aflatoxins because the fungus has more access to the food source, resulting in easier growth and production of toxins. Since different researches have shown the repellent properties of nectar by neem plant, this study investigates the reduction of aflatoxin production caused by Aspergillus on pistachios using the formulation of neem extract. Considering that in this study the active ingredient is extracted from various compounds and formulated and stabilized in inert oil, it can be said that the results show the effect of pure Azadirachtin on the fungus and toxin production, while in other studies, mostly the mixture of compounds in the extract is used to investigate their effects on the fungus.

2. Materials and Methods

Initial Preparation of Neem Seeds

Fresh neem seeds were collected from August to October 2017 from Bandar Abbas in Hormozgan province. After collection and washing with distilled water, the seeds were shaded for 5 days at 30-40 ° C to dry completely. The kernels were then separated dried, then ground and pulverized by an electric shredder, model T-001 (Dessini Company in Italy).

Preparation of Extract

To prepare the extract according to the patented EP 0 834 254 B1 and optimized invention; 100 g of neem kernel was ground into very fine pieces and stirred in 250 ml of water for 10 hours at 30^{o} C. In the next step, the solid phase (neem seed residues) was separated from the liquid phase by paper filter. 25 ml of ethyl acetate was added to the resulting aqueous and placed at room temperature for 6 hours and then the ethyl acetate phase was separated. In the second stage, 20 ml of ethyl acetate was added and after stirring, the solution was kept at laboratory temperature for 4 hours to complete the extraction operation. The solutions were then mixed and their volume was reduced to one-tenth at vacuum at 45 ° C in a rotary evaporator (Eppendorf Model 5301, Germany). Immediately, 400 ml of normal hexane was added to the hot solution, and then Azadirachtin containing sediment was formed after 30 minutes. After centrifugation (Gallen camp, England), the sediment was placed at 30 ° C for 12 hours to dry completely [10].

Formulation of Neem Seed Extract

To formulate the extract, as the obtained powder was not soluble in water, 3.15 g of the powder containing extracted Azadirachtin was mixed with 25 ml of sunflower oil for 30 minutes by horizontal shaker (Gallen Kamp-England), then it was slowly added to 72 g of stirring twin 20 surfactant and stirred for 30 minutes at 150 rpm to obtain a completely uniform solution.

Cultivation of Fungal Strains

Aspergillus flavus isolate was prepared from the fungal collection of the Plant Diseases Research Department of the Iranian Plant protection Research Institute in Tehran and the fungi were grown on the sloping medium of Potato Dextrose Agar (PDA) (Merck Germany).

Fungi grown in the laboratory were used as fungal storage [11].

Determining the Effective Amount of Neem Seed Extract

In order to determine the level of toxinogenicity of this fungus when it is exposed to different concentrations of Azadirachtin under test to measure the amount of aflatoxin produced by this fungus , and also to analyze the activities of growth inhibition based on minimum inhibition concentration, Zero, 500, 1000, 1500, and 2000 microliters of diluted extract were selected at a ratio of 1/100 (volume of extract formulated to water) and added into Erlenmeyer of 250 ml containing 5 pieces of fungal cloned agar from PDA solid culture medium, and 30 ml of sterile liquid PDB medium., The Erlenmeyer was transferred to a 28 ° C oven equipped with a homogeneous stirrer and then placed in an incubator (Germany memmert) at a constant temperature of 28 +_2° C for 9 days. After filtering the culture medium with Whatman No. 1 paper, the sample was filtered and refrigerated until HPLC analysis [11].

Making a Standard Solution

After preparing standard solutions for each aflatoxin, their concentration was determined using UV visible light spectrophotometer (Varian, CARY 100, USA) and AOAC method number 97/22, and then stored at -20 ° C [12]. These standards were used to prepare mixed standard solutions for HPLC testing.

A solution of a standard mixture of aflatoxins at a concentration of 1000 ng/ml was then made as the storage standard. Solutions of aflatoxin standard with concentrations of 0.4 to 7.2 ng / ml were made as HPLC working standard [13].

To determine the amount of Azadirachtin in the extract, the standard solution of Azadirachtin (> 95% purity) (from Sigma Company) was prepared in methanol (0.5 mg / ml) and stored at -20 °C. A certain amount of the initial standard solution was diluted with the mobile phase immediately before use and solutions containing Azadirachtin were made at 1-25 µg /ml as the HPLC working standard and 25 µl of it was injected into the HPLC [14].

Chromatographic Conditions in Determining the Amount of Azadirachtin

The amount of Azadirachtin was determined according to Kaushik method [15] from instrument, connected to UV detector, model 2489 and solvent isocratic mobile phase consist Water : methanol (60:40 vol: vol) with a flow rate of one milliliter per minute and the reverse phase column (Waters Nova-pak® C-18, 3.9 × 250 mm, 4 µm particle size) at 40°C was used. The detection was performed with an ultraviolet detector at a wavelength of 214 nm. The calibration curve was depicted using the prepared standards and the correlation coefficient was calculated by drawing the relevant curve, and the concentration of Azadirachtin in the extract was calculated using the area under the curve.

Chromatographic Conditions in Determining the Amount of Aflatoxin B1

To monitor the amount of aflatoxins, the reverse phase liquid chromatography of Breeze model of water company connected to fluorescence detector of water[@], model 2475 was used in excitation and emission waveslenght of 360 and 450 nm and pre-column derivatization with bromine (Br) by electrochemical method and using a cobra cell device.

The calibration curve was drawn using the prepared aflatoxin standards and the correlation coefficient was calculated by drawing the corresponding curve, and the aflatoxin concentration in the samples was calculated using the area under the curve for each sample.

