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Original Article

An Investigation on Measurement Means of Citrinin Toxin Quantity by Toxigenic *Aspergillus* Species in Biomass, Using ELISA

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ABSTRACT

Objective: Citrinin is a mycotoxin produced by several species of the genera *Aspergillus* and *Penicillium* and occurs mainly in food products and animal feed. Some scientific reports show a link between Citrinin and nephrotoxic and possibly a carcinogenic effect for humans. **Methods:** Samples collected by settling plates, in northern Iran and pure culture isolation performed till the toxin measurement to be done in cell extracts (biomass) which prepared by merging culture in separated prepared culture media incubation. The amount of toxin measured by extracting solutions (50% Acetone, 25% Alcohol Methyl, 25% Alcohol Ethyl and PBS washing proposed) using Direct Competitive ELISA. **Results:** In conducted research, the relative distribution per obtained *Aspergillus* species isolates for biomass preparation showed that the most frequent were *A.ostianus*, *A.fumigatus*, *A.niveus*, *A.niger*, *A.awamori* and *A.parasiticus* respectively with a prevalence (28.6%), according to averaged Citrinin, indicated that the potent species was *A.niger* (2009.3ppb) whereas the lowest observed by *A.wentii* (18.46 ppb). According to maximum Citrinin limits (200ppb) in food products and animal feed including especially related to the *Aspergilli* and *Penicilli*, as the result of our measurements and the performed statistical analysis, the maximum amount of Citrinin in the cell extracts, respectively were determined (2009.3ppb) and always produced by *A.niger*, that were too much more than the universal and local standards allowance enable us to introduce it for all biotechnologic or toxicologic research mentions eventhought as a scientific opinion on the risks for public and animal health related to the presence of Citrinin in food and feed at the targeted geographic area.

1. INTRODUCTION

Citrinin as a secondary metabolite [C₁₃H₁₄O₅, IUPAC: (3R, 4S)-4,6-dihydro-8-hydroxy-3,4,5-trimethyl-6-oxo-3H-2-benzopyran-7-carboxylic acid; is a polyketide mycotoxin produced by several species of the genera

Aspergillus, *Penicillium* and *Monascus*, Some of the citrinin producing fungi are also able to produce the mycotoxins ochratoxin A or patulin. Citrinin is generally formed after harvest under storage conditions and it occurs mainly in grains, but can also occur in other products of plant origin and also in spoiled dairy

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products. In addition, citrinin is found as an undesirable contaminant in red mould rice (RMR) which is used as a food preservative and colourant in Asian foods (EFSA, 2011a). Citrinin can be synergistic with the ochratoxin A to attenuate the activity of RNA synthesis in kidney tissue (Poupko et al., 1997). Citrinin has a conjugated, planar structure which gives its natural fluorescence, the highest fluorescence is produced by a non-ionized citrinin molecule at pH 2.5 (Franco et al., 1996). For the growth of the citrinin producing fungi on grain it is necessary to have a humidity of at least 16.5 – 19.5% and is practically insoluble in cold water but soluble in aqueous sodium hydroxide, sodium carbonate, or sodium acetate; in methanol, acetonitrile, ethanol, and most other polar organic solvents (Xu et al., 2006). One of the most serious problems to confront the quality of food and feed, is the presence of mycotoxins (Hussein and Brasel, 2001). Citrinin is a nephrotoxic mycotoxin produced by several species of the genera *Aspergillus*, and also has been reported that Citrinin may be implicated in human disease, in particular, there are limited evidence for the carcinogenicity that was concluded by the International Agency for Research on Cancer (IARC, 1993). During research for antibiotic agents in the middle of the last century, interest in Citrinin arose when its broad antibacterial activity was identified. However, interest decreased when its mammalian toxicity was demonstrated (Ambrose and DeEds, 1946). A large number of Citrinin derivatives have been isolated from different fungal species in search of antitumor compounds indicating that Citrinin might be a precursor of novel active compounds (Du et al., 2010). Thus many efforts devoted to the study of the origin and propagation of the nature and risks of Citrinin in food and feed and now prevention of contamination or Citrinin pollutions in human and animal food completely is not allowed. Filamentous fungi, particularly *Aspergillus* Citrinin-producing species are the most important found in abundance as a food source contamination, so we decided to measure Citrinin mean by ecotypic strains of the genus *Aspergillus* in the fungal biomasses.

