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Effect of Novel Zein Coating Combined with Different Antioxidants (Thymol and Carvacrol) on the Aflatoxin Production of Peanut

Asma Afshari[1], Mahdi Piroozehpoor[2], Abdollah Jamshidi[2], Mohammad Azizzadeh[3], Atefeh Sarafan Sadeghi[4*]

1. Department of Nutrition, Faculty of Medicine, Mashhad University of Medical Sciences, Mashhad, Iran
2. Department of Food Hygiene and Aquaculture, Faculty of Veterinary Medicine, Ferdowsi University of Mashhad, Mashhad, Iran
3. Department of Clinical Sciences, Faculty of Veterinary Medicine. Ferdowsi University of Mashhad, Mashhad, Iran
4. Department of Nutrition Sciences, Varastegan Institute for Medical Sciences, Mashhad, Iran

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ABSTRACT

Introduction: Aflatoxin contamination in agricultural crops leads to health hazards and is detrimental to the economy. Despite the improvement in processing, handling, and storage, aflatoxin contamination remains a severe issue in the peanut industry. The present study aimed to investigate the effect of corn-zein coating combined with thymol and carvacrol (edible coatings) on aflatoxin production in peanut.

Methods: Analysis of aflatoxins B_1, B_2, and total aflatoxin was performed in 11 treatments for coated and uncoated peanuts, which were stored at room temperature for 90 days. The treatments included zein, thymol (500, 1,000, and 1,500 ppm), and carvacrol (5,000, 10,000, and 15,000 ppm), which were incorporated with thymol and carvacrol at three concentrations. The experiments were also conducted on control samples.

Results: Significant differences were observed between the coated and uncoated treatments ($P<0.05$). Mean aflatoxins B_1, B_2, and total aflatoxins increased in the treatments with the highest concentration of the antioxidants compared to baseline on day 90. On the other hand, the other treatments showed a declining trend compared to the control.

Conclusion: According to the results, zein coating yielded optimal results in terms of preventing aflatoxin formation. Therefore, it is recommended that zein be used as a proper coating for peanut in order to promote its health benefits.

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Introduction

There is constantly a high demand for nuts as a healthy nutritional choice considering their proven cardioprotective effects (1). Peanut (*Arachis hypogea L.*) is a legume crop mostly grown for its edible seeds, which is categorized as a grain legume and oilseed due to its high oil content (2). Considering that peanut seeds possess almost 45% oil, 30% protein, 10% carbohydrate, 3.0% fiber, and 2.5% ash, they are known to be an abundant source of energy and protein for human diet and are consumed in considerable amounts (3). However, peanut is considered to be the main substrate for fungal species such as *Aspergillus* species, especially *A. flavus* and *A. parasiticus*, which could produce aflatoxins. Aflatoxins are a family of natural toxins with carcinogenic, mutagenic, and teratogenic compounds in animal species and humans. Aflatoxin B_1 is the most potent natural carcinogen, which has been classified as a Group 1 human carcinogen by the International Agency for Research on

* *Corresponding author:* Atefeh Sarafan Sadeghi, Department of Nutrition Sciences, Varastegan Institute for Medical Sciences, Mashhad, Iran. Tel: 00985135091160; Email: atefeh_sarafan@yahoo.com

Cancer (IARC) (4, 5).

Consumers demand the enhanced quality and freshness of food products, which has resulted in the development and application of edible coatings (6). Edible coatings are the substances that are added to the exterior layer of foodstuffs, so that the final product (e.g., proteins, starch, and gums) would be appropriate for consumption (7). This method of food preservation improves the stability of foodstuffs, while preventing moisture loss and oxygen diffusion. It is also implemented as a vehicle for various additives, such as antioxidants (8-10).

Zein contains a class of alcohol-soluble proteins knows as prolamins, which are found in corn endosperm and employed as tough, glossy, hydrophobic, greaseproof, edible coatings. Zein and its resins have unique features compared to the plant-based proteins that are used in the preparation of edible coatings. They reduce the migration of fats, prevent discoloration by decreasing oxygen and carbon dioxide transfer, creating resistance to microbial attacks with excellent flexibility, causing compressibility, and combining with active ingredients (e.g., antioxidants) (11-13).

