ISSN: 0976-3104
SPECIAL ISSUE (ARBS)
Nejati et al.

THE IIOAB JOURNAL

ARTICLE OPEN ACCESS

AN INVESTIGATION ON TOXIGENICITY AND ANALYTICAL COMPARISON OF OCHRATOXIN AND CITRININ IN LABORATORY CULTURE MEDIA

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ABSTRACT

One of the most serious problems facing the quality of food and feed, is the presence of mycotoxins which are produced by different species of the genus Aspergillus. Ochratoxin and citrinin (CIT) are of the greatest concerns as they are highly toxic and carcinogenic compounds. Samples were collected by settling plates, in northern Iran, and pure culture isolation was performed upto toxin measurement in culture media, which had been prepared by mergingculture in separatly prepared culture media incubation. The amount of toxin was measured by extracting solutions using Direct Competitive enzyme-linked immunosorbent assay (ELISA).The greatest amount of CIT was produced by A.niger (1655.91ppb), and the highest amount of ochratoxin produced by A.carbonarius (8.007ppb) that played an important role among all the conducted isolates. In contrast the lowest amount of CIT was produced by A.foetidus (0.060ppb), the lowest ochratoxin was produced by A.spIV isolates (1.835ppb). Considering the maximum limits CIT (200ppb) and for ochratoxin (5ppb) in food and feed including abbreviated new drug applications (ANDAs), the maximum amount of CIT which was produced by A.niger was much more than the universal standard limits. The greatest amount of ochratoxin produced by A.carbonarius and also was more than the standard limits. Since methods for controlling mycotoxins are highly preventive, the analytical comparison of toxins, including aflatoxin, ochratoxin and CIT was intended to be elucidated in this study.

Received on: May 5th 2016
Revised on: May 27th 2016
Accepted on: 25nd – June -2016
Published on: 10st– July-2016

KEY WORDS

Aspergillus, Citrinin (CIT), Ochratoxin, Toxigenic activity Culture media

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INTRODUCTION

Fungal growth is one of the main causes of food spoilage, which not only makes great economic losses, but also threatens human and animal health, particularly through the synthesis of mycotoxins. Citrinin is a polyketide mycotoxin produced by several species of the genera *Aspergillus*, *Penicillium* and *Monascus*. Some of the fungi producing citrinins (CITs) are also able to produce mycotoxins ochratoxin A (OTA) or aflatoxin. CIT is generally formed after harvest under storage conditions and it occurs mainly in grains and cereals. It can also occur with other products of plant origin and in spoiled dairy products. However, in various studies on OTA, references have been made to the concomitant occurrence of CIT in a given food or feed materials, Meanly when CIT was found in a sample, it always occurred with OTA [1]. CIT has a conjugated, planar structure which gives it's a natural fluorescence, the highest fluorescence is produced by a non-ionized CIT molecule at pH 2.5 [2]. For the growth of the CIT, which produces fungi on grain, it is necessary to have a humidity of at least 16.5 – 19.5%. This CIT is practically insoluble in cold water and soluble in aqueous sodium hydroxide, sodium carbonate, or sodium acetate, methanol, acetonitrile, ethanol, and most other polar organic solvents [3]. One of the most serious problems facing the quality of food and feed, is presence of mycotoxins [4]. CIT is a nephrotoxic mycotoxin produced by several species of the genera *Aspergillus*, and it has also been reported to be involved in human disease. However, there are limited evidences for the carcinogenicity that was concluded by the International Agency for Research on Cancer. During studying antibiotic agents in mid 20th century, the interests in CIT arose when its broad antibacterial activity was identified. However, this interest decreased when its mammalian toxicity was demonstrated [4]. A large number of CIT derivatives have been isolated from different fungal species in search of antitumor compounds indicating that CIT might be a precursor of novel active compounds [5]. Fungal growth and mycotoxin production during storage are generally spot processes significantly affected by crop variety agronomic

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ISSN: 0976-3104

practices and weather conditions during harvest, post harvest, drying, cleaning and storage and processing conditions as well as the toxigenic potential of the mold species. Consequently, the distribution of mycotoxins in a lot of agricultural products is heterogeneous. This sampling is the largest source of variability associated with mycotoxin analysis and the most crucial step in obtaining reliable results [6].