2. MATERIALS AND METHODS

The following sample agenda for the closed (Indoor) and opened (Outdoor) positions based on the CBS rules was performed, fifty square acres of agricultural areas and processing plants sampled by settle plates in a group setting, were taken by six plates containing Malt extract agar, Yeast extract agar, Czapek yeast extract agar, Czapek agar, Sabouraud dextrose agar and potato dextrose

agar confounded with chloramphenicol (100ppm) were used to withdraw a sample group. All plates at $2 \pm 25^{\circ}\text{C}$ and were incubated aerobically and consistently in the range of 3, 7 and 15 days were checked to withdraw and the culture plates are subcultured in the tubes that containing agar slant bottles from growth media of Malt extract agar, Yeast extract agar, Potato dextrose agar, Corn meal agar, Sabouraud dextrose agar, Czapek yeast agar, Czapek dox agar and incubated with the previous program. Of *Aspergillus* colonies on selective agar plates that containing Czapek dox agar, Czapek yeast extract agar (with and without %20 sucrose), Malt extract agar and Czapek dox agar (with and without %20 sucrose) according to ICPA identification rules grown at $2 \pm 25^{\circ}\text{C}$, after 3, 7 or 14 days were reviewed and provided slide cultures for each sample on substrates of Czapek dox agar and Czapek yeast extract %20 sucrose to provide normative growth model. Preparing extracts obtained from isolates grown in liquid medium to perform more motivate and abundant extract of each isolate grown were taken on Czapek extract broth and a 50ml Falcon tubes containing liquid medium Czapek dox broth that contains 2 percentage of malt extract to enhance growings, tubes with 200RPM at $3 \pm 25^{\circ}\text{C}$ and in the light - darkness were incubated then after a 7-14 days float or sink in a liquid mass on the same field small Germ tubes using centrifugation with 3000RPM for 15 min precipitated then separated with sterile filter paper from the growth medium till each to be harvested. The mass was dried for 48 hours in a desiccator and then 2g of biomass was harvested, dried and then 2g of each major mold was transferred in a 15ml falcon tubes three times in a row (every $1 \pm 5\text{min}$), mixed with 5 ml of liquid nitrogen and a stirring device glass tube (Pearls) and every 25 min combination. Falcon tubes with 5ml of sample buffer and added cold acetone 1ml, centrifuged with 3000RPM for 15min separation takes place supernatant of coarse sediment removal and other tubes were kept in the notation for synchronization, the size of each protein mixture obtained from *Aspergillus* isolates was performed and all samples were measured by the Bradford method to size 0.5mg/ml aligned the concentrated sample dilution and the diluted samples were again concentrated by this method until all the juice extract samples with 0.5 mg per ml of protein.

A competitive enzyme immunoassay for the quantitative analysis of Citrinin in cereals and feed (RIDA SCREEN® FAST assay) were used to quantification of mycotoxin citrinin formed by the *Aspergillus* species are able to produce Citrinin and/or Ochratoxin A, therefore both mycotoxins often appear together, is possible to detect this mycotoxins both rapidly and with accuracy. Firstly filtered the extracts through whatman No.1 filter, diluted 1 ml of the deionized water and used 50 μl of the filtrate per well in the tests as the basis of the antigen-antibody reaction in the microtiter wells coated with the aimed mycotoxins followingly standards, respectively sample

solution and anti- mycotoxins antibodies, were added concomitantly. Free and immobilized mycotoxins compete for the mycotoxins antibody binding sites (competitive enzyme immunoassay) after a washing step secondary 100µl antibodies labeled with peroxidase were added to bind to the bound anti- mycotoxins antibodies while any unbound enzyme conjugated secondary antibodies were then removed in washing step, 100µl substrate/chromogen were added to the wells, bound enzyme conjugate (secondary antibodies labeled with peroxidase) converts the chromogen into a blue color product then turning by addition of the 100µl stop solution leads to a color change to yellow. The measurement is made photometrically at 450nm, at the absorbance lead inversely proportional to the mycotoxins concentration in the sample.

