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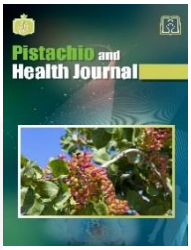

ORIGINAL ARTICLE

Incidence of *Aspergillus* species and Ochratoxin production potential in the main pistachio orchards of Iran

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Background: Aspergillus species not only cause damage to the color of pistachio fruit but also reduce the value of this product since they produce toxic secondary metabolites such as aflatoxins, ochratoxins, and fumonisin.

Materials and methods: In order to identify *Aspergillus* species associated with pistachio, 300 samples of fresh fruits, dried fruits, leaves, and fresh old wastages were collected from the main pistachio production area of Iran (in Kerman, Yazd, Semnan, and Ghazvin provinces). Fungal isolation was performed using single conidial: 220 isolates were identified based to morphological charachteristics. 22 species belonged to four subgenus and eight sections were identified according to morphological characteristics. The OTA-producing ability of the *Aspergillus* section *Circumdati* isolates was determined by the thin layer chromatography (TLC) method.

Results: The results showed that *Aspegillus tubingensis* was the most prevalent species in pistachio orchards. Most isolates of *A. ochraceous and A. melleus* are OTA producer *in vitro*, but isolates of *A. nakazawae* and *A. muricatus* were not able to produce OTA.

Conclusion: higher incidence of *A. tubingensis* as a not-dangerous mycotoxins producer species can ecologically lead to competition between this species and mycotoxigenic aspergillii and cause a decrease in mycotoxins contamination of pistachio nut.

Keywords: *Aspergillus*; Iran; Mycotoxins; Pistachio orchards

1. Introduction

Pistachio nuts (*Pistacia vera*, Anacardiaceae) have long been cherished as the symbol of health since ancient times. Pistachio is enriched with many health-benefiting nutrients. Pistachio kernels are a rich source of many antioxidant chemical substances, such as carotenes, vitamin-E, and antioxidant compounds. They are the source of minerals such as copper, manganese, potassium, calcium, iron, magnesium, zinc, and selenium. Regular consumption of pistachio in the diet can help lower total as well as bad LDL-cholesterol and increase good HDL-cholesterol levels in the blood [1]. Pistachio is the only edible fruit of 11 species in the genus *Pistacia*. The pistachio is originated from western Asia, where still found growing wildly in Turkey, Syria, Iran, Iraq, India, Lebanon, Palestine, southern Europe, Asia, and Africa [2]. Iran is one of the main producers of pistachio in the world. Most of this product is exported to other countries. The European Union (EU) and China has traditionally been one of the major destinations for Iran's pistachio. Contamination of agricultural products with mycotoxins including aflatoxins, ochratoxin, and fumonisin is one of the major challenges encountered by producers [3]. Species of the *Aspergillus* are cause of various plant and food secondary rot, with the consequence of possible accumulation of mycotoxins (a secondary metabolite that has powerful teratogenic, mutagenic and hepatocarcinogenic effects [4]. They can contaminate agricultural products at different stages, including pre-harvest, harvest, processing, and handling. Changes due to spoilage by *Aspergillus* species can be of, nutritional, and qualitative nature, including pigmentation, discoloration, rotting, development of off-odors, and off-flavors [4]. This fungus is common all over the world as an air and soil mycoflora found in live and dead animal and plant organisms. It is particularly interested in infecting nut kernels and oily plants. Peanut, corn, wheat, rice, pistachio, and almond are the main products infected by this fungus. Iran has about 316,000 hectares of pistachio orchards and produces about %57 of the world pistachio. More than %60 of the world pistachio export is done from Iran to other countries, well showing the economic significance of this product for the country. Iran is also recognized as the most important producer and exporter of pistachio in the world [5]. Contamination of pistachio nut by