In the food industry, there is an increasing demand for utilizing bio-preservatives, including phenolic compounds, which may be effective alternatives to synthetic additives (14). Moreover, it has been long recorded that some bio-preservatives have multiple biological effects, such as antimicrobial properties (15). In particular, carvacrol and thymol are mainly found in thyme and oregano essential oils (16). These isomers and phenolic monoterpenes have exhibited significant antibacterial effects *in-vitro* (17). Carvacrol has been shown to have antifungal, antitoxigenic, insecticidal, anti-parasitic, and desirable flavoring properties (18). With its high antifungal activity and low minimum inhibitory concentration (MIC), thymol has been observed to be an ideal antimicrobial substance (19). The antimicrobial activities of carvacrol and thymol have been previously studied, and the findings have confirmed their protective effects against fungal and bacterial strains (20).

The present study aimed to investigate the feasibility of reducing aflatoxin production in peanut using edible zein coating combined with carvacrol and thymol as natural antioxidants.

Material and methods

Experimental Materials

Regular-grade corn-zein was supplied by Freeman Industry (Tuckahoe, NY). Peanut samples were obtained from Edalat Company (website: www.edalatnuts.com/Iran) and stored in polyethylene plastic sheeting in a refrigerator until the examinations. Thymol, carvacrol, and glycerol were purchased from Sigma (Sigma-Aldrich GmbH, Steinheim, Germany; website: www.sigmaaldrich.com). Other chemicals, including ethyl alcohol and citric acid, were ordered from Merck (website: http://www.merck.com).

Preparation of Corn-Zein

At this stage, 10 grams of corn-zein powder was dissolved in 100 milliliters of aqueous ethanol solution (95%) (10% zein). The mixture was heated to the temperature of 70-80°C in an electric oven equipped with a magnetic mixer to completely dissolve in ethanol. In addition, 4.7 milliliters of glycerol was added to the mixture as a plasticizer, and the solution was gently mixed for five minutes. Afterwards, the mixture was cooled to the temperature of 50°C to add 1.2 grams of citric acid. Finally, the temperature of the solution reaches 25°C (21).

Preparation of Antimicrobial Solutions

Stock solutions of thymol were prepared in a hydroalcoholic diluent (10% ethanol). All the stock solutions were freshly prepared before use and sterilized by filtering through the membranes (pore size: 0.20 µm; Minisart, Sartorius, Goettingen, Germany). The solutions were prepared to produce the three final concentrations of the active compound (500, 1,000, and 1,500 ppm).

Carvacrol stock solution (Sigma, Poole, UK) was prepared in a hydroalcoholic diluent (10% ethanol) and sterilized by filtration (0.2 µm). The solutions were utilized to produce the final concentrations of 5,000, 10,000, and 15,000 ppm. An additional sample was also prepared without any antioxidant complex and applied as control (10, 22).

Storage Conditions and Sampling

The peanuts were sprayed with various

Table 1. Coating treatments implemented in this study

Treatment code	Coating material	Antioxidant	Concentration of antioxidant (ppm)
Z	Zein	None	Not applicable
ZT1	Zein	Thymol	500
ZT2	Zein	Thymol	1000
ZT3	Zein	Thymol	1500
ZC1	Zein	Carvacrol	5000
ZC2	Zein	Carvacrol	10000
ZC3	Zein	Carvacrol	15000
ZT1C1	Zein	Thymol+ Carvacrol	500+5000
ZT2C2	Zein	Thymol+ Carvacrol	1000+10000
ZT3C3	Zein	Thymol+ Carvacrol	1500+15000
CON	None	None	Not applicable

coating solutions for 30 seconds. Following that, they were placed in an oven at the temperature of 35°C for five hours to remove excessive moisture, and the treatments were packed. Various treatments used in the present study are shown in Table 1.

An uncoated treatment was considered as control. After coating and drying, 100 grams of the samples were packaged in polyethylene plastic bags (diameters: 27×28 cm). The samples were stored at room temperature (25°C). The samples of each treatment were evaluated at baseline and after 90 days of storage.

High-Performance Liquid Chromatography (HPLC) to Determine the Aflatoxin Levels in the Samples

- ***Sample Preparation***

Extraction of aflatoxins was carried out using the method proposed by the Association of Analytical Communities (AOAC) (method 991.31) (23). The peanut samples were ground using a Waring blender (Watertown, MA, USA), and 25 grams of ground peanut was used in each test. Following that, five grams of Merck-grade sodium chloride (Merck, Germany) was added to 125 milliliters of methanol and water (ratio: 70:30).

