DETECTION OF OCHRATOXIN B1 AND OCHRATOXIGENIC FUNGI IN DRIED VINE FRUITS OF RAISIN USING LC-MS/MS TECHNIQUE

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ABSTRACT
This study aimed to monitor the Mycobionta of raisin samples obtained from Duhok governorate used for grape juice preparation, occurrence of ochratoxigenic microfungi and incidence of ochratoxin B1. Aspergillus carbonarius and Aspergillus niger groups were the major agents responsible for ochratoxins contamination worldwide. Ochratoxigenic isolates of Aspergillus were revealed by based culture methods, which include fluorescences spectra in the range of wavelength (365-435 nm) and pigment production after exposition to the ammonium hydroxide. All tested of Aspergillus carbonarius showed ochratoxigenic potential and the occurrence of A. niger was higher than non Ochratoxigenic isolates. Natural contamination of grape juice with ochratoxin B was detected by LC-MS/MS technique, out of 15 juice samples 10 were found to contamination with OTB1.

KEY WORDS: Grape juices, Ochratoxin B1, LC-MS/MS.

INTRODUCTION
Ochratoxins are one of the most important mycotoxin groups are secondary metabolites generated via several fungi in genera of Aspergillus and Penicillium which colonize diversity of crops in the fields and or during storage as a consequence of compound ecological and environmental conditions such as humidity, temperature and susceptibility to fungal contamination, (CAST, 2003), and are weak organic acids consist of a derivative of an isocumarin. Several Aspergillus species have been described as producers of ochratoxins belong to section Nigri, Circumdati and Flavi, in temperate region Penicillium species such as Penicillium verrucosum, P. viridicatum (Pitt, 1987), P. nordicum, P. aurentagriseum have been described as producer of Ochratoxins. The family of Ochratoxins consist of three members A, B and C which differ slightly from each other in chemical structure. Ochratoxin A is one of the most related mycotoxins with its presence in food and feed products existence regulated in many countries (Petzinger and Ziegler, 2002). The Ochratoxin A has teratogenic (reproductive) immunosupressant and carcinogenic effects and a clear connection has been shown between nephropathy and exposure to OTA in human and animals. Ochratoxin B (fig. 1) is the nonchloronited analogue of OTA is considered to be much lower toxicity and is more extensively metabolized and more rapidly eliminated than OTA, OTC is ethyl ester of OTA (Renzulli et al., 2004). On Dried vine fruits of raisin, Aspergillus and Penicillium species are predominant present (Valero et al., 2005), in particular the predominance of Aspergillus species on raisin is notified worldwide including Italy and Spain (Abarca et al., 2003) Brazil (Iamanaka et al., 2005), Argentina (DaRocha Rosa et al., 2002) and California (Palumbo et al., 2011). Obviously Aspergillus s species are present worldwide in all the grape products and under all environmental conditions. The aim of present work to survey the incidence of OTB1 in raisin using LC/MS-MS technique.
MATERIALS AND METHODS

Sources of raisin (Zibib) samples:

20 samples used for production of zibib juice was take up from native shops of fruit juice . The lower sample size was 500 gram . All samples was stored in sterilized paper bags and stocked in refrigerator at 4°C. Samples were all processed during seven days after collection.

Preparation of grape juice:
The grape juice was qualified as described by MacDonald et al., (1999). Fifty gram of dried vine fruit was homogenized in blender (moulinex) on high speed with 250 ml of sterilized distilled water for ten minute , and one minute rest each three minute, in order to prevent sample heating. Then three ml of this juice were taken after passing through Millipore filter (0.23) µm and mixed with seven ml of methanol before storing in sterilized Eppindrof vial in refrigerator for detection of OTB1.

Isolation of fungi from dried vine fruits samples:

Dichloran Rose Bengal Chloramphenicol Agar (DRBC) (Fluka, Germany) were used for the enumeration and isolation of contaminated fungi (King et al.,1997). Thirty dried vine fruits chosen randomly from each sample and treated with 2 % sodium hypochlorite solution for two minute then rinsed with sterile distilled water then plated onto DRBC agar and incubated in darknes for seven days at 25 ºC.

