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Studying the Effects of Elements on Early Splitting of Pistachio Nuts and the Effects of Phenolic Compounds on Aflatoxin Control

[1]Hossein Afshari and [2]Hossein Hokmabadi

[1]Islamic Azad University, Damghan Branch, Dameghan, Iran
[2]Pistachio Research Institute, P.O. Box 77175-435, Rafsanjan, Iran

Abstract: In order to study the effects of elements on early splitting and the effects of phenolic compounds and gallic acid on the control of aflatoxins, an experiment was conducted at Rafsanjan Pistachio Research Station. At harvest time fruits of 15 clusters of each variety (Ahmadaghaii, Kalleghuchi, Ohadi) pollinated freely were collected randomly and their phenolic compounds and gallic acid contents were determined. Also from tree of each variety huge numbers of early-split, regular crack and healthy fruits were collected and their green hulls were examined for their macro and micro elements. The tests were conducted by factorial in the form of fully random blocks and the results were analyzed based on Duncan classification by SAS and Sigma Plot in three replicates. The highest amount of nitrogen (2.15%) was found in the hulls of healthy fruits and its minimum was observed in the hulls of early-split and irregular crack fruits (1.94% and 1.97%). Maximum magnesium was observed in hulls of healthy fruit and minimum of it was found in hulls of early-split ones (0.10%). The highest rate of phenolic compounds was found in green hulls of Ohadi fruits (1398mg/100g.) and the lowest rate was observed in green hulls of Ahmadaghaii (1131 mg/100g.) variety. Results indicated that an increase in the phenolic compounds content causes a reduction or control of aflatoxins production in pistachio fruits.

Key words: Aflatoxin · Pistachio · Green hulls · Phenolic compounds · Gallic acid

INTRODUCTION

The growth of pistachio in orchards is related to its soft hull and shell skin. When the fruit matures the soft and hard skins are separated and the splitting of the shell is a significant factor in the marketability of pistachio nuts [1]. Usually the green hulls are not split before harvest to protect the kernels against pets and pollutants. Nevertheless, in fruits experiencing early splitting the green hulls are split along the crack of the hard shell and the kernel is exposed to moulds. Aflatoxins are an important group of mycotoxins produced by *Aspergillus flavus* and *Aspergillus parasiticus* [1-3]. In 1979 Mojtahedi *et al.* [4] reported that fruits of Iranian pistachio orchard were polluted. Doster and Michailidis [5] studied the early splitting phenomenon and its relationship to *Aspergillus fungi* in two years. They reported pistachio fruits having split green hulls were marked with 2 week intervals. They also found, depending on the orchard, 15 to 48% of the early splitting pistachio fruits were formed 4 weeks and 10-30% of them were formed 2 weeks before harvest.

Nutritional factors are probably among the causes of early splitting in pistachio fruits [6] found on the impact of macro and micro elements in green pistachio hulls on the early splitting of 3 pistachio cultivars of Akbari, Kalleghuchi and Momtaz that early splitters, natural fruits and those having growth cracks had different contents of those elements. There was a significant relationship between Mg and Zn concentrations of different green hull types. Mineral sulfur had a significant correlation with early-splitting of pistachios; it showed that early splitters had low sulfur content. Mg concentration in cracked pistachios had a sharp rise. Thus we may be able to control this phenomenon through finding any deficiency in the amount of nutrients contributing to early splitting and spraying them on the fruits. It is unlikely that post harvest processing inhibits microorganism growth and controls aflatoxins completely [7]. However a good strategy will be to search for other natural factors contributing to resistance against *Aspergillus* populations and inhibition of aflatoxin biosynthesis [8]. Such methods will ensure product safety and may even be used for choosing cultivar or genetically bred sorts [9].

Corresponding Author: Hossein Afshari, Islamic Azad University, Damghan Branch, Dameghan, Iran

Recent study has shown that walnut shells produce lower aflatoxin levels than pistachio or almond nut [10]. Though walnut shells are harder they are destroyed by pests so that source of this resistance should be their photochemical structures rather than physical protection [10]. A study by Mahoney and Rodriguez [7] shows included pistachio fruits with and without the soft hulls contaminated with Aspergillus. Test results indicated pistachio with green hulls had lower levels of aflatoxin contamination [7]. Antioxidant properties and elimination of mycotoxins by phenolic compounds, particularly when exposed directly, have been established by many researchers [11-13]. The inhibition properties of phenolic compounds on aflatoxin may be due to the inhibitory activities of enzymes [12]. The goal of this study is to investigate the role of different elements in the early splitting phenomenon and the impact of phenolic compounds and gallic acid on the control of aflatoxins.

