JOURNAL OF FOOD QUALITY & HAZARDS CONTROL
SHAHID SADOUGHI UNIVERSITY OF MEDICAL SCIENCES & HEALTH SERVICES

Journal of Food Quality and Hazards Control 3 (2016) 87-92

Impacts of Dehydroacetic Acid and Ozonated Water on *Aspergillus flavus* Colonization and Aflatoxin B_1 Accumulation in Iranian Pistachio

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HIGHLIGHTS

- Dehydroacetic acid reduced *Aspergillus flavus* more effectively than ozonated water in Iranian pistachios.
- The least AFB_1 level was detected in dehydroacetic acid treated pistachio group (1.5±0.21 µg/kg).
- Application of dehydroacetic acid is recommend to pistachios industries for AFB_1 prevention.

Article type
Original article

Keywords
Pistacia
Aflatoxins
Aspergillus flavus
Dehydroacetic Acid
Ozone

Article history
Received: 18 Mar 2016
Revised: 7 May 2016
Accepted: 14 Jun 2016

Acronyms and abbreviations
AF=Aflatoxin
DHA=Dehydroacetic Acid
OW=Ozonated Water
PBST=Phosphate Buffered Saline and Tween 20
PDA=Potato Dextrose Agar
HPLC=High Performance Liquid Chromatography
OD=Optical Density
LOD=Limit of Detection
LOQ=Limit of Quantitation

ABSTRACT

Background: Aflatoxins (AFs) are the most prevalent carcinogenic mycotoxins, produced mainly by *Aspergillus flavus* and *A. parasiticus*. The general purpose of the present study was to use Dehydroacetic Acid (DHA) and Ozonated Water (OW) to control *A. flavus* growth and accumulation of Aflatoxin B_1 (AFB_1) in Iranian pistachios.
Methods: Three treatment pistachio groups were separately immersed in DHA (1 N) and OW (10 mg/L), and also combination of DHA and OW. Then, each group was contaminated with 1×10^3 *A. flavus* cell suspensions. Also, sterile distilled water as well as cyclohexamide solutions were considered as negative and positive control, respectively. The solution inhibitory characteristics were assessed by enumeration of viable as well as cultivable fungal conidia on the samples. High Performance Liquid Chromatography (HPLC) technique was used for AFB_1 determination. Data were analyzed by One way ANOVA and Tukey T-tests using SPSS, Inc, Chicago, IL software (v. 16.0).
Results: DHA and combinations of DHA and OW groups which exhibited no growth of *A. flavus*, had the most antifungal activity ($p<0.05$) in comparison with the other groups. The highest AFB_1 contamination content was seen in negative control samples (6.19±0.33 µg/kg) and also OW treated ones (6.1±0.32 µg/kg). The least AFB_1 level was detected in DHA treated group (1.5±0.21 µg/kg) and combinations of DHA and OW (1.59±0.21 µg/kg). However, no AFB_1 was found in positive control samples.
Conclusion: We found that DHA showed antifungal and inhibitory activities more than OW in Iranian pistachios. Application of DHA is recommend for Iranian food industries as a potential mean for prevention of the fungi growth and AFB_1 accumulation in pistachios nuts.

Introduction

Aflatoxins (AFs) are known as potent sources of health risks to both humans as well as animals which can induce mutagenic, carcinogenic, and teratogenic effects in body. These mycotoxins are naturally produced by *Aspergillus*

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To cite: Esmaeilzadeh Nooghi M., Jafari A.A., Sedighi Khavidak S., Jafari H. (2016). Impacts of dehydroacetic acid and ozonated water on *Aspergillus flavus* colonization and aflatoxin B_1 accumulation in Iranian pistachio. *Journal of Food Quality and Hazards Control*. 3: 87-92.