OTA is a toxin naturally produced by several species of *Aspergillus* and *Penicillium*. These mold species are capable of growing in different climates and on different plants.Thus, contamination of food crops with OTA can occur worldwide. This toxin has a nephrotoxic, immunosuppressive, teratogenic and carcinogenic properties [7]. Many studies have shown that cereals and cereal products are the main sources of OTA [8,9]. It has also been detected in other products such as green coffee, milk, wine and grape juice [10]. OTA can be found in a wide range of human foods. The toxicology and human health risks of OTA have been assessed at both European and international levels by the European Commission Scientific Committee on Food (SCF) and the Joint FAO/WHO Expert Committee on Food Additives (JECFA), who have established tolerable intakes of OTA from food [11]. The kidney is the major site of OTA-induced toxicity, where it acts principally on the middle (S2) and terminal (S3) segments of the proximal convoluted tubules [12]. OTA has been shown to be nephrotoxic in all monogastric species tested, although there are species differences in sensitivity to nephrotoxic effects [13]. Once absorbed, OTA readily binds to serum albumin and is distributed in the blood predominantly in a bound form [14]. OTA binds strongly to human serum albumin and shares a common binding site with other known anionic compounds, including warfarin, naproxen and phenylbutazone, giving rise to the possibility of OTA–drug interactions [15]. Clinical chemistry parameters have been used to monitor the effects of OTA on renal function. Studies have shown that OTA exposure can lead to increased urine volume, blood urea nitrogen [15], urinary glucose, and proteinuria. As well as to reductions in the activity of OTA 313 enzymes such as alkaline phosphatase, leucine aminopeptidase and γ -glutamyl transferase, in the kidney [16]. The biochemistry of OTA results primarily from its structural similarity to the essential amino acid, phenylalanine (Phe). The principal effect appears to be inhibition of protein synthesis, although secondary effects, such as inhibition of RNA and DNA synthesis have also been implicated in its mechanism of action. The chemical structure of OTA consists of a 5'-chlorinated-3,4-dihydro-3-methyliscumarin moiety linked to L-Phe [17]. A number of epidemiological studies have identified OTA as a likely etiological agent responsible for a fatal kidney disease primarily affecting rural populations in the central Balkan peninsula, including Bosnia and Herzegovina, Bulgaria, Croatia, Romania, Serbia and Slovenia. The disease, referred to as Balkan endemic nephropathy (BEN), is characterized by tubular degeneration, interstitial fibrosis and hyalinization of the glomeruli and a slowly developing impairment of renal function with a progressive decrease in kidney size [18]. Although OTA is a contaminant of food and feed worldwide, it has been detected at high levels in a wide range of foods and in human blood samples taken from areas where BEN is endemic [19]. OTA has been implicated in the development of cancers of the human urinary tract because of the higher incidence of urinary tract tumors in humans in regions where BEN is endemic [20]. The mechanism by which OTA induces tumor formation remains controversial and is an area of active study. Formation of DNA adducts is thought by some researchers to be an important event in the tumorigenicity of OTA and it is postulated that OTA or one of its metabolites may act through a genotoxic mechanism involving direct covalent binding to DNA. However, numerous studies yet have investigated the transformation of OTA into a reactive intermediate and its possible role in tumorigenesis [21]. Data on the possible effects of OTA on the immune system are limited. A number of *in vivo* and *in vitro* studies suggest that OTA may affect both humoral and cell-mediated immunity, although the reported effects were generally observed at higher doses than those capable of causing nephrotoxicity.At very high doses (0.5–80 mg/kg body weight) OTA has been shown to cause gross changes to organs of the immune system, such as reductions in thymus size in mice, rats and chickens [22] and necrosis of cells in the spleen and lymph nodes in rats [23].

Scientific evaluations now generally become the basis for recommendations regarding the international regulation on mycotoxins (ochratoxin and CIT) by the Codex Committee on Food Additives and Contaminants (CCFAC) as well as the European Union. Regulations may include guidelines regarding maximum residue levels or procedural guidelines aimed at prevention by using a Hazard Analysis of Critical Control Points (HACCP), or a combination of them. The overall process needs to be transparent and should aid in the development of harmonized mycotoxin regulations control procedures. Ideally, such guidelines are acceptable to countries producing as well as those importing food commodities [24].

