Enzyme-linked Immunosorbent Assay and High-performance Liquid Chromatography analysis of Ochratoxin A in *Zataria multiflora* and *Foeniculum vulgare* in Ahvaz (Iran)

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Abstract

Background: Contamination of ochratoxin A as a nephrotoxic agent can occur in a wide range of nutrients including medicinal plants. The aim of this study was to determine the amount of ochratoxin A (OTA) on high consumption medicinal herbs of Avishan Shirazi (*Zataria multiflora*) and Fennel (*Foeniculum vulgare*) in Ahvaz (Iran) using the enzyme-linked immunosorbent assay (ELISA) and high-performance liquid chromatography (HPLC) methods and compare the results of the two methods. Materials and Methods: In this study, the amount of OTA in high consumption medicinal herbs of Avishan Shirazi and Fennel (30 specimens) was investigated using HPLC and competitive ELISA methods, which were prepared randomly from groceries. Data analysis was performed using the SPSS20 software. Results: The level of OTA measured using ELISA and HPLC was significantly lower than the limit in all samples. Furthermore, despite higher contamination of fennel in both methods, no significant difference was observed between the studied plants (\(P > 0.05\)). There was a high correlation between HPLC and ELISA in determining the amount of OTA in Avishan Shirazi \((r^2 = 0.937)\) and fennel \((r^2 = 0.977)\); however, the values of OTA measured using ELISA method was higher compared to the HPLC method. Conclusion: Due to the good association between ELISA and HPLC methods, ELISA can be used to determine the contamination of OTA in plants especially as a screening method. However, chromatography methods, such as HPLC, are required in order to eliminate possible false results especially for very low amounts of toxin.

Key words: Ochratoxin A, medicinal herbs, enzyme-linked immunosorbent assay, high-performance liquid chromatography

INTRODUCTION

Over the years, natural medicines, especially medicinal plants, and the raw materials have been used in the pharmaceutical industry.¹² Fennel (*Foeniculum vulgare*) of the Apiaceae family contains several chemical compounds in its structure including anethol, pinene, camphene, limonene, phephandrene, pectin, calcium oxalate and starch that used in food, beverage, cosmetic, and sanitary industries.³⁴ Avishan Shirazi (*Zataria multiflora* Boiss) belonging to Lamiaceae family, which contains the chemical compounds of thymol (the main components of the plant), carvacrol, borneol, rosmarinic acid, menthen, and tannin is widely used as tea, spice and is used as a disinfectant for gastrointestinal and urinary tract, as well as diuretics and anti-inflammatory drugs in traditional medicine.⁵⁶ Many plants are produced in tropical and subtropical regions. Warm and wet climate, long drying time, and farmers’ ignorance may cause considerable qualitative

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problems including contamination with mycotoxins. Among mycotoxins, ochratoxin A (OTA), and aflatoxin B1 are common in a wide range of foodstuffs including medicinal plants.[7] OTA, phenylalanine-derived isocoumarin-based chlorine, with a molecule weight of 403.83 daltons and the molecular formula of C20H18NCLO6, is produced by Aspergillus and Penicillium species. Renal toxicity, carcinogenicity, and suppression of the immune system from the toxic effects of the mycotoxins are proven.[3] Methods commonly used in the determination of mycotoxins are high-performance liquid chromatography (HPLC), thin-layer chromatography (TLC), and enzyme-linked immunosorbent assay (ELISA).[8] Considering that medicinal herbs are often consumed without processing or raw materials, they are important for the health of the consumer. Therefore, the aim of this study was to determine the amount of OTA on high consumption of medicinal herbs using ELISA and HPLC methods and compare the results of the two methods.

MATERIALS AND METHODS

Reagents, equipment and sampling

Hydrochloric acid, phosphoric acid, and OTA (1 mg, solid powder) were purchased from Merck company. Furthermore, immunoaffinity OTA columns (Neogen, USA), competitive ELISA kit (Europroxima, Netherlands), and other HPLC chemicals (Samchon, Korea) were used. In this study, medicinal plants (15 samples of Avishan Shirazi and 15 samples of Fennel) were randomly collected from the groceries of Ahwaz and were kept in suitable conditions and away from environmental contaminations until the test.