species especially *A. flavus* and *A. parasiticus* that contaminate a wide range of foods and feeds (Eslami et al., 2015; Heshmati, 2010; Heshmati and Milani, 2010; Mason et al., 2015; Williams et al., 2004). AFs are secondary fungal metabolites mixes, including aflatoxin B_1 (AFB_1), AFB_2, AFG_1, and AFG_2 in nature and several synthesized forms such as AFM_1 and AFM_2 in dairy products. However, maximum acceptable levels of AFs in food have been regulated in many countries. It is predicted that over five billion people in the world are exposed to dietary AFs. Also, there are a synergism effect between AFs and chronic hepatitis B virus for developing cancer in endemic area resulted up to 30 times higher risk of liver cancer in individuals exposed to each risk factor alone (Liu et al., 2012; Rahmani et al., 2011; Strosnider et al., 2006). Various sources of human foods, agricultural commodities, fruits, and tree nuts in tropical and subtropical areas of the world may contaminate with AFs (Bandyopadhyay et al., 2007). Maize, peanut, cottonseed, tree nut, pistachio nut, spice, etc. are reported as the most common agricultural commodities with the risk of AFs contamination (Patel et al., 1989).

The main global pistachio market is provided by Iran which 47% come from Iran and 25% come from USA (Van Egmond and Jonker, 2004). In Iran, pistachios are considered as a major crop covering approximately 210000 tons of annual production of dried nuts, having an important role in the agricultural economy of several areas in central parts of Iran. There are many reports in literature for natural occurrence of AF in pistachio nuts marketed in Iran and the other countries. Dini et al. (2013) revealed that 23.4% Iran's pistachio samples were infected with AFB_1 with the mean value of 2.18±13.1 ng/g. In another study, total AF was detected in 28.3% Iranian pistachio samples, having AFB_1 with average content of 5.9 ng/g (Cheraghali et al., 2007). A screening survey using biomarker in Iranian population carried out by Mason et al. (2015) showed that AF was found in 15 of 70 (21%) urine samples indicating high dietary intake level of AFB_1. They found a significant relationship between AFM_1 levels in excreted urine samples and consumption of nuts. Presence of AF in pistachio samples of Algeria was also reported by Fernane et al. (2010). In similar work in Qatar, high AF contamination prevelnce rate in marketed pistachio was reprted by Abdulkadar et al. (2000). It was shown by Arino et al. (2009) that the incidence rate of AFB_1 in pre-packed and bulk pistachios sold in Spain was 19% and 50%, respectively. These researchers indicated that all positive samples were originated from Iran, while pistachios from USA, Turkey and Spain had no AF. Thus, it is obvious that in last decade, high level of AFs contamination in Iranian pistachios has caused the loss of its global market. Therefore, finding more applied and practical methods for AFs control in exported pistachio is necessity.

There are several physical, chemical, and novel biological methods in literature, which were investigated in order to control of AF producing fungi in food and tree nuts commodities (Dalie et al., 2010; Farzaneh et al., 2012; Rahaie et al., 2010; Rahaie et al., 2012; Rastegar et al., 2017; Velluti et al., 2003; Yin et al., 2008). Dehydroacetic Acid (DHA, $C_8H_8O_4$), as a pyrone derivative, is an organic and safe compound with fungicidal and bactericidal effects. DHA is used in food industry to give a taste of pickles, showing potentially antifungal and antimycotoxigenic properties (Durakovic et al., 2010). Ozonated Water (OW) as an antifungal agent is used for degradation of AFB_1 in dried figs (Arita et al., 2005; Zorlugenc et al., 2008). However, there are no comprehensive data about efficacy of DHA and OW in reduction of AFB_1 in pistachio. The aim of present study was to use DHA and OW to control *A. flavus* growth as well as AFB_1 accumulation in Iranian pistachio.

Materials and methods

Preparation of inoculums

In this experimental study, AF producing strain of *A. flavus* (PTCC 5004) was obtained from Iranian Centre of Industrial Bacterial and Fungal Collection in a lyophilized vial. The fungi was cultured on slants of Potato Dextrose Agar (PDA; Oxoid, UK), and stored at 25 °C. The fresh isolated colonies of *A. flavus* were used for inoculation of PDA plates and incubated for 10 days at 25 °C until they were well sporulated. A spore suspension of 0.5 McFarland (1×10^6 CFU/ml) was harvested in sterile Phosphate Buffered Saline and Tween 20 (PBST).