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Aspergillus and mycotoxins are the most serious challenge for pistachio production, consumption, and exportation all around the world. Factors affecting infection of pistachio nuts to mycotoxin include pistachio cracking (especially early splitting), environmental factors, cultural practices, frequency and time and type of irrigation, animal manures, and harvesting time [6]. *Aspergillus* species not only cause physical damages to pistachio nuts but also reduce the quality of pistachio since they produce toxic secondary metabolites, such as aflatoxins, ochratoxins, and fumonisin [6]. Ochratoxin A (OTA) was discovered as a secondary metabolite of a strain of *Aspergillus ochraceus*. OTA exhibits intestinal fragility, nephrotoxicity, immunosuppression, teratogenicity, carcinogenicity, and cytotoxicity in hepatic cell lines [7]. The OTA inhibits carboxypeptidase A, renal phosphoenolpyruvate carboxykinase, phenylalanine tRNA synthetase, and phenylalanine hydroxylase activity. Formation of free radicals has been considered as one of the mechanisms for the carcinogenic/toxic effects of OTA [8]. Fungi from two genera are able to produce ochratoxins. In the genus *Penicillium*, OTA is produced by *P. verrucosum* and *P. nordicum*, and in the genus *Aspergillus* by *A. ochraceus*, *A. melleus*, *A. auricomus*, *A. ostianus*, *A. petrakii*, *A. sclerotiorum*, and *A. sulphureus*, all in section *Circumdati* (formerly the *A. ochraceus* group). *A. alliaceus* and *A. albertensis*, recently shown to be more closely related to section *Flavi*, have also been described as OTA producers [9]. Some members of *Aspergillus* section *Nigri*, such as *A. niger* var. *niger* and *A. carbonarius* have been reported as ochratoxigenic fungi [7]. Several food products worldwide face the risk of OTA contamination, including coffee, cereal grains, processed foods, beer, grapes, wine, cocoa, nuts, and dried fruits [10]. A survey on contaminant fungal flora of some nuts showed that 49% of market pistachio has fungal contamination, and *Penucillium*, *Mucor* and *Aspergillus* with 26, 24, and 20% were found to be the most dominant spoilage agents, respectively [11]. This study was conducted to evaluate the incidence and prevalence of *Aspergillus* species in the main pistachio orchards of Iran and OTA production potential of section *circumdati* isolates *in vitro*.

2. Materials and Methods

2.1. Samples

Three hundred samples of fresh and dried pistachio, pistachio fresh leaf (from various parts of the tree), fresh and old hull, and hard skin were collected from pistachio orchards in Kerman, Yazd, Semnan and Ghazvin provinces as the main pistachio production provinces. The number of samples was determined based on the cultivation area of each province. All samples were brought to the laboratory for further studies. Sampling was conducted during August to November 2007. Samples were placed separately on sterile filter papers and kept in a dark incubator at 25°C. After two to three weeks, single conidial heads were chosen according to their phenotype and subculture repeatedly on Malt Extract Agar (Sigma-Aldrich, Germany) media until complete purification. Morphological identifications were carried out according to following references [12-14].

2.2. Ochratoxin analysis

Fifteen isolates from section *Circumdati*, of which 10 isolates of *A. ochraceus*, two isolates of *A. melleus*, three isolates of *A. nakazaweae*, and one isolate of *A. muricatus*, were analyzed for OTA production. To assay ochratoxin production, isolates of section *Circumdati* (most probable species for Ochratoxin production) were grown in 100-mL flasks containing 50 mL (YES; yeast extract, sucrose) liquid medium. Cultures were incubated without agitation for 14 days at 30°C in the dark place. Ochratoxin A was extracted by adding 5 mL of chloroform. OTA-producing ability of the *Aspergillus* isolates was determined by the thin layer chromatography (TLC) method. [15-19].

3. Results

In this study, 220 isolates were recovered from the pistachio and related samples. Twenty two species belonged to four subgenus and eight sections were identified according to morphological charachteristics. The most common subgenus were *Aspergillus* subgenus *Circumdati* (74%) (Fig. 1). Eight Sections of all the 22 sections of *Aspergillus* were included in this study. These Sections are *Flavi* (27%), *Nigri* (25%), *Circumdati* (18%), *Aspergillus* (12%), *Nidulantes* (12%), *Cremei* (3%), *Terrei* (2%), and *Fumigati* (1%), respectively Fig. 1).