MATERIALS AND METHODS

performed. Fifty square acres of agricultural areas and processing plants sampled by settling plates in a group The following sample agenda for the closed (indoor) and open (outdoor) positions based on the CBS rules was setting, were taken by six plates

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ISSN: 0976-3104

containing malt extract agar, yeast extract agar, Czapek yeast extract agar, Czapek agar, Saboraud dextrose agar and potato dextrose agar confounded with chloramphenicol (100ppm) were used to withdraw a sample group. All plates were aerobically incubated at 2 ± 25 C° and consistently checked in the range of 3,7 and 15 days to be withdrawn. The culture plates were subcultured in the tubes containing agar slant bott from the growth media of malt extract agar, yeast extract agar, potato dextrose agar, corn meal agar, Saburod dextrose agar, Czapek yeast agar and Czapek doxs agar and incubated according to the previously mentioned program. Of Aspergillus colonies on selective agar plates those containing Czapek doxs agar, Czapek yeast extract agar (with and without 20% sucrose), malt extract agar and Czapek doxs agar (with and without 20% sucrose) according to ICPA identification rules grown at 2 ± 25 C°,after 3 ,7 or 14 days were reviewed and each sample was provided with slide on substrates of Czapek doxs agar and Czapek yeast extract of 20% sucrose to provide a 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 then a 50ml Falcon tube containing liquid medium Czapek doxs broth with 2% of malt extract to enhance growings, and tubes with 200RPM at 3 ± 25C° and in the light - darkness were incubated 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 48h in a desiccator and then 2g of biomass was harvested, dried and then 2g of each major mold was transferred in to a 15ml Falcon tube three times in a row (every 1± 5min), mixed with 5 ml of liquid nitrogen and a stirring glass Pearls every 25 min combination. Falcon tubes with 5ml of sample buffer and added cold acetone of 1ml, were centrifuged with 3000RPM for 15min. Separation toke 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 and all samples were measured by the Bradford method. To size 0.5mg/ml the concentrated sample dilution was aligned and the diluted samples were again concentrated by Bradford method until all the juice extract samples with 0.5 mg/ml of protein were attained.

A competitive enzyme immunoassay for the quantitative analysis of CIT in cereals and feed (RIDA SCREEN® FAST assay) was used to quantify of mycotoxin CIT formed by the *Aspergillus* species. *Aspergillus* species are able to produce CIT and/or OTA, therefore both mycotoxins often appear together and it would be possible to detect the mycotoxins rapidly and with accuracy. Firstly the extracts were filtered through whatman No.1 filter, 1ml of the deionized water was diluted and 50µl of the filtrate per well was used in the tests as a basis for the antigen-antibody reaction in the microtiter wells coated with the aimed mycotoxins Afterwards, the standards, respective sample solutions and anti- mycotoxins antibodies, were added concomitantly. Free and immobilized mycotoxins competed for the mycotoxins antibody binding sites (competitive enzyme immunoassay) after a washing step. Secondary antibodies of 100µl labeled with peroxidase were added to bind to the bound anti- mycotoxins antibodies while no unbound enzyme conjugated. Secondary antibodies were then removed in washing step, 100µl substrate/chromogen was added to the wells, bound enzyme conjugate (secondary antibodies labeled with peroxidase) converted the choromogen into a blue color product and then turning by addition of 100µl stop solution lead to a color change to yellow.The measurement was made photometrically at 450nm, and the obsorbance lead inversely proportional to the mycotoxins concentration in the sample.

RESULTS AND DISCUSSION

The measurement revealed that the mean CIT was produced by the studied species, with most of the toxin produced by *A.niger* (1655.91 ppb) and at lower levels by *A.niveus* (951.81ppb), *A.ochraceus* (883.29ppb), *A.parasiticus* (873.01ppb), *A.fumigatus*(868.25ppb), *A.ostianus* (577.84ppb), *A.af.flavus* (522.44ppb), *A.flavus*(522.42ppb), *A.alliaceus* (311.73ppb), *A.sojae* (245.99ppb), *A.awamori*(216.27ppb), *A.terreus* (198.8ppb), *A.af.terreus* (198.5ppb), *S.ornate* (53.33ppb), *A.melleus* (44.54ppb), *A.unguis* (32.39ppb), *A.wentii* (8.04ppb), *A.candidus* (2.74ppb), *A.carbonarius*(1.62ppb),*A.foetidus* (0.061ppb), *A.*SP3, *A.*sp*IV*, *A.*sp*V*,*A.af.nidulans* and *A.*sp*VI* species did not produce CIT in culture media [**Figure-1**].