The homogenized mixture was blended at high speed for two minutes and filtered through fluted filter paper (Vicam, Germany). Afterwards, 15 milliliters of the filtered extract was blended with 30 milliliters of distilled water and filtered through Whatman glass microfiber filter (Whatman, UK). At the rate of 1-2 drops per second, 15 milliliters of filtered diluted extract was passed through the affinity column (Vicam, Germany). The aflatoxin accumulated in the column by passing one milliliter of high-performance liquid chromatography (HPLC)-grade methanol through the column and adding one milliliter of purified water to the elute. Afterwards, total aflatoxins were determined using HPLC (24).

At the next stage, methanol (100%), acetonitrile (100%) (Merck, Germany), and double distilled water were filtered through a nylon membrane filter (0.45 mm) (Whatman, UK). The mobile phases used in quantification were prepared at the temperature of 24°C and flow rate of 0.4 ml/min, as follows:

ACN/H2O/MeOH: 8/54/28

- ***Mobile Phase Preparation***

Aflatoxin levels were measured using an HPLC system (Knauer, NY, Germany) consisting of a fluorescence detector (RF/10AXL). Separation was carried out on the C18 column (Merck, Darmstadt, Germany). Data acquisition and processing were performed using Empower 2 Chromatography Data Software (Waters, Milford, USA).

Statistical Analysis

Each treatment experiment was conducted in triplet. Data analysis was performed in SPSS version 21 using paired t-test to assess the differences between the mean values of each treatment. In addition, analysis of variance (ANOVA) and Duncan's multiple range tests were applied to determine the changes during the tests and differences in variables, respectively. In all the statistical analyses, P-value of less than 0.05 was considered significant.

Results

Aflatoxins are poisonous, carcinogenic byproducts of certain fungi, which adversely affect various crops, such as peanuts (5). Therefore, new approaches should be used to

preserve peanuts in order to diminish the debilitative economic and health effects of aflatoxins and improve the value of the peanut industry.

In the present study, various treatments of zein coating with antioxidants (thymol and carvacrol) reduced the production of aflatoxin B_1 in peanut grains compared to the control. According to the information in Table 2, the mean production of aflatoxin B_1 increased significantly in the control samples during 90 days.

The results of the treatments against aflatoxin B_1 production were as follows (Table 2):

Z>ZC2>ZT1C1>ZC1>ZT1>ZT2C2>ZT2>ZT3>ZT3C3 >ZC3>CON

According to the information in Table 2, the treatment containing zein-based edible coating without antioxidant was the most effective method, which resulted in the major inhibition of aflatoxin production. Moreover, the mean aflatoxin B_1 in the treatments with the highest concentration of antioxidants increased after 90 days compared to the baseline. On the other hand, a declining trend was observed in the other treatments compared to the control. Our findings indicated that carvacrol alone might be able to cope better with aflatoxin B_1 production compared to thymol and the combination of carvacrol and thymol.

A significant difference was observed between the first and last day of the experiments in all the treatments in terms of aflatoxin B_1 production, with the exception of ZT1C1 ($P<0.05$). In addition, the most significant difference in this regard was denoted between the treatment with zein coating at the highest concentration of the antioxidants and control on day 90 ($P<0.05$).

According to the obtained results, various treatments with zein coating and antioxidants (thymol and carvacrol) decreased the production of aflatoxin B_2 in the peanut grains compared to the control. According to the information in Table 3, the mean production of aflatoxin B_2 increased in the control samples during 90 days.

The results of the treatments against aflatoxin B_2 production were as follows (Table 3):

Z>ZT1>ZC2>ZC1>ZT2>ZT2C2>ZT1C1>ZT3>ZT3C 3>ZC3>CON

As can be seen in Table 3, the treatment containing zein-based coating without antioxidants was the most effective treatment, resulting in the major inhibition of aflatoxin production. According to our findings, the mean aflatoxin B_2 in the treatments with the highest concentrations of the antioxidants increased after 90 days compared to the baseline, while in the other treatments, a declining trend was denoted compared to the control. Furthermore, carvacrol had no significant difference with thymol regarding the inhibition of aflatoxin B_2 production, and using carvacrol and thymol independently yielded better results than their combination.

In the present study, a significant difference was observed between the baseline and day 90 in terms of aflatoxin B_2 in all the treatments, with the exception of ZT1C1 ($P<0.05$).