Standard OTB preparation:
The standard OTB was obtained from Aldrich Sigma (Milan, Italy) was dissolved in acetonitrile at 1mg/ ml and stored at 4°C in the dark until uses .To prepare the working standard for LC-MS/MS analysis, the OTB stock solution was equally pipetted and transferred to vials, and it was diluted with mobile phase . The final concentration of OTB were 1mg/ml.

Fungal Identification:
For the identification of species belong to the genus Aspergillus , an pure colonies was grown on the following media according to the Klich (2002) , Czapeck yeast extract agar incubated for 7 days at 25 ºC. (CYA25), Czapeck yeast extract agar incubated for 7 days at 37 ºC. (CYA37), Czapeck yeast extract agar with 20 % sucrose incubated for seven days at 25 ºC. (CY20S), Malt Extract Agar (MEA) incubated for seven days at 25 ºC.and dox solution Agar (CZ) incubated for seven days at 25 ºC.

Preparation and ingredients of the four above media was mentioned in Klich (2002). Each medium were supplemente with 50 mg / L Chloramphenicol (SDI) to inhibit bacterial growth. For each cultures four plates was used, two of (CYA) and one for each of (CY 20S) and MEA . Each plate inoculated on the center then incubated in the dark for seven days. One CYA is incubated at 37 ºC.. The rest are incubated at 25 ºC.. All species were identified according to the keys and descriptions provided by (Klich ,2002; Samson et al., 2004 ; Frisvad et al., 2004 ; Abarca et al.,2004 ; De Vries et al., 2005; Serra et al.,2006b; Samson et al., 2007; Perrone et al., 2008 ;Samson and Noonim et al., 2008).

High – Performance liquid chromatography –Tandem Mass Spectrometry (LC/MS-MS) equipment and parameters for Determination of Ochratoxin B1.

This was done at Princess Haya center biotechnology. The University of Sciences and Technology, Jordan.

MS Detection and Chromatographic separation was performed by using the Agilent 1200 Rapid Resolution LC and 6460 Triple Quadrupole Mass Spectrometer. All samples were injected directly and analyzed without further preparation of samples.

For the LC method 5m MoL Ammonium acetate ( pH: 3.2) and methanol was used as mobile phases in gradient mode. The Column (ACE 5C 18 (50 ×2.1 mm) was kept at 55 ºC with a flow/ rate of 0.4 ml/min. The total...
analysis time was set to 25 minutes. The ESI source with agilent Jet stream Technology were coupled with the mass spectrometer.

Determination of the optimal MRM transition for all analysts was carried out by flow injection of standards at concentration levels of 1 ng/ml using Mass optimizer, the automated MRM Software Development Method.

RESULTS AND DISCUSSION

The filamentous fungal genera isolated from dried vine fruits after surface disinfection on Dichloran Rose Bengal Chloramphenicol DRBC media are shown in table 1. A total of 20 filamentous Genera as well as to non sporulating mycelia and yeasts was isolated.

| No. | Fungi                              | % frequency on DRBC |
|-----|------------------------------------|---------------------|
| 1   | Aspergillus aculeatinus             | 8.83                |
| 2   | A. auricomus Saito                 | 3.0                 |
| 3   | A. awamori Nakaz                   | 10                  |
| 4   | A. brasiliensis Frisvad, Varga & Samson | 5.3             |
| 5   | A. carbonarius Thom (Bainier)       | 61.6                |
| 6   | A. fia ves Link                    | 18.83               |
| 7   | A. laitcofeatus Samson & Frisvad    | 2.1                 |
| 8   | A. melleus Yukawa                  | 1.3                 |
| 9   | A. japonicus Saito                 | 1.67                |
| 10  | A. foeldus Thom & Raper            | 6.17                |
| 11  | A. fumigatus Fresen                | 10                  |
| 12  | A. niger Tiegh. nom. cons.         | 90                  |
| 13  | A. ochraceus K.Wilh                | 2.3                 |
| 14  | A. sclerotionurum Huber            | 3.4                 |
| 15  | A. ostinus Wehmer                  | 3.2                 |
| 16  | A. parasiticus Speare              | 10                  |
| 17  | A. tubingensis Schober             | 3.3                 |
| 18  | A. sclerotioniger Samson & Frisvad | 10                  |
| 19  | A. westerdijkai                    | 2.5                 |
| 20  | P. aurantiogresum Di erckx         | 2.5                 |
| 21  | P. revicompactum Dierckx           | 7                   |
| 22  | P. citrinum Thom                   | 10.3                |
| 23  | P. chrysogenum Thom                | 3                   |
| 24  | P. expansum Link                   | 12                  |
| 25  | P. glabrum (Wehmer) Westling       | 8.5                 |
| 26  | P. verrocosum Dierckx              | 3                   |
| 27  | Alternaria alternata Keissi(Fr.)   | 7.3                 |
| 28  | Cunninghamella echinulate          | 12                  |
| 29  | Curvularia sp. Boedijn             | 1.6                 |
| 30  | Emer icella nidulans Vuill(Eidam)  | 10                  |
| 31  | E. quadrilineata Thom & Raper      | 10                  |
| 32  | Eurotium h erboirorum              | 10                  |
| 33  | Fusarium solani Link               | 5                   |
| 34  | Geotrichium candidum Link.Fr.      | 3.0                 |
| 35  | Monoascus spp. Tiegh.              | 1.6                 |
| 36  | Monilia (Pres                      | 10.0                |
A total of 43 species assigned to 18 genera as well as to yeasts and mycelia sterilia were identified.