MATERIALS AND METHODS

The present study was conducted in 2006 at Iran's Pistachio Research Institute (IPRI) and included three commercial pistachio cultivars known as Kalleghuchi, Ohadi and Ahmadaghaii.

Studying the Elements Found in the Green Hulls of Early Splitting, Cracked Hull and Intact Pistachios of Kalleghuchi, Ohadi and Ahmadaghaii Cultivars: At harvest time we took plenty of early-split, cracked hull and intact fruits of each cultivar from freely pollinated trees and used 5 grams of their green hulls for our tests in three replications to determine their macro and micro elements. In order to determine the rate of highly consumed elements. We used kjahdal(nitrogen), Olsen (phosphor) and flame photometry (potassium). But for elements of low consumption rates and metals we had to covert the plant sample into a mineral substance, which was done by dry ashing method. The procedure included putting a powdered plant sample inside a furnace at 550°C for 6 hours so that it turned into ashes. Then 10 ml 2N HCl was added to the whitened ash and the mixture was placed for half an hour on a Bin Marie (water bath). The produced extract was then taken to 100 ml by distilled water. The extract was used for measurement of potassium, calcium, zinc, iron, manganese, magnesium and phosphorus. In case of calcium we used flame photometry while magnesium, manganese, zinc and iron were treated by atomic absorption instruments. The required solutions were prepared and the calibration for each element was found according to the relevant wave length and finally readouts were taken (2, 21). The test was done by factorial in the form of a fully-randomized design. Results were analyzed by Duncan grouping by SAS.

Studying Phenolic Compounds in the Green Hulls of 3 Cultivars: Kalleghuchi, Ohadi and Ahmadaghaii and Their Effects on the Control of Aflatoxin: At harvest time we took the fruits of all three cultivars from trees with free pollination by random picking method. Total phenolic compounds and the amount of gallic acid - which is a phenolic compound itself- in the green hull were determined. Also kernels of available fruits in each cluster were tested for aflatoxin.The concentration of phenolic compounds in the extracts were determined according to the folin-ciocalteu method [14] and the results were expressed as tannic acid equivalents per gram dry weight of sample (TAE/gdw). The pistachio hull extracts were dissolved in a mixture of methanol and water (2:1v/v). Samples (0.2 ml) were mixed with 1.0 ml of 10- fold- diluted folin-ciocalteu reagent and 0.8 ml of 7.5% sodium carbonate solution; after the mixture had been allowed to stand for 30 min at room temperature, the absorbance was measured at 765 nm using UNICAM 8620 UV- Vis spectrophotometer. The estimation of phenolic compounds in the extracts was carried out in triplicate and the results were averaged [15].

Determining Gallic Acid Content of Phenolic Compounds: Duplicate analysis were performed for every sample from each collection and time period. Methanolic HCl was added to ground pistachio (20 mg) and stirred at 100°C for 1 h. After cooling, aliquots (200 μL) were evaporated to dryness under N2 at 40°C, redissolved in methanol (1.0 ml) and filterd through 0.2 μm nylon syring filters(Gelman). Aliquots(10 μL) were analyzed for methyl gallate and ellagic acid by reversed- phase HPLC on a 250 mm *4.6 mm i.d. Vydac201sp104 C18 coloumn, using a gradient from 100% water containing 0.3% TFA to 100% methanol over 25 min at a flow rate of 1.0 ml/min, with detection at 252 and 280 nm using an Agilent 1100 series diode array detector [9,16].