According to the measurements average amounts of ochratoxin and CIT were produced by *Aspergillus* species, *A.niger*, *A.niveus*, *A.ochraceus*, *A.parasiticus*, *A.fumigatus*, *A.ostianus*, *A.af.flavus*, *A.flavus*, *A.alliaceus*, *A.sojae*, *A.awamori*, *A.terreus*, *A.af.terreus*, *S.ornate*, *A.melleus*, *A.wentii*, *A.candidus*, *A.carbonarius* and *A.foetidus* isolates which produced both ochratoxin and citrinin. This showed that there was a significant relationship among *Aspergillus* species enabling them to produce more than one toxin at a time.

Considering the conducted studies and the results obtained by ELISA and relative distribution, the number of samples per obtained *Aspergillus* species isolates for culture media was determined, and the most frequent ones were identified to be *A.foetidus*, *A.ostianus*, *A.*sp*V*, *A.unguis*, *A.candidus*, *A.awamori*, *A.melleus* , *A.wentii*, and *A.niveus* with a prevalence of (36%) as the most frequent the measurements of mycotoxins averaged CIT, ochratoxin in culture media, indicated that the most CIT was produced by *A.niger* (1655.91ppb), for ochratoxin the highest concentration was produced by *A.carbonarius* (8.007ppb). In contrast, the lowest CIT (0.061ppb) produced by *A.foetidus*, the lowest ochratoxin (1.835ppb) was prepared by *A.*sp*IV* isolates. According to maximum CIT limits (200ppb) and maximum Ochratoxin limits (5ppb) in Europe, Asia, America eventually Latin America, New Zealand, Africa, Canada and the Middle East for food products and animal feed including, wheat, corn, barley, rice, flour, Black wheat, oats, red rice, fruit and nuts, especially related to the genus *Aspergillus* and *Penicillium* were determined.

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ISSN: 0976-3104

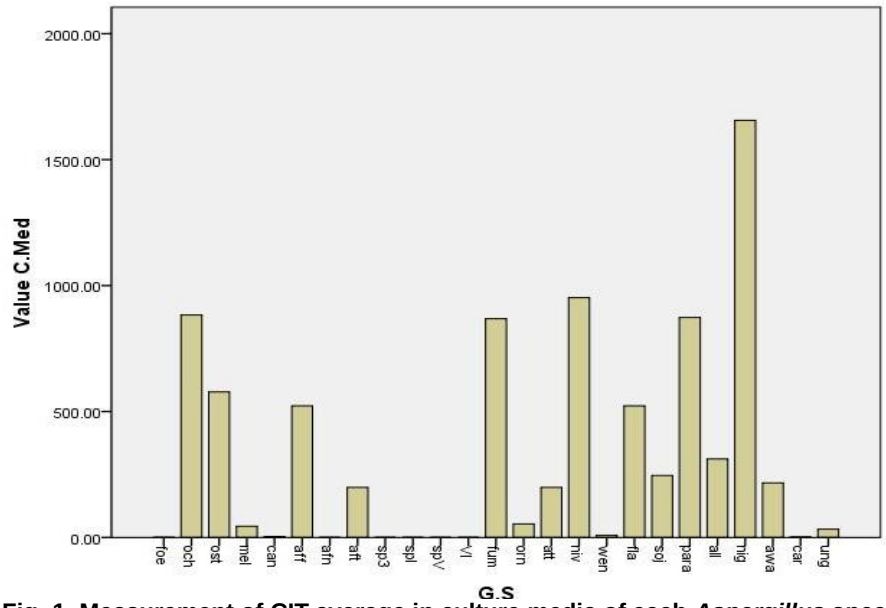

Fig: 1. Measurement of CIT average in culture media of each *Aspergillus* species.

The measurement revealed that the mean ochratoxin was produced by studied species, with most of the toxin produced by *A.carbonarius* (8.007ppb) and at lower levels by), *A.melleus* (6.14ppb), *A.niveus* (5.033ppb), *A.ostianus* (4.619ppb), *A.spV* (4.285ppb), *A.awamori* (4.241ppb), *A.ochraceus* (3.897ppb), *A.foetidus* (3.645ppb), *A.niger* (3.535ppb), *A.spVI* (3.285ppb), *A.alliaceus* (3.243ppb), *A.flavus* (3.225ppb), *A.af.nidulans* (3.149ppb), *A.Fumigatus* (3.133ppb), *A.wentii*(3.055ppb), *S.ornata* (2.959ppb), *A.af.terreus* (2.941ppb), *A.*sp3(2.733ppb),*A.parasiticus* (2.725ppb), *A.terreus* (2.551ppb), *A.af.flavus* (2.349ppb), *A.candidus* (1.967ppb), *A.sojae* (1.939ppb), *A.spIV* (1.835ppb) and *A.unguis* species did not produce OTA in culture media [**Figure-2**].