3. RESULTS

According to frequency of Citrinin- producing fungal species in the studies of cell extracts (biomass) and the samples population of various species of *Aspergillus* were studied and collected from the north of Iran, the maximum frequency was related to: *A.ostianus*, *A.fumigatus*, *A.niveus*, *A.niger*, *A.awamori*, *A.parasiticus* with a frequency of 6 samples (28.6%), *A.ochraceus*, *A.terreus*, *A.carbonarius*, *A.unguis*, *A.foetidus* with a frequency of 5 samples (23.8%), *A.nidulans*, *A.wenti*, *A.VI*, *A.melleus* with a frequency of 4 samples (19%), *A.sojae*, *A.nomius*, *A.candidus*, *A.alliaceus* with a frequency of 2 samples (9.5%) and also *S.ornate*, *A.flavus* species with a frequency of 1 sample 4.8% (Fig. 1).

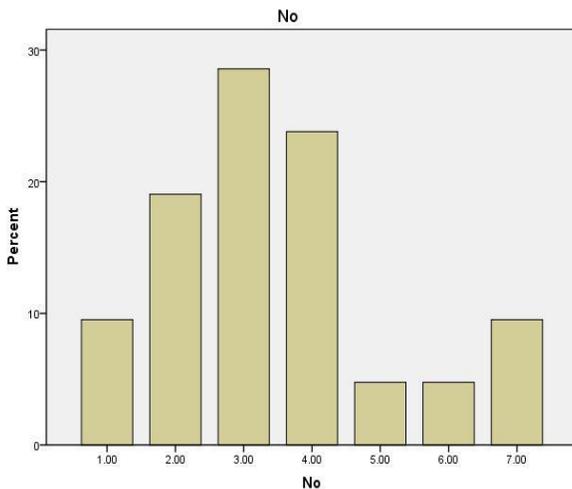


Fig. 1 -Distribution percentage of the studied isolates belonging to the genus *Aspergillus*.

In the measurement of the Citrinin mean produced among the studied species, most of the toxin produced by *A.niger* (2009.3 ppb) and after that *A.sojae* (765.84ppb) and *S.ornata* (553.84ppb), *A.terreus* (498.84 ppb), *A.fumigatus* (456.74ppb), *A.unguis* (428.53ppb), *A.parasiticus* (360.3ppb), *A.flavus* (322.3ppb), *A.melleus* (301.34 ppb), *A.alliaceus* (189.6 ppb), *A.ostianus* (117.41ppb), *A.carbonarius* (56.92 ppb), *A.foetidus* (36.91ppb), *A.candidus* (27.3 ppb), *A.wentii* (18.46ppb) and *A.ochraceus*, *A.awamori*, *A.VI*, *A.niveus*, *A.af.nidulans* and *A.af.nomius* species has not produced Citrinin (Fig. 2).

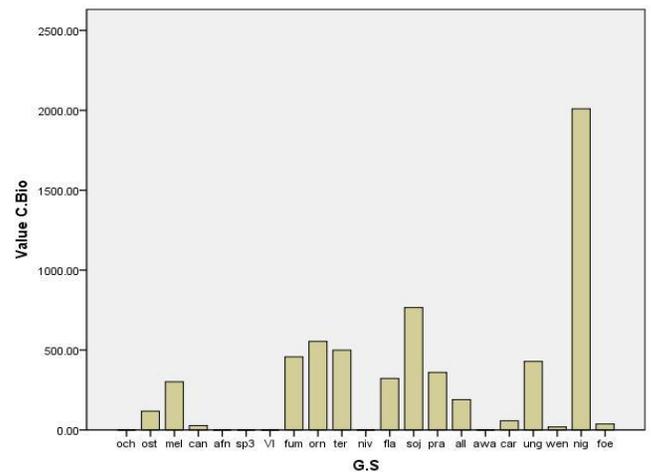


Fig. 2 -Measurement of Citrinin average amount in the biomass of each *Aspergillus* species.

According to Figure 3 of the our 21 species of *Aspergillus* researched, 6 species of *A.ostianus*, *A.candidus*, *A.alliaceus*, *A.carbonarius*, *A.wentii*, *A.foetidus* produced Citrinin (0-250 ppb) and *A.melleus*, *A.fumigatus*, *A.terreus*, *A.flavus*, *A.parasiticus*, *A.unguis* (250-500 ppb), *S.ornata* (500-750ppb), *A.sojae* (750-1000ppb), *A.niger* (2000-2500ppb) and *A.ochraceus*, *A.awamori*, *A.VI*, *A.niveus*, *A.af.nidulans*, *A.af.nomius* species has not produced Citrinin.