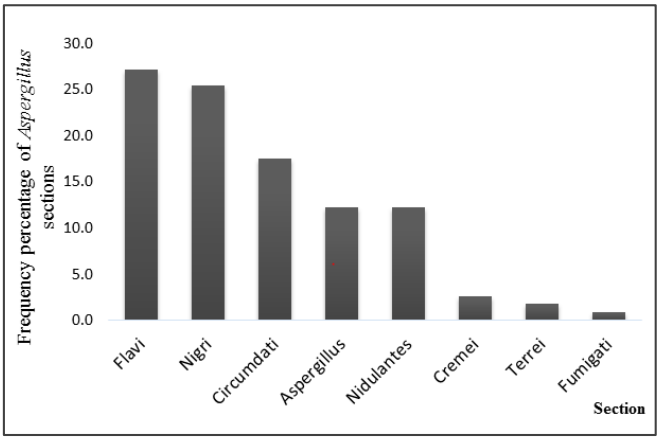

Fig. 1. Incidence of *Aspergillus* sections in pistachio production area of Iran

Altogether 22 species were identified in recovered isolates. They included *A. alliaceus*, *A. flavus*, *A. parasiticu,s*, *A. tamarii* (section *Flavi*), *A. japonicas*, and *A. tubingensis* (section *Nigri*), *A. ochraceus*, *A. nakazawae*, *A. melleus*, and *Neopeteromyces muricatus*, (*section Circumdati*), *A. dimorphicus and Chaetosartorya chrysella* (section *Cremei*), *A. terreus* (section *Terrei*), *A. fumigatus* (section *Fumigati*), *A. sydowii*, *A. versicolor* ،*Aspergillus* sp. *Emericella quadrilineata*, (section *Nidulantes*), *E. repens*, *E. rubrum*, *E.*

chevalieri, and *E. amstelodami* (section *Aspergillus*). Of the identified species, *A. tubingensis* (24%), *A. flavus* (21%), and *A. ochraceous* (12%) were the most prevalent species, respectively (Fig. 2).

Aspergillus tubingensis and *A. flavus* were isolated from all studied aeries, *Aspergillus* sp. *A. terreus*, *A. fumigatus*, and *A. alliaceus* were isolated just from Kerman province. *A. dimorphicus* and *A. versicolor* were isolated only from Yazd province, and *A. muricatus* just from Semnan province (Fig. 3).

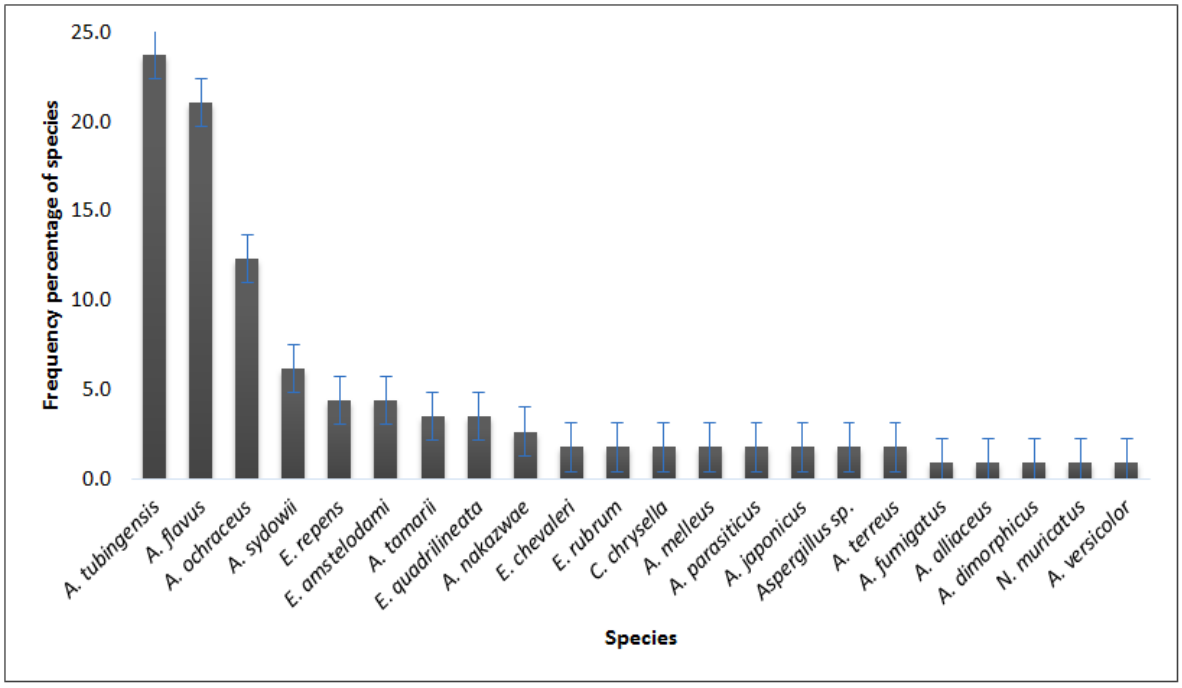

Fig. 2. Incidence of *Aspergillus* species in main pistachio production area of Iran