Determining Aflatoxin Contents in Kernels of 3 Cultivars: The aflatoxin tests were carried out by anindependent accredited laboratory based on AOAC Official Method 999.07 [17]. For 50 g oftest portion 5 g NaCl and 120 ml MeOH and 100 ml hexane is added and blended in high-speed blenderfor 3 min. The mixture is filtered, 20 ml filtrateis mixed with 130 ml distilled water, 75 ml withdrawnand passed by flow rate of 2-3 ml/min from conditioned immunoaffinity column

(EASIEXTRACT, R-Biofarm). The column is washed with 10-20 ml water and dried applying vacuum; 500 ml methanol is passed by gravity, after 1min another 1500 ml methanol is passed; air pressure applied to collect remainder MeOH. For quantification, 200 ml is injected to reverse-phase high-performance liquid chromatography with post-column derivatization using kobra cell and fluorescence detector. LOD was 0.3 and 1.2 mg/kg for aflatoxin B1 and total aflatoxins respectively. LOQ was 1 and 2 mg/kg for aflatoxin B1 and total aflatoxins, respectively. Small samples were homogenized using ultra-turrax prior to extraction. In case the filtered sample was not enough for final quantification of possible higher levels, it is noted in the results that the actual amount is more than the given amount. All the given amounts for aflatoxin concentrations in text are the total amount of aflatoxins in samples, which in nearly all cases is the same as or near aflatoxin B1 concentration [6].

The experiment was done in a complete-randomized design and results were analyzed based on Duncan grouping by SAS and Sigma Plot software applications.

RESULTS AND DISCUSSION

Studying the Elements Found in Green Hulls of Early Splitting, Cracked Hull and Intact Pistachio Fruits of Kalleghuchi, Ohadi and Ahmadaghaii Cultivars: Based on information obtained from variance analysis presented in Table 1 the levels of elements such as phosphorus, potassium, calcium, iron, zinc and boron were statistically significant at a 1% level in the green hulls of the said three cultivars. Nitrogen and magnesium were significant at a 5% level in the hulls of intact, early splitting and growth cracked (cracked hull) fruits. The highest nitrogen content (2.15%) was observed in green hulls of intact fruits and it's the lowest rate was seen in green hulls of early splitting and cracked hull ones (19.4 and 1.97%) (Table 2). The highest rates of magnesium (0.13%) was seen in green hulls of intact fruits while its minimum rate was seen in green hulls of early-splitters (0.10%) (Table 2). There was no significant difference between the interaction of cultivar and fruit types on the elemental levels at 5%. Growth split (cracked hull) pistachios, This kind of hull split takes place in less than 15 days of harvest and is characterized by ragged brown edges and much wider splits than early splits and random orientation of split on hull [4]. In Californian orchards, it is estimated that the incidence of decay by aflatoxin-producing fungi in growth split nuts was substantially lower than that of early-split nuts [5]. It was reported earlier that the *Aspergillus* moulds were found at much higher

Table 1: Studying the Amount of Elements in Green Hulls of 3 Pistachio Cultivars

	Elements					
Variety	B (ppm)	Zn (ppm)	Fe (ppm)	Ca (%)	K (%)	P (%)
Ahmadaghaii	140b	12a	47c	1.3a	7.5a	0.18b
Kalleghuchi	185a	10b	85a	1b	7.5a	0.18a
Ohadi	181a	10b	66b	0.9b	7.1b	0.22a

Table 2: Studying the Amount of Nitrogen and Magnesium in Green Hulls of 3 Pistachio Cultivars

	Elements	
Fruit Type	Mg (%)	N (%)
Intact	0.13a	2.15a
Early Split	0.10b	1.94b
Growth Crack	0.12a	1.9b

Figures in each column were compared. Numbers with similar letters have no significant statistical difference (α=1%)

frequencies in Iran compared to California and pistachio litter is introduced having an important role in the infection of nuts by increasing the amount of Aspergillus inoculum in pistachio orchard [1]. Early-split pistachios, As noticed before, this group was the main source for high contaminations in pistachio nuts because the fungus has the most time for establishment and development of contamination when compared to other kinds of split [6]

The hulls, as the outermost layer of the pistachio fruit, is covered with a cuticle which provides a protective barrier against pathogen attack. Workers have isolated cutinases from pathogenic fungi which degrade plant cuticle and provide a point of entry into the host plant. Wound pathogens, such as *A. flavus* lack the ability to penetrate the cuticular layer. They gain entry into the host plant through breaks in the cuticle caused by abrasions or insects. Pistachio hulls with intact cuticle are resistant to *A. flavus* colonization [7] The lower aflatoxin content of kernels with hulls compared with hulled kernels is also probably the result of the aflatoxin-inhibitory effect of the hulls [1].