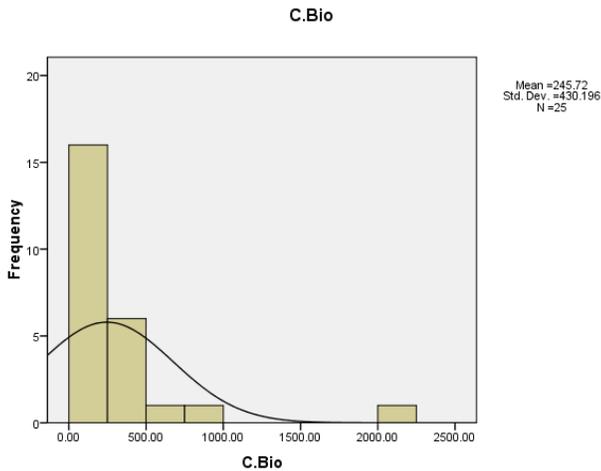


Fig. 3 - Diagram normal curve Citrinin mean values measured in the cell extract (biomass).

According to Figure 4, distribution of toxin levels measured in cell extracts of the studied species does not follow the normal.

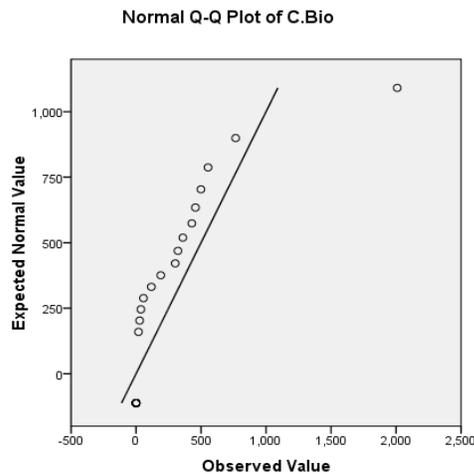


Fig. 4 -Normal Distribution Chart of Citrinin in biomass using Q-Qplot

According to Figure 5, distribution of toxin levels measured in cell extracts of the studied species does not follow the normal.

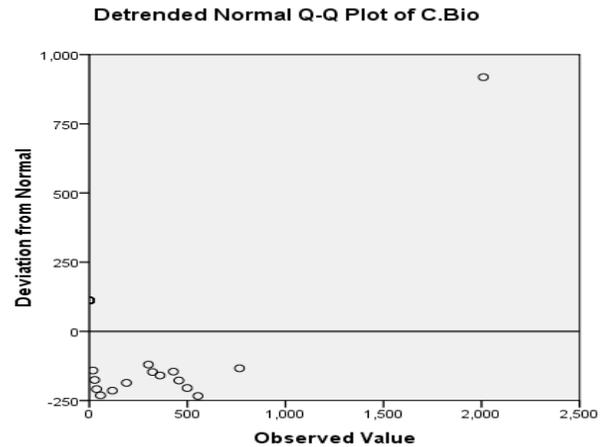


Fig. 5 - Normal Distribution Chart of Citrinin in biomass using Q-Qplot

4. DISCUSSION AND CONCLUSIONS

In studies conducted in this research and the results obtained by ELISA and relative distribution, the number of samples per obtained *Aspergillus* species isolates Prepared for cell extracts (biomass), the most frequent were *A.ostianus*, *A.fumigatus*, *A.niveus*, *A.niger*, *A.awamori* and *A.parasiticus* respectively with a prevalence (28.6%) as the most frequent, according to measurements of mycotoxins averaged Citrinin in biomass, indicated that the most of species produced Citrinin was *A.niger* valued (2009.3 ppb) in contrast the lowest Citrinin produced by *A.wentii* (18.46ppb). According to the maximum Citrinin limits at (200ppb) in Europe, Asia, America eventually Latin America, New Zealand, Africa, Canada and the Middle East in food products and animal feed especially related to the genus *Aspergillus* and *Penicillium* and etc were determined, as the result of our measurements performed in this study and the statistical analysis, the maximum amount of Citrinin in the cell extracts, respectively was (2009.3 ppb) and always produced by *A.niger*, that were too much more than the universal and local standards allowance enable us to introduce it for all biotechnologic or toxicologic research mentions eventhought as a scientific opinion on the risks for public and animal health related to the presence of Citrinin in food and feed at the targeted geographic area. The Q-Q plot normal distribution curve for Citrinin mean values measured in cell extracts (biomas) samples, showed that the observed tend to right skewness is statistically significant, therefore, does not follow a normal distribution pattern (Fig 4-Fig.5).