Preparation of experimental groups

Two kg of freshly de-hulled dried pistachios was purchased from Noogh, Kerman province of Iran. Three treatment groups of 500 g pistachios samples were then separately immersed for 15 min at room temperature in 1 N DHA, OW water at the final concentration of 10 mg/L (O_3aq), and combination of DHA (1 N) and OW water (10 mg/L). Also, sterile distilled water and 500 mg/L cyclohexamide solutions (as gold standard for preventing of any fungal growth) were considered as negative and positive control groups, respectively. The immersed samples were then incubated on a 100 rpm reciprocal shaker (Labtron, Iran) for about 2 h at room temperature to treat with the studied chemicals. In this research, all experimental analyses were carried out in triplicate.

Fungi growth inhibitory test

Each pistachio sample separated in five 100 g groups and conveyed in a flask contained 250 ml yeast extract broth (Oxoid, UK), inoculated with 5 ml *A. flavus* conidial suspensions having a final content of 5000 CFU/ml, and after that incubated at room temperature for 3 days on a reciprocal shaker. After that, 5 g of each sample was put in a Petri dish with humid sterile gauze, transferred to incubator at 25 °C for 7 days. All samples were first evaluated for fungal growth, washed with PBST for evaluation of Optical Density (OD) using spectrophotometer apparatus (Perkin-Elmer, Germany). In order to viable conidial counting, 10 µl of each sample was put in 90 µl of normal serum and then spread on saboraud dextrose agar (Oxoid, UK) plates having chloramphenicol with concentration of 50 mg/L. Next, the probable isolated fungi colonies were evaluated and determined as viable conidia cell counts per milliliter (CFU/ml).

AFB_1 analysis by High Performance Liquid Chromatography (HPLC)

After fungi growth inhibitory test, each sample was washed with PBST, dried at 50 °C for 24 h and then sent to Farough reference laboratory (Tehran, Iran) for determination of AFB_1 using HPLC method with immune-affinity column (Jalili, 2016).

Briefly, 5 g homogenized sample was extracted with 0.5 g NaCl and 30 ml methanol:water (2:8) by a high speed blender and the fat was then removed using n–Hexan. The AFs was eluted with a flow rate of 1 ml/min from column and detected at the excitation at wave lengths of 365 and emission at 435 nm with the injection volume of 20 ml. A separate calibration curve was established for each AF in each sample.

Setting of calibration curve and determination of the Limit of Detection (LOD) as well as extraction recovery were carried out according to Siahi Shadbad et al. (2012). The LOD was defined as the analytic concentration that gives a signal equal to: y_b+3.3sb where y_b is the signal of the blank and sb is its standard deviation. Similarly, the Limit of Quantitation (LOQ) was defined as: y+10 sb.

Statistical analysis

Data were analyzed by SPSS, Inc, Chicago, IL software (v. 16.0) with consideration of 0.05 as statistically significant level. Mean values of colonization rate, spectrophotometry and also AF were compared statistically using One way ANOVA as well as Tukey T-tests.

Results

The mean viable cell count and the OD values of treated pistachios have been showed in Table 1. Similar to positive control samples, DHA, and combinations of DHA and OW groups which exhibited no growth of *A. flavus*, had the most antifungal activity (p<0.05) in comparison with other groups. However, lesser antifungal effect was also seen in OW treated samples. The untreated negative control group revealed the highest (p<0.05) viable *A. flavus* conidia (33000±4381.8 CFU/ml) and OD values (0.341±0.215) among all the studied groups. Results of *A. flavus* colonization on each treated and control groups have been illustrated in Fig. 1 to Fig. 4.

There are significant relations (p<0.05) in AFB_1 levels among various treated and control groups. The highest AFB_1 contamination content was seen in negative control samples (6.19±0.33 µg/kg) and also OW treated ones (6.1±0.32 µg/kg). The least AFB_1 level was detected in DHA treated group (1.5±0.21 µg/kg) and combinations of DHA and OW (1.59±0.21 µg/kg). However, no AFB_1 was found in positive control samples.

Discussion

In this research, we showed high inhibition effects of DHA and its combination with OW for *A. flavus* growth and its AFB_1 production. Anti-mycotoxigenic effects of DHA have been previously shown by Durakovic et al. (2010), Durakovic et al. (2011), and Durakovic et al. (2012) in maize, yogurt, as well as soybean, respectively. However, in the present study we chose Iranian pistachio as a new food model to evaluate the antifungal effects of these chemical compounds. Our results were in accordance to the previous mentioned researches in the other food models.