Ochratoxin A analysis by high-performance liquid chromatography

To extract OTA, 100 mL of the methanol/phosphate buffer (50/50) is added to 20 g of each herbal sample and mixed. Then, after passing the mixture with a filter, 30 mL of PBS was added to each 20 mL. The supernatant was passed (2 to 3 mL/min) from a special immunoaffinity column of OTA. After washing the column with water and ochratoxin separation with HPLC methanol, it was evaporated with nitrogen gas and dissolved in the mobile phase. Finally, 200 μl of the solution was injected into the HPLC apparatus, and the amount of toxin contained was analyzed using 18 C column (4.6 mm × 150 mm, particle size: 5 μm), a fluorescence detector (excitation wavelength 333 nm and emission 460 nm), the moving phase (acetonitrile, water, and glacial acetic acid with 49.5: 49.5:1 ratio), and flow rate of 1.5 mm/min. For calibration curve, different concentrations of standard solution of OTA (0.25, 0.5, 1, 2.5 and 5 μg/L) were prepared and injected into HPLC. Determining the accuracy of the extraction method by adding dry vacuum standards to the filtered sample in triplicate, and the repeatability of the method by inoculation of different concentrations of OTA (six repetitions per day) was investigated. To confirm the regenerative, each of the concentration (6 consecutive days, three times) was injected into the device, and their CV was calculated by Solution-LC software. Diagnosis of OTA in plant samples was performed by comparing their peak level with the standard, and the amount of toxin was also measured by measuring the peak area during retention time and comparing it with the calibration curve.[10]

Ochratoxin A analysis by enzyme-linked immunosorbent assay

Samples, reagents, and standards were prepared and analyzed according to the instructions of the competitive enzyme immunoassay of OTA (Europroxima, OCH5121). In brief, 5 g of homogenized sample with 10 mL of 5.0 M phosphoric acid (10 min), and then, with 20 mL of dichloromethane (10 min) were mixed. After 5 minutes of centrifugation (2000 rpm), the dissolved supernatant (containing phosphoric acid) was removed, and the underlying phase (dichloromethane) was filtered. Then, 12 mL of the solution was dried at 50°C, and the residue was dissolved in 1.5 mL of extraction buffer. n-hexane (2 mL) was added to it, mixed, and centrifuged (2000 rpm). The n-hexane layer was removed and diluted 50 μl of the underlying layer with 200 μl of diluent buffer. Finally, 50 μl of prepared samples and 50 μl of standard solutions (0.25, 0.5, 1, 2 and 5 μg/kg) in two repetitions were entered in the anti-mouse antibody-coated enzyme-linked immunosorbent assay wells. 25 μl of conjugate (OTA horseradish peroxidase) and 25 μL of mouse monoclonal anti-ochratoxin A were added to wells and incubated (1 h in dark at 37°C C). After washing, 100 μl of the substrate solution was added to each well and placed at room temperature (30 min). Finally, after adding 100 μl of the stop solution to each well, the wells were immediately absorbed at 450 nm. Plants OTA values based on percentage of maximal absorbance of each sample were obtained directly from the calibration curve.

Statistical analysis

Data analysis was performed using the SPSS20 software. In the statistical analysis, one-sample t-test was used to compare the results with the maximum limit, independent-samples t-test was used to compare the contamination of plants, and Pearson correlation was used to examine the consistency of the two methods of analysis.