It should be noted that the previous studies, the Enzyme linked immunosorbent assays (ELISA) for Citrinin detection have been reported in wheat, barley, maize, RMR, and other grains, with LODs ranging from 2 to 15000 $\mu\text{g}/\text{kg}$ that according to our analysis by ELISA methods, Citrinin is produced in the range of (18.46-

2009.3 ppb) in cell extracts by *A.niger* (2009.3ppb) and *A.wentii* is 18.46ppb (Li et al., 2010). In studied on the analysed wheat samples (for food use) from the Czech Republic shortly after harvest. There was only one sample positive for Citrinin, which had a low content not exceeding the LOQ (1.5µg/kg), the authors also analysed barley samples destined for malt production. One of the samples was contained the highest Citrinin content (93.6 µg/kg), barley and wheat for feed use were also analysed and Citrinin was found in only few barley samples up to a concentration of 13.2 µg/kg (Polisenska et al., 2010). The occurrence of Citrinin was reported in Indian groundnuts infected with *A. flavus* and *A. terreus*, it is similar to our analysed by ELISA methods, Citrinin is produced by *A. flavus* (322.03ppb) and *A. terreus* (498.84ppb) in some cell extracts contaminated samples (Subrahmanyam and Rao, 1974). In Spain, samples of aromatic and/or medicinal herbs sold were screened, using an ELISA (LOD=16.5µg/kg) and found 61% of samples contaminated with Citrinin up to 355µg/kg in ginkgo leaves (Kononenko and Burkin, 2008). Occurrence of Citrinin in fruit (apple, cherry, black currant and grape) and vegetable (tomato) juices sampled from retail stores was analysed in Germany, using ELISA (LOD = 0.08 µg/kg) and traces of Citrinin (maximum 0.2 µg/L) could be found in 4 out of 55 samples (Dietrich et al., 2001). In one research, Citrinin detected in grains for feed use (LOD = 10 µg/kg). Wheat, barley and maize contained Citrinin in 5%, 4% and 2% of the samples, with maximum values of 144, 998, and 953 µg/kg (Kononenko and Burkin, 2008). Also in Citrinin investigated in grains for food use with LC-MS/MS, Citrinin was detected in one wheat sample at a concentration of 0.19 µg/kg (Tabata et al., 2008). In occurrence of 3 mycotoxins (Aflatoxin B1, Citrinin and Ochratoxin A) in one third rice samples investigated from 5 provinces of the central region of Vietnam, using HPLC with fluorescence detection (LOD= 0.11 and LOQ=0.35µg/kg). Citrinin was detected in 13% of the samples at concentrations up to 0.42µg/kg (Nguyen et al., 2007). Citrinin found in only a few samples of grains at concentrations from 100 to 300 µg/kg and also detected Citrinin in 10% samples of Egyptian kidney beans at a concentration of 370 µg/kg and also analysed fruit (apple, cherry, black currant and grape) and vegetable (tomato) juices sampled from retail stores in Germany using ELISA (LOD = 0.08 µg/kg) and traces of citrinin (maximum 0.2 µg/L) could be found in 4 out of 55 samples. Citrinin has been detected (LOD = 40 -100 µg/kg) in 2 grape samples (both at 70 µg/kg), one fig sample (60 µg/kg) and one pear sample 50 µg/kg (Aziz et al., 2006). And also Citrinin incidence was monitored in cereal samples intended for use as food and feed from villages in Bulgaria where BEN had occurred. Samples were analyzed for Citrinin using an ELISA with an LOD = 5µg/kg (Vrabcheva et al., 2000). For high consuming toddlers, children and adults, Citrinin concentration is between 9 and 53 µg Citrinin/kg and between 19 and 100 µg Citrinin/kg for average consumers,

respectively (EFSA, 2011a). For animals, risk characterization was based on the estimate of the Citrinin concentration in grains that would result in exceedance of the NOAEL of 20 µg/kg b.w. per day which ranged between 640 and 1173µg/kg. The CONTAM Panel concluded that the impact of uncertainties on the risk assessment is large, and more data regarding the toxicity and the occurrence of Citrinin in food and feed in Europe are needed to enable refinement of the risk assessment. The CONTAM Panel concluded that this study is suitable to identify a no-observed-adverse-effect level (NOAEL) of 20µg Citrinin/kg b.w. per day for nephrotoxicity (EFSA, 2011b).

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