Nitrogen accounts for a significant part of the protoplasm and a major group of enzymes and catalysts increasing the speed of plant metabolism processes. It is present in the structures of nucleoproteins, amino acids, amino sugars, poly peptides, etc. Chlorophyll too is a nitrous compound. Nitrous non-protein compounds such as cellulose and pectin, which are very active biologically yet are used in cell membranes to reinforce tissues, play a very critical role in plant structures [18]. So the cracking of the shells of early splitting pistachio shells followed those cracked irregularly may be due to nitrogen

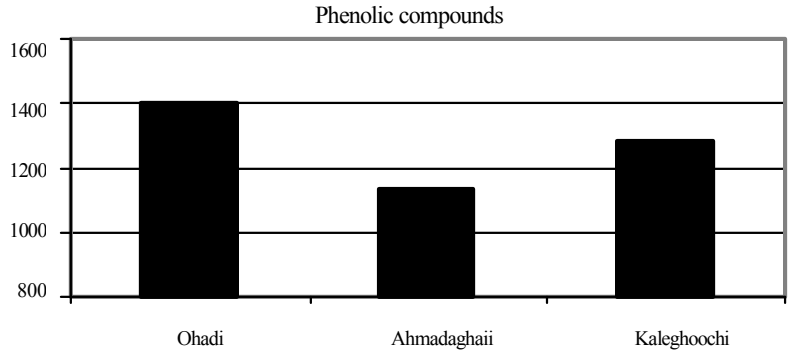

Fig. 1: Penolic compounds in hulls of 3 cultivars

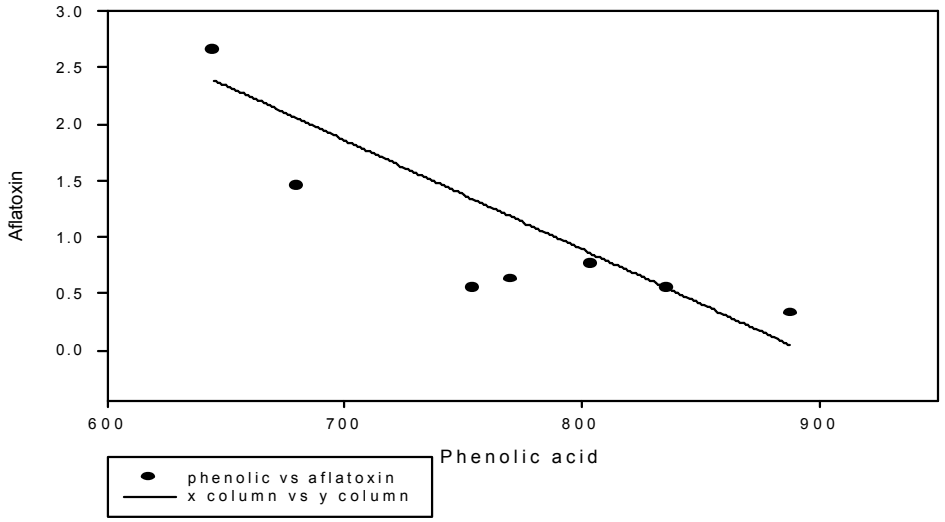

Fig. 2: Relation between aflatoxin of kernels and phenolic compounds of hulls

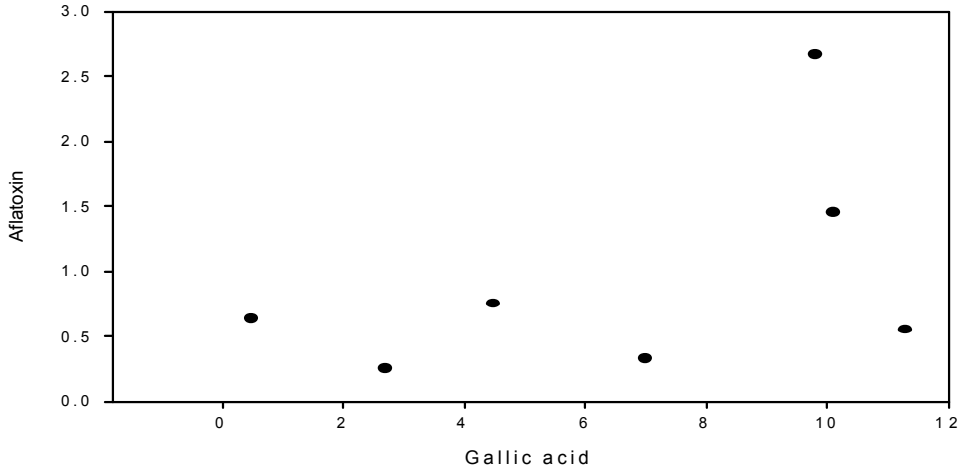

Fig. 3: Relation between aflatoxin of kernels and Gallic acids of hulls

deficiency. Since any sort of *in vivo* or *in vitro* stress directly influences the fruit hull, the need for resistance in the green hulls of pistachio fruits is obvious. The hull's resistance depends on its cell membranes and the polymers forming it. On the other hand, the quantity and resistance of fruits are determined by their hulls. For the same reason, the enzyme of cell membranes controlling the fruit growth should be concentrated in the same spot [19]. Early split pistachios with shriveled hulls contain substantially more aflatoxin than pistachios with early split, fleshy hulls. Shriveled, desiccated hulls may lack the moisture required for aflatoxin colonization, thus limiting growth to the kernel. High aflatoxin levels could accumulate in these kernels since *A. flavus* would not be exposed to the aflatoxin inhibitory effect of the hulls [6].

When the fruits ripen the amount of protein pectin methyl esterase (PME) and mRNA increases; intact and early splitting samples are different in this regard. The enzyme is more active in the soft hulls of intact fruits and causes higher solvability of the pectins, which in turn brings about a decline in the viscosity of matrix compounds and give the hull more elastic power against the pressure of the bony shell. On the other hand, the hulls of early splitters have lower activity of the enzyme which reduced pectin solubility and increased sedimentation at the membranes, which make them more sensitive to pressure. Tayman and Hanada [19] demonstrated that PME activity in precarp of tomato fruits, in which genes were displaced, changed the