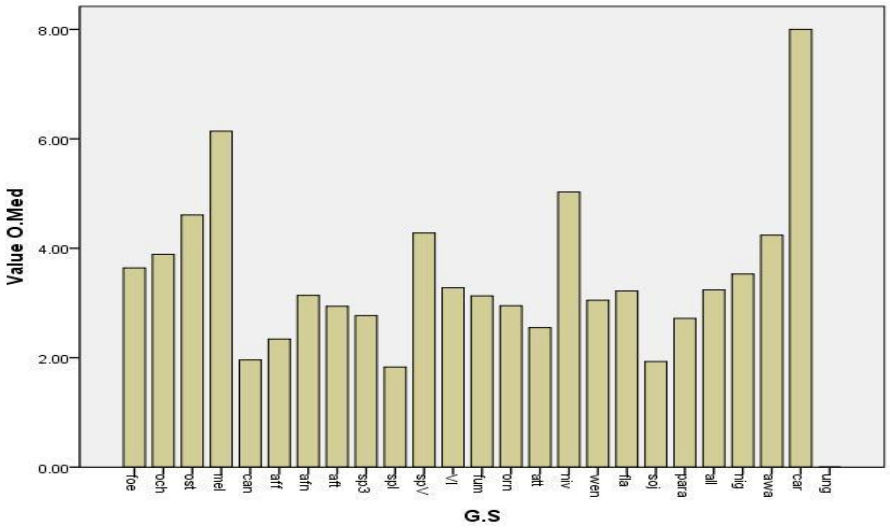

Fig: 2. Measurement of ochratoxin average in culture media of each *Aspergillus* species.

According to the previous researches in relation to the measurement of ochratoxin and CIT and the subsequent analysis, OTA produced by species in *A.carbonarius*, *A.ochraceus* and *A.niger* in grain samples were examined, however, our recent measurements performed in this study revealed that *A.niger* (3.535ppb), *A.carbonarius* (8.006ppb), *A.ochraceus* (3.897ppb) produced OTA in culture media [25]. Pioneer studies were carried out in order to determine the possible presence of OTA and CIT in game sausages, semi-dry sausages and fermented dry-meat products, randomly taken from individual producers and the market. OTA was quantified using ELISA,

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ISSN: 0976-3104

while CIT was quantified using high performance liquid chromatography (HPLC)-fluorescence detector. In 90 samples, the fungi most frequently isolated from dry-cured meat products were of *Penicillium* species, while *Aspergillus* was isolated from only one sample. As much as 68.88% of the samples were positive for mycotoxins. Finally, the analysis of different types of meat products resulted in the OTA identification in 64.44%, CIT identification in 4.44% of the samples. The maximum OTA concentrations established in the commercial sausage samples equalled to 7.83 µg/kg. Although OTA was detected in all the three types of products at different percentage, mutual differences were not statistically significant [26,27]. In Spain, samples of aromatic and/or medicinal were herbs sold screened, using an ELISA (LOD = 16.5µg/kg) and 61% of them were found to be contaminated with CIT (up to 355 µg/kg in ginkgo leaves), in two samples the highest concentrations of CIT contamination, the toxin co-occurred with OTA, AFB1. In another study in different storage centers in India, seed samples of medicinal plants were found to have curative properties for various human diseases, and 20% of samples were contaminated with CIT at a concentration between 10 and 760 µg/kg [28]. One of the first comprehensive studies on the occurrence of Citrinin was done on grain samples associated with lung problems in farmers and silo operators were collected from farm storages and analyzed. The grain had been stored under damp conditions, resulting in heating and spoilage. After development of an appropriate screening method, more samples were found to contain CIT (0.07 to 80mg/kg). The contaminated samples included wheat, oats, barley and rye. All samples positive for CIT were also contaminated with OTA. Studies In Europe have been mainly carried out in Southeastern European countries, where the occurrence of CIT has been linked to the so-called Balkan endemic nephropathy [29]. In a study on wheat samples (for food use) from the Czech Republic were analyzed shortly after harvest. In the mentioned study, there was only one sample positive for CIT, which had a low content, not exceeding the limit of quantification (LOQ) 1.5µg/kg. The same samples had an OTA content of 4.7µg/kg. The authors also analyzed barley samples destined for malt production. One of the samples was offered to a malt house, but not accepted due to a higher content of admixtures and impurities and a moldy smell. This sample contained the highest CIT content (93.6µg/kg) and also contained OTA (31.4µg/kg). Barley and wheat for feed use were also analyzed by these authors and CIT was found in only few barley samples a concentration of 13.2 µg/kg [30]. In another study on CIT in grains for food use with through LC-MS/MS method. In the mentioned study, CIT was detected in one wheat sample at a concentration of 0.19µg/kg, together with OTA and also in two buckwheat samples at concentrations of 0.55 and 0.62µg/kg, together with OTA, in one third of rice samples [31]. A methodology was described by Molinié et al. (2005) for simultaneous extraction/purification of OTA and CIT with a recovery for CIT of 80% and an LOD of 0.5 µg/kg. They confirmed that for breakfast cereals, it Citrinin was presenting its content was higher than that of OTA. They analyzed samples, of which 69% were contaminated with OTA at 0.2-8.8µg/kg and 18% were contaminated with CIT in the range of 1.5_42 µg/kg. When CIT was found in a sample, it always occurred with OTA.The sample with the highest CIT concentration (42µg/kg) contained OTA at a concentration of 4.1µg/kg. The (ELISAs) for CIT detection have been reported in wheat, barley, maize, RMR, and other grains, with LODs ranging from 2 to15000 µg/kg. According to the analysis using ELISA methods, CIT was produced in the range of (0.060-1655.91ppb) in the culture media by *A.niger* (1655.91ppb) and by *A.foetidus* (0.060ppb) [32].