There are some attempts reported for control of AFs in crops and tree nuts either by prevention of AFs producer fungi or detoxification of toxin by the other compounds. Rodriguez and Mahoney (1994) revealed the effectiveness of four surfactants, including triton X-100, tergitol NP-10, triton X-301, and latron for inhibition of AFB_1 production by 96% to 99%. Patel et al. (1989) found that combination of hydrogen peroxide and gamma radiation can reduce inactivate AF in artificially contaminated ground nuts. Similarly, Akbas and Ozdemir (2006) used ozone treatments for the degradation of AFs in pistachio kernels as well as ground pistachios. These researchers reported that AFB_1 and total AFs were reduced by 23% and 24%, respectively, when pistachio kernels were treated by 9 mg/L concentration of ozone for 420 min.

Table 1: Viable *A. flavus* conidia enumaration and OD values in analyzed pistachios in different treated and control groups

Treatment solutions	Viable *A. flavus* conidia (CFU/ml)		Optical density value	
	Mean	Standard deviation	Mean	Standard deviation
1 N DHA	0	0	0.129	0.008
1+1 DHA and OW	0	0	0.143	0.012
OW (10 mg/L)	8716.7	796.03	0.308	0.017
Cyclohexamide (positive control)	0	0	0.113	0.005
Distilled water (negative control)	33000	4381.8	0.341	0.215

Fig. 1: Colonization of *A. flavus* in positive control pistachio group

Fig. 2: Colonization of *A. flavus* in DHA treated pistachio group

Fig. 3: Colonization of *A. flavus* in OW treated pistachio group

Fig. 4: Colonization of *A. flavus* in negative control pistachio group

In the present study, it was found 73.6% reduction in the growth of *A. flavus* in OW treated pistachios in comparison with negative control, indicating the lower antifungal properties than the other test groups that showed complete fungi growth inhibition. However, in spite of DHA, OW did not reduce AFB_1 production and therefore it had not any effective compound in order to AF reduction in pistachio samples. Our results were in accordance with Zorlugenc et al. (2008) that showed the effectiveness of OW for reducing impact of OW on viable fungi without any effect on reduction of AFB_1 production. However, our finding was not in agreement with the results of Bashiri et al. (2013) that reported 32-47% AFB_1 reduction in pistachio treated with 4 mg/ml ozone. It seems that this controversy may be resulted from the 1 N choleric acids which added to OW to regulate the pH

value at 5 as they reported in their paper. In the current study, when 10 mg/ml ozone was added to water, its pH was normally 5 and it was not necessary to add choleric acids. There are some studies in literature that showed gaseous ozone is more useful than OW in order to reduction of AFB_1 in dried figs, red pepper, and poultry feeds (Inan et al., 2007; Torlak et al., 2016; Zorlugenc et al., 2008). Also, OW concentration and exposure time are two important factors that could affect the antifungal effect of ozone in food models. Inan et al. (2007) showed reduction of 80% and 93% of AFB_1 production in red pepper after exposures to 33 as well as 66 mg/L ozone for 60 min, respectively. As a limitation of our study, the OW concentration (10 mg/L) and the time of OW exposure (15 min) in the present work was less than the results of research by Inan et al. (2007) that could be probably the reason of controversy. In a similar study published recently in 2016, gaseous ozone was used for reduction of AFB_1 and microorganisms in poultry feed. The samples were separately treated with constant concentrations of 2.8 as well as 5.3 mg/L of ozone up to 240 min. Significant reductions were shown in the AFB_1 level and fungal population that is in disagreement with the results of present study (Torlak et al., 2016).

Conclusion

We found that DHA had antifungal effect and AFB_1 inhibitory activities more than OW in Iranian pistachios. Application of DHA is recommend using in Iranian food industries as a potential means for prevention of the growth and AFB_1 accumulation in pistachios nuts.

Conflicts of interest

All authors of this manuscript state that they had no conflicts of interest.

Acknowledgements

This article is extracted from an MS student thesis from Ashkezar Branch of Islamic Azad University. Authors would like to thanks Ashkezar Branch of Islamic Azad Univesity for their financial support and also would like to thanks Mrs. M. Ghafoorzadah for her kind help in the laboratory works.

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