Aspergillus species showes the wide spectrum (19 species), then followed by Penicillium (seven species), Eurotium (two species), Rhizopus (two species) whereas the remaining genera were represented by one species each.

Aspergillus niger and A. carbonarius has been founded as the most frequently isolates from dried vine fruits with percentage occurrence of 90% and 61.6% respectively, followed by A. flavus (18.83%) Penicillium brevicompactum (12.3), P. expansum (12).

Black aspergilli (Aspergillus section Nigri) were represented by 9 species. These includes A. aculeatus, A. awamori, A. brasiiliensis, A. carbonarius, A. foetidus, A. japonicus, A. lactofoeatatus, A.niger, A.sclerotioniger, A.tubingensis.

Several other mycological surveys in different parts of the world confirmed the predominance of black aspergilli contaminated dried vine fruits (Iamanaka et al., 2005; Romero et al., 2005; Valero et al., 2007, Palambo et al., 2011 and Somma et al., 2012).

Penicillium was second in number of species isolated from dried vine fruits and was represented by 7 species. Penicillium expansum, P. citrinum and P. brevicompactum were the most frequent species with 12%, 10.3% and 7.0% frequency of occurrence respectively. Hakobyan et al., (2010) identified 12 Penicillium species from dried vine fruits in Armenia.

**Abilities of Ochratoxigenic strains of Aspergillus on culture based method.**

The incidence of the ochratoxigenic strains of six species of the black aspergilli that were isolated from dried vine samples shows in Table 2. The most frequently was A. niger 25% showed a potential for ochratoxin production. However, three out of 293 isolates (1%) of A. niger from dried vein fruits in Argentina proved to be ochratoxigenic as reported by Romero et al., (2005). In most other previous studies the percentage range from 0.6 to 185% (Sage et al., 2002; Serra et al., 2003).

All strains of A. carbonarius were positive for ochratoxin (100%). Romero et al., (2005) working on dried vine fruits in Argentina stated that although A. carbonarius was less common comparable to A. niger, but, however, a high proportions (96%) of the screened isolates were ochratoxigenic. Several other authors in different parts of the world demonstrated the consistent ability of A. carbonarius to produce ochratoxin (Abarca et al., 2003; Pietri et al.,2001; Mohammed et al., 2010 ) who found percentages of ochratoxigenic potential for A. carbonarius strains ranges from 25-100%. The results also showed that none of the A. japonicus isolates were ochratoxigenic. This was revealed also by ( Batista et al., 2003) and Serra et al.,2005 A. tubingensis A.sclerotioniger and A.westerdijikii also showed positive result 65 %,85 % and 25 %, respectively.

Table (2): Ability of some isolates of Aspergillus section Nigri isolated from dried vine fruit to produce Ochratoxin B1 in vitro.

| Isolated Fungi | No. of isolates | Positive isolates | % of positive isolates |
|----------------|----------------|-------------------|------------------------|
| A. carbonarius | 85             | 85                | 100                    |

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Detection of OTB1 contaminated naturally dried vine fruit by LC-MS/MS.