For chromatographic separation, the reverse phase column (Waters Nova-pak® C-18, 3.9 × 250 mm, 4 µm particle size) was used at 40 °C. According to the method provided by Iranian National Standard No. 6872, the solvent isocratic washing system: methanol: acetonitrile (20:20:60 volumetric: volumetric) was used as a moving phase with a flow rate of one milliliter per minute [16].

Extraction and Purification of Aflatoxins by IAC Method

Aflatoxins extraction was done using immunoaffinity columns in accordance with National Standard 6872. For this purpose, after diluting 3 ml of the liquid solution medium, 21 ml of phosphate salt buffer was passed through the immunoaffinity column, then the column was washed with 15 ml of phosphate buffer (pH = 7.6) and dried by air flow. Finally, 1.5 ml of HPLC purity methanol was added to the column to collect the adsorbed aflatoxins in a dark vial, then dried in vacuum, then the dried sample was diluted in a mobile phase and 100 µl of it was injected into the HPLC [17].

3. Results

Validation of the Extraction and Identification Method of Aflatoxins

TheLimit of detection, Limit of quantification , accuracy and precision of the test were obtained by extracting a contaminant-free sample at 10 and 5 ng/g levels in 6 iterations, the results of which are shown in Table 1.

Table1: Competency Parameters of Aflatoxins Detection Method

	Mean Rec SPK 10 ppb% (n=6)	RSD%	Mean Rec SPK 5 ppb% (n=6)	RSD%
Aflatoxin B1	84%	6%	91%	9%
Aflatoxin B2	85%	8%	99%	10%
Aflatoxin G1	82%	5%	99%	13%
Aflatoxin G2	85%	9%	95%	7%

	Aflatoxin B1	Aflatoxin B2	Aflatoxin G1	Aflatoxin G2
LOQ (ng/g)	1.15	0.23	1.03	0.19
LOD (ng/g)	0.38	0.08	0.34	0.06

Determining the effective amount of neem extract

Zero, 500, 1000, 1500, and 2000 Microliters of diluted extract on A. flavus aflatoxygenic fungi in immersed liquid culture resulted in 4 to 9% increase in growth by measuring the dried mycelium mass compared to the control group (Table 2).

Table 2: The effect of different dilutions of neem extract formulation on the growth percentage of fungal mycelium and aflatoxin production

Determining the Amount of Azadirachtin in the Formulation and the Amount of Aflatoxin Reduction

Dilution (microliter per 100 ml of water)	AFB2 Reduction %	AFB1 Reduction %	Fungal mycelium growth (n=3)
Control	0%	0%	100%
500	9.12± 5.14	11.36± 8.22	100± 8.61
1000	23.41± 8.53	17.38± 3.26	101±5.14
1500	20.45± 7.79	18.47± 4.83	100±6.59
2000	25.30± 5.66	19.72± 4.37	100±3.74

The results of chromatographic injection showed that the amount of Azadirachtin used in the formulation was 1.26 ± 0.2 wt%, and the use of this formulation in all dilutions was accompanied by a slight reduction in the production of aflatoxin B1. Due to the lack of significant differences in the reduction of aflatoxin B1, dilution of one ml of the extract was used as an effective formulation to evaluate the reduction of aflatoxin in pistachios. The results of the present study showed that formulated neem seed extract can reduce the production of aflatoxin B1 by more than 17±3.4% and the amount of aflatoxin B2 was reduced by 23±8.5 using Aspergillus in vitro.

4. Discussion and Conclusion

Previous research has shown that the extract does not inhibit the growth of the fungus and also increases the growth of the fungus. In this study, the formulated extract containing Azadirachtin did not prevent the growth of fungus. The results of this study were completely consistent with the results of Zeringue et al. in which the addition of neem oil, as in the present study, increased mycelial growth and slightly decreased aflatoxins [18]. In other studies, researchers found similar results using neem oil. For example, Rodriguez et al. found that a dose of 0.5% neem oil could reduce the amount of aflatoxin production by fungi in the laboratory [19]. One of the most important benefits of the formulation used in this study was the purity of Azadirachtin compared to neem oil and, in addition, unlike neem oil, which cannot be mixed with water, it is easily mixed with water and spread in the environment. It is important to note that insects open the way for fungi to enter the plant by causing physical damage to the crop, and one of the most important causes of contamination of agricultural products with fungal diseases or

mycotoxins is the activity of insects and the damage caused to the tissue. Due to the fact that Azadirachtin is well known as an insect repellent, its use, in addition to reducing the production of toxins by the fungus, will reduce the activity of the fungus, and in turn the possibility of fungus penetration into the product and its contamination, and as a result, by using the formulated compound, in addition to the dispersing properties of insects, which was expressed in previous research on neem extract and oil, it is possible to prevent the damage caused by pistachio contamination with aflatoxin.

Based on the results of the study, it can be concluded that there is not significant difference between the extracts obtained in different studies and also the Azadirachtin formulated in this study on fungal growth and Azadirachtin has no effect on preventing fungal growth but the most important difference between the studies conducted on neem extract and the amount of Azadirachtin extracted in this study is the reduced production of Azadirachtin by fungi. In other studies, the researchers found that aqueous extract of neem significantly reduced aflatoxin production, while purified Azadirachtin in this study had little effect on reducing aflatoxins. Therefore, the reduction of aflatoxin in other studies can be attributed to other substances in the extract, which along with Azadirachtin synergistically prevent the production of aflatoxin by fungi, and requires further research.

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