Table 2. Comparison mean and standard error (SE) of aflatoxin B_1 between different treatments of peanut

Treatments	Aflatoxin B_1	
	Baseline Mean ± SE	90 day Mean ± SE
Z	1.91 ± 0.02 [a]	0.98 ± 0.02[e]
ZT1	1.55 ± 0.01[d]	1.14 ± 0.02[e]
ZT2	1.67 ± 0.02[c]	1.32 ± 0.01[e]
ZT3	1.80 ± 0.0 [b]	16.32 ± 0.04[d]
ZC1	1.80 ± 0.01[c]	1.08 ± 0.01[e]
ZC2	1.81 ± 0.01[b]	0.89 ± 0.01[e]
ZC3	1.72 ± 0.02 [c]	38.54 ± 0.03 [b]
ZT1C1	1.48 ± 0.02[e]	1.39 ± 0.02 [e]
ZT2C2	1.53 ± 0.02[d,e]	1.15 ± 0.01[e]
ZT3C3	1.92 ± 0.00[a]	21.84 ± 0.08 [c]
CON	1.72 ±0.02 [c]	355.38 ± 0.71[a]

Means followed by the different superscript alphabets in the same column are significantly different ($P < 0.05$)

Table 3. Comparison mean and standard error (SE) of aflatoxin B_2 between different treatments of peanut

Treatments	Aflatoxin B_2	
	Baseline Mean ± SE	90 day Mean ± SE
Z	0.54 ± 0.01 [a]	0.11 ± 0.00[f]
ZT1	0.48 ± 0.01[b]	0.14 ± 0.00[f]
ZT2	0.40 ± 0.00[c,d]	0.13 ± 0.00[e]
ZT3	0.39 ± 0.02 [c,d]	1.81 ± 0.01[c]
ZC1	0.39 ± 0.00[c,d]	0.10 ± 0.01[f]
ZC2	0.44 ± 0.00[c]	0.10 ± 0.00[f]
ZC3	0.43± 0.01 [c]	8.16 ± 0.03[a]
ZT1C1	0.37 ± 0.00[e]	0.32 ± 0.03 [f]
ZT2C2	0.37 ± 0.02[d]	0.16 ± 0.00[f]
ZT3C3	0.59 ± 0.00[a,b]	3.62 ± 0.00[d]
CON	0.50 ±0.02 [a,b]	15.27 ± 0.03[b]

Means followed by the different superscript alphabets in the same column are significantly different ($P < 0.05$)

Table 4. Comparison mean and standard error (SE) of total aflatoxin between different treatments of peanut

Treatments	Total aflatoxin	
	Baseline Mean ± SE	90 day Mean ± SE
Z	2.4± 0.02 [a]	1.09 ± 0.01 [f]
ZT1	2.03 ± 0.02 [c]	1.29 ± 0.01 [f]
ZT2	2.07 ± 0.02 [c]	1.4 ± 0.01 [e,f]
ZT3	2.19 ± 0.01 [b]	18.15 ± 0.03 [d]
ZC1	2.09 ± 0.02 [c]	1.19 ± 0.02 [f]
ZC2	2.24 ± 0.01 [b]	0.99 ± 0.00 [f]
ZC3	2.06± 0.03 [c]	46.69 ± 0.02 [b]
ZT1C1	1.85 ± 0.02 [e]	1.72 ± 0.11 [c]
ZT2C2	1.95 ± 0.01 [d]	1.36 ± 0.00 [e,f]
ZT3C3	2.4 ± 0.01 [a]	25.41 ± 0.02 [e]
CON	2.2 ±0.02 [b]	605.69±0.63 [a]

Means followed by the different superscript alphabets in the same column are significantly different (P < 0.05)

Moreover, a significant difference was denoted between the treatment with zein coating at the highest concentration of the antioxidants compared to the control on day 90 (P<0.05).

According to the findings of the current research, various treatments with zein coating and antioxidants (thymol and carvacrol) decreased total aflatoxin production in peanut grains compared to the control. As can be seen in Table 4, the mean total aflatoxin production surged in the control samples during 90 days.

The results of the treatments against total aflatoxin production were as follows (Table 4):

Z>ZC2>ZT1>ZT2>ZT2C2>ZT1C1>ZC1>ZT3>ZT3C3>ZC3>CON

According to the information in Table 4, treatment with zein-based coating without antioxidants was the most effective method, which resulted in the major inhibition of total aflatoxin production. On the other hand, the mean total aflatoxin increased in the treatments with the highest concentration of the antioxidants on day 90 compared to the baseline, while the trend was declining in the other treatments compared to the control.