An accurate, sensitive and precise method using LC-MS/MS for the detection of OTB1 in dried vine fruits has been applied to confirm the previous data. Results showed out of 15 samples was contaminated with OTB1 (Table 3, Figure 1). Samples contaminated with *A. carbonarius* *A. sclerotioniger*, *A. westerdijikii* and *A. tubingensis* showed the highest contamination level, whereas samples contaminated with *A. japonicus*, *A. awamori* and *A. aculeatinus* were negative. These results are in line with (Tjamoss *et al.*, 2004; Bau *et al.*, 2005; Serra *et al.*, 2005 and Somma *et al.*, 2012).

It is interesting to note that the majority of fungi associated with naturally contaminated samples were potentially ochratoxins-producing species as described by (Cabanas *et al.*, 2010) *A. carbonarius* was observed in several studies to be the main source of ochratoxins contaminations in dried vine fruits (Chulze *et al.*, 2006).

We conclude that raisins are more frequently contaminated with different levels of OTB1 than are other dried fruits and that *Aspergillus* species are the likely source of that contamination therefore, regular raisins consumption may contribute to exposure of humans to Ochratoxins.

### Table (3): negative and positive contaminated samples with OTB1 and their fungal associated.

| Samples name | Analyte peak area (counts) | Analyte peak height (cps) | Fungi Associated with samples |
|--------------|---------------------------|---------------------------|------------------------------|
| Sample 1     | 0.0                       | 0.0 (-ve)                 | *A. awamori*, *A. flavus*, *A. faetidus* |
| Sample 2     | 0.0                       | 0.0 (-ve)                 | *A. awamori*, *A. japonicus*, *A. faetidus*, *A. niger* |
| Sample 3     | 0.0                       | 0.0 (-ve)                 | *A. sclerotiorum*, *A. faetidus*, *A. parasiticus* |
| Sample 4     | 1251531.2                 | 303641.9 (+ve)            | *A. westerdijikai*, *A. carbonarius*, *A. ochraceus*, *A. tubingensis*, *A. lacticoffeatus* |
| Sample 5     | 1963.6                    | 481.4 (+ve)               | *A. niger*, *A. carbonarius*, *A. sclerotioniger*, *A. aculeatinus* |
| Sample 6     | 1022392.3                 | 126907.9 (+ve)            | *A. niger*, *A. carbonarius*, *A. sclerotium*, *A. ochraceus* |
| Sample 7     | 896709.4                  | 155446.6 (+ve)            | *A. niger*, *A. brasiliensis*, *A. carbonarius*, *A. ochraceus*, *A. tubingensis* |
| Sample 8     | 0.0                       | 0.0                       | *A. niger*, *A. foetidus*, *A. auricomus* |
| Sample 9     | 2964178.2                 | 592308.5 (+ve)            | *A. niger*, *A. melleus*, *A. tubingensis*, *A. lacticoffeatus* |
| Sample 10    | 529110.3                  | 94738.9 (+ve)             | *A. niger*, *A. carbonarius*, *A. sclerotium*, *A. ochraceus*, *A. ostinus*, *P. verrocosum*, *A. tubingensis* |
| Sample 11    | 1552.0                    | 266.9 (+ve)               | *A. niger*, *A. lacticoffeatus*, *P. verrocosum* |
| Sample 12    | 1150318.0                 | 187792.6 (+ve)            | *A. niger*, *A. ostinus*, *A. carbonarius*, *A. ochraceus* |
| Sample 13    | 1451164.9                 | 227965.3 (+ve)            | *A. sclerotioniger*, *A. carbonarius*, *A. niger*, *A. tubingensis*, *A. lacticoffeatus* |
| Sample 14    | 2060421.3                 | 472911.8 (+ve)            | *A. niger*, *A. carbonarius*, *A. sclerotium*, *A. ochraceus* |
| Sample 15    | 0.0                       | 0.0 (-ve)                 | *A. auricomus*, *A. japonicus*, *A. aculeatinus* |

(+ve) Positive, (-ve) Negative.
Fig. (2): OTB1 detection from grape vine fruits by LC-MS/MS.
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