RESULTS

Ochratoxin A detection by enzyme-linked immunosorbent assay and high-performance liquid chromatography methods

The concentration of OTA was obtained by comparing the standard calibration curve in both methods. The limit of detection (LOD) and limit of quantification of OTA in the
studied plants were calculated 0.0758 and 0.25 μg/kg, respectively [Figure 1: HPLC chromatogram for an OTA standard]. The results of the HPLC method showed high repeatability, and regenerative of this method. OTA analysis of Z. multiflora in HPLC method showed the toxin in 13.33% of the samples were below the LOD, 26.66% were no detected (ND), and 60% of samples had between 0.1 and 0.97 μg/kg contamination. In ELISA method, ochratoxin contamination in Z. multiflora in 13.33% was below the LOD, 13.33% was ND, and 73.33% of the samples showed a contamination range of 0.078–1.65 μg/kg [Table 1]. OTA analysis of fennel in HPLC method showed that the toxin in 13.33% of the samples were below the LOD and 88.66% of the samples were in the range of 0.12–0.89 μg/kg. In ELISA method, ochratoxin contamination in fennel in 6.66% was below the LOD and 93.33% of the fennel samples showed a contamination range of 0.09–0.97 μg/kg [Table 2]. Although according to the results, fennel samples contamination was significantly higher in both methods; statistical analysis (independent sample t-test) showed no significant difference between fennel and Avishan Shirazi (P > 0.05). Analysis of the results using one-sample t-test showed the level of OTA in studied samples using both methods was significantly lower than the EU maximum limit (20 ppb or 20 μg/kg) (P < 0.05), which indicates the low contamination of these plants.

Enzyme-linked immunosorbent assay and high-performance liquid chromatography correlation in ochratoxin A analysis

Pearson correlation assay was used to check the agreement of ELISA and HPLC methods. The results showed a high correlation (P < 0.01) between HPLC and ELISA in OTA determination in Z. multiflora (r² = 0.937) and fennel (r² = 0.977). However, the values of OTA measured using ELISA were higher than HPLC method [Figure 2].

**DISCUSSION**

The contamination of various foodstuffs and agricultural commodities with mycotoxins is a major problem in the areas, where climate, agriculture, and maintenance are favorable for fungal growth and toxin production.[12] Bugno et al. conducted a study on the microbial and fungal contaminations of medicinal plants in Brazil, and showed that most of the fungal contaminations belonged to Aspergillus and Penicillium species, of which 21.97% were toxigenic (42.9% aflatoxin and 22.4% ochratoxin).[13] HPLC, TLC, and ELISA are common assays for determining mycotoxins. The TLC method has a relatively high coefficient of variation and is used only when mycotoxin contamination is higher than the current control limit. Chromatographic techniques such as HPLC are very expensive and time-consuming. On the other hand, TLC and HPLC methods require a solid-phase extraction or immunoaffinity column.[18,9] Therefore, it is now preferable to use immunological methods such as ELISA due to the need for a low sample size, the possibility of evaluating the high sample, and the simpler purification procedure. In addition, these methods are fast, simple,

In this study, the OTA contamination level in all medicinal plants was determined below the maximum limit using ELISA and HPLC methods. Noonim et al., in a study, to identify the toxinogenicity of Aspergillus species isolated from coffee beans in Thailand showed the presence of OTA in 98% of samples in the range of 0.6–27 μg/kg. Rizzo et al. examined the contamination of various toxigenic fungi on 152 samples of medicinal herb and aromatic plant, which 52% of them were contaminated with different species of Aspergillus that 26% of the isolates produced OTA in the concentration range of 0.12–9 μg/kg. In the present study, a significant correlation was found between HPLC and ELISA in comparison to OTA values. Of course, the values of OTA measured in samples using ELISA method were higher than HPLC. These differences can be related to different methods of extraction and antibody specificity. On the other hand, due to the possibility of antibody cross-reaction with similar compounds of mycotoxins, immunoassay methods such as ELISA may sometimes show higher values or false positives in comparison with chromatography.

Consequently, extensive studies on the validity and precision of ELISA in a wide range of commodities are necessary before the commercial use. Jakšić et al. also showed a good agreement between three methods of ELISA, TLC, and HPLC for evaluating the contamination of raisins to OTA in Serbia. Flajs et al. in determine the concentration of OTA in red wine in Croatia using HPLC and ELISA methods, showed there is a good correlation (r = 0.821) between these methods especially in high concentrations of OTA. It can conclude ELISA can be used as a convenient and screening method to determine the presence of OTA in plant because it can analyze a large number of specimens in a short time; however, chromatography methods, such as HPLC, are required to eliminate possible false results especially for very low amounts of toxin.

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