CONCLUSION

Mycotoxins are very hazardous to animals and humans. Mycotoxicoses cause severe problems. The risk of contamination by mycotoxins such as citrinin, ochratoxin is an important food and feed safety concern. Domestic animals are exposed to mycotoxins, significant caution shall be carried on using animal products such as milk, eggs and meat. According to the measurements average amounts of ochratoxin and citrinin were produced by *Aspergillus* species, there was a significant relationship among *Aspergillus* species taxonomic criteria enabling them to produce more than one toxin at a time, and also a significant relationship was seen among isolates which produced both of citrinin and ochratoxin as well. After development of an appropriate screening method, a significant relationship was seen among *A.niger*, *A.niveus*, *A.ochraceus*, *A.parasiticus*, *A.fumigatus*, *A.ostianus*, *A.af.flavus*, *A.flavus*, *A.alliaceus*, *A.sojae*, *A.awamori*, *A.terreus*, *A.af.terreus*, *S.ornate*, *A.melleus*, *A.wentii*, *A.candidus*, *A.carbonarius* and *A.foetidus* isolate which produced both of CIT and ochratoxin as well. Considering the conducted studies and the results obtained by ELISA and relative distribution, the number of samples per obtained *Aspergillus* species isolates for culture media was determined, based on the measurements and the statistical analysis, the maximum amount of CIT in culture media (1655.91ppb) was produced in *A.niger*. In fact *A.niger* was the most important species and the highest CIT producer causing it to exceed the universal and local standard limits. The greatest amount of ochratoxin produced by *A.carbonarius* (8.007ppb) was more than the standard limits. When CIT was found in a sample, it always occurred with OTA confirmed that it Citrinin was presenting its content was higher than that of OTA. It can be concluded that since methods for controlling

THE IIOAB JOURNAL
ISSN: 0976-3104

mycotoxins are largely preventive, those mycotoxins that are known or suspected to cause human disease have been focused in this study.The occurrence of 2 mycotoxins (CIT and OTA) was investigated in mycobiome samples collected from 3 provinces of the northern region. Our results confirm that there is a great need to measure the average amount of OTA, and CIT were produced by native *Aspergillus* species and showed too, there was a significant relationship among *Aspergillus* species enabling them to produce more than one toxin at a time.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

ACKNOWLEDGEMENT

This study was supported by the Islamic Azad University, Lahijan Branch. The authors would also like to thank the technical and financial supports and are grateful for laboratory and statistical analysis assistance done by Seyyed Hamed Shirazi Beheshtiha and Soheyl Shokri Fashtali as well as the scientific supports.

FINANCIAL DISCLOSURE

None.

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THE IIOAB JOURNAL
ISSN: 0976-3104

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