A significant difference was observed between the baseline and day 90 of the experiments in all the treatments in terms of total aflatoxin (P<0.05). Additionally, the significant difference between the treatment with zein coating was denoted at the highest concentration of the antioxidants and the control on day 90 (P<0.05).

According to the information in Table 5, the difference between the baseline and day 90 of the experiments in the control was most significant with all the treatments in terms of the production of aflatoxin B_1, B_2, and total aflatoxin.

Table 5. Comparison of Mean and Standard Error (SE) of Differences (Day 90-Baseline) in Various Treatments of Peanut for Aflatoxin B_1, B_2, and Total Aflatoxin

Treatments	Aflatoxin B_1 (Day 90-Baseline) Mean±SE	Aflatoxin B_2 (Day 90-Baseline) Mean±SE	Total Aflatoxin (Day 90-Baseline) Mean±SE
Z	-0.93±0.04 [f]	-0.43±0.01 [h]	-1.39±0.04 [h]
ZT1	-0.41±0.03 [e,f]	-0.34±0.00 [g]	-0.74±0.04 [e,f,g]
ZT2	-0.34±0.00 [e,f]	-.0.26±0.00 [g, f]	-0.61±0.01 [e,f]
ZT3	14±0.05 [d]	1.42±0.03 [d]	15.95±0.01 [d]
ZC1	-0.71±0.02 [e,f]	-0.29±0.01 [g]	-0.090±0.04 [f,g,h]
ZC2	-0.92±0.02 [f]	-0.33±0.00 [g]	-1.25±0.02 [g,h]
ZC3	36.82±0.05 [b]	7.73±0.04 [b]	44.63±0.04
ZT1C1	-0.96±0.02 [e]	-0.04±0.01 [e]	-0.13±0.01 [e]
ZT2C2	-0.37±0.01 [e,f]	-0.20±0.02 [f]	-0.59±0.02 [e,f]
ZT3C3	19.91±0.08 [c]	3.12±0.02 [c]	22.97±0.10 [c]
CON	353.66±0.69 [a]	14.76±0.05 [a]	603.44±0.064 [a]

Mean values followed by different superscript alphabets in the same column are significantly different (P<0.05).
In the treatments with a negative difference, aflatoxin B_1, B_2, and total aflatoxin decreased, and in those with a positive difference, they increased over time.

Discussion

According to the results of the present study, treatment with zein coating without the antioxidants had an optimal impact on the inhibition of aflatoxin production. Similar to our study, Tihminlioglu et al. evaluated the inhibitory effects of corn-zein coating on food packaging applications, concluding that oxygen permeability reduced by nearly four times in the coating treatments (25, 26). In another research, Carlin et al. utilized zein coating to assess *listeria monocytogenes* growth on cooked sweet corn. According to the obtained results,

the population of the bacteria was 10-fold lower in the coated samples compared to non-coated sweet corn, which demonstrated the inhibitory effects of zein coating (27). This finding could be explained based on the reduced availability and accessibility of the nutrients on the surface. Corn-zein is a natural protein coating, which is used as an excellent oxygen and moisture barrier in nuts and other foods considering its tough, hydrophobic, and greaseproof properties and resistance to microbial attacks. However, this finding must be established through further experimentation to evaluate whether zein only acts on aflatoxin production or on fungal growth as well (28, 29).

According to the current research, the medium and minimum concentrations of the antioxidants had more significant effects on aflatoxin inhibition comparatively. Comparison of the results of the present study with the findings of Rasooli et al. indicated that the minimum concentration of thyme essential oil resulted in fungal growth inhibition (30).

Since aflatoxins are carcinogenic and teratogenic substances that act in a cumulative manner, any procedure leading to the reduction of their concentration in foods could be considered important in the agriculture industry.

Conclusion

According to the results, the levels of aflatoxin B_1, B_2, and total aflatoxin changed significantly within and between the study groups during storage. Furthermore, the treatments containing zein coating yielded optimal result in terms of preventing the production of aflatoxins compared to the other treatments. Therefore, it is recommended that zein coating be utilized in peanuts in order to promote their health benefits.

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Conflict of interest

None declared.

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