concentration and distribution of cations to soluble and banded form, Mg^{2+} being the most affected ion. Magnesium (Mg) plays the role of an activator in numerous plant enzymes and in this case we can refer to its role in activating phosphorus carriers involved in the absorption of elements [18]. The results related to magnesium induced changes in the hulls of early splitting pistachios accord with those of Tayman and Handa [19] on magnesium induced changes in genetically modified tomato fruits, in such a way that low PME activity in samples of early splitter hulls changes the grouping of soluble and banded Mg^{2+}. In natural fruits the decline in hull activity towards preserving 2 valence cations at ripening time is evident, which is due to high solubility of pectins in the cell membranes at that time [19, 18]. Disintegration of pectins by polygalactoronase enzyme and the decline of Ca and Mg are effective in the softening of the fruits. They cause such changes in the hulls of intact fruits more than those of early splitters and that brings about more softening and less viscosity of the cell membrane polymers so that it demonstrates higher resistance against pressures applied by the bony shell. On the other hand, due to lower PME and thus PG activity, the hulls of early splitting samples slightly lose their softness and due to their higher viscosity the membranes have no choice but to tear apart against the pressures.

Studying Phenolic Compounds in the Green Hulls of Three Cultivars: Kalleghuchi, Ohadi and Ahmadaghaii and Their Impact on Aflatoxin Control: Based on the results of variance analysis as showed in Figure 1, the amounts of phenolic compounds in the green hulls of tested fruits of three cultivars were significantly different at a 5% level. Yet the difference was not observed in gallic acid in the tested samples of the same 3 cultivars.

According to Fig. 2 among all pistachio clusters in which phenolic compounds were measured, aflatoxin was detected only in the kernels of 7 clusters. Data produced by the linear function $y=8.6149-0.0096x$ were fitted. It was observed that the increase of phenolic compounds in the samples caused a reduction in aflatoxin level following the same linear function (R^2=0.87). In other words it is 87% certain that we place a point of phenolic compounds instead of x on Diagram 2 and it estimates the aflatoxin content of the sample. Based on Fig. 3 dispersion of the points of aflatoxin against gallic acid does not follow a specific trend and there is no function for it either.

Some fungi, particularly *Aspergillus and Penecilium*, have the capacity to grow in an environment containing phenolic compounds as the only source of carbon and may even be cultured in high levels of tannic substances. Among such fungi a special enzyme was extracted from Aspergillus flavus IF05839 family [9]. This extra-cellular fungi enzyme hydrolyzes the ester chains of tannic acid, glucose 1 galat and methyl galat in order. Hydrolyzable tannic substances include a hydrocarbon such as ester glucose with gallic acid ring or hexahydroxy difenic acid with simple structure. It is possible that more complex structures are producible through the connection of extra chains of gallic acid rings with primary ester acids. Valigomeres may be produced from the pairing of oxid etiumonomeres. The presence of the intracellular fungi enzyme is probably useful for the fungi in accessing the glucose rings of tannic substances which is needed for the feeding of *Aspergillus* flavus. The ability of this fungi in hydrolyzing tannic substances indicates that one or more hydrolytic compounds exist that inhibit aflatoxin production [9]. Mahoney and Rodriguez [7] reported *A. flavus* growth on the hull-containing medium is much more luxuriant, with consistently more dense myceial growth and sporulation, than the growth on pistachio

agar alone. Nevertheless, even with the enhanced growth of *A. flavus*, aflatoxin production wasinhibited 71% on the hull-containing medium. Radial growth and sporulation of *A. flavus* were restricted on pistachio agar containing the pistachio hull extract and aflatoxin production was inhibited 99.9%. Carry *et al.* [20] showed in their study that biosynthesis of aflatoxins is inhibited by hydrolysable tannins, such as gallic acid found in green hulls of pistachio fruits and that Fungal Tannase enzyme is responsible for the inhibition of aflatoxin production. They also asserted that culturing fungi in environments containing tannic acid (TA) would reduce their growth. Mahoney and Molyneux [9] also contaminated pistachio fruits both with and without their green hulls with *A. flavus* and demonstrated that only phenolic compounds found in green hulls of pistachio nuts were effective in reducing aflatoxin levels and that was due to the effect of phenolic compounds on enzyme activities [14]. The effect of phenolic compounds found in the green hulls of three pistachio cultivars tested in this study in terms of inhibition of aflatoxin production accords with findings of some other researchers [8, 12]. Gradziel and Wang [21] observed that seed coat could delay *A. flavus* colonization of almond kernels for up to 3 days. Wounded kernels with seed coat also contained an average of 50% less aflatoxin than wounded kernels without seed coats after 7 days, but after 10 days wounded kernels with and without seed coats had similar levels of aflatoxin. Thus, even for kernels with cuticular lesion, which are susceptible to rapid and extensive *A. flavus* colonization, the presence of a seed coat can delay aflatoxin production for several days.

Nut kernel are known to contain high level of phenolic compounds and the aqueous compounds would be converted into volatile derivatives. The only compound detected was methyl gallat, present in trace amounts (data not shown). In sufficient to account for the potent reduction of aflatoxigenecity produced by the extracts. It therefore appeared to be unlikely that the active constituents were discrete low molecular weight compounds. The fact that the activity was extractable by solvent exhibiting a range of polarities and that some of the inhibitory activity was not extractable, suggested that a complex of relatively high molecular weight substances was responsible. The most likely class of compounds was hypothesized to be hydrolizable tannins because pistachio hulls is well established as having high levels of these compounds, which are responsible for the astringency of the nuts [9]. Pistachio hulls contain other phenolic compounds like quinic acid and are much simpler in structure than the other tannins. The stereochemistry of quinic acid eliminates any possibility of dimerization of the gallic acid moieties which would lead to hexahydroxydiphenic acid and subsequently ellagic acid on hydrolysis; gallic and quinic acids are therefore the only possible hydrolysis products [22]. In addition to these compounds, pistachio hulls contain anacardic acids, consisting of an *o*-dihydroxybenzoic acid moiety substituted with a series of lipophilic side chains [13]. The common plant constituent, caffeic acid, a known inhibitor of 5-lipoxygenase (IC_{50} 3.7 μM) and 12-lipoxygenase (IC_{50} 5.1 μM) is also present, together with its quinic acid ester, chlorogenic acid, a non-inhibitor of lipoxygenases [23]. Many of the polyphenolics present in tree nuts are quite common phytochemicals and readily available either from commercial sources or by extraction and isolation from the nuts themselves. Some researchers showed that complexity range of phenolic compounds in pistachios are responsible for inhibitory effect of aflatoxins [22]. Tannins may react with enzymes, inactivating them. Vαzquez-Barrios *et al.* [24] reported that pectinases from fungi could be inactivated by tannins. There are several other examples of tannins inactivating enzymes. Tannins could also form complexes with carbohydrates, alkaloids, vitamins and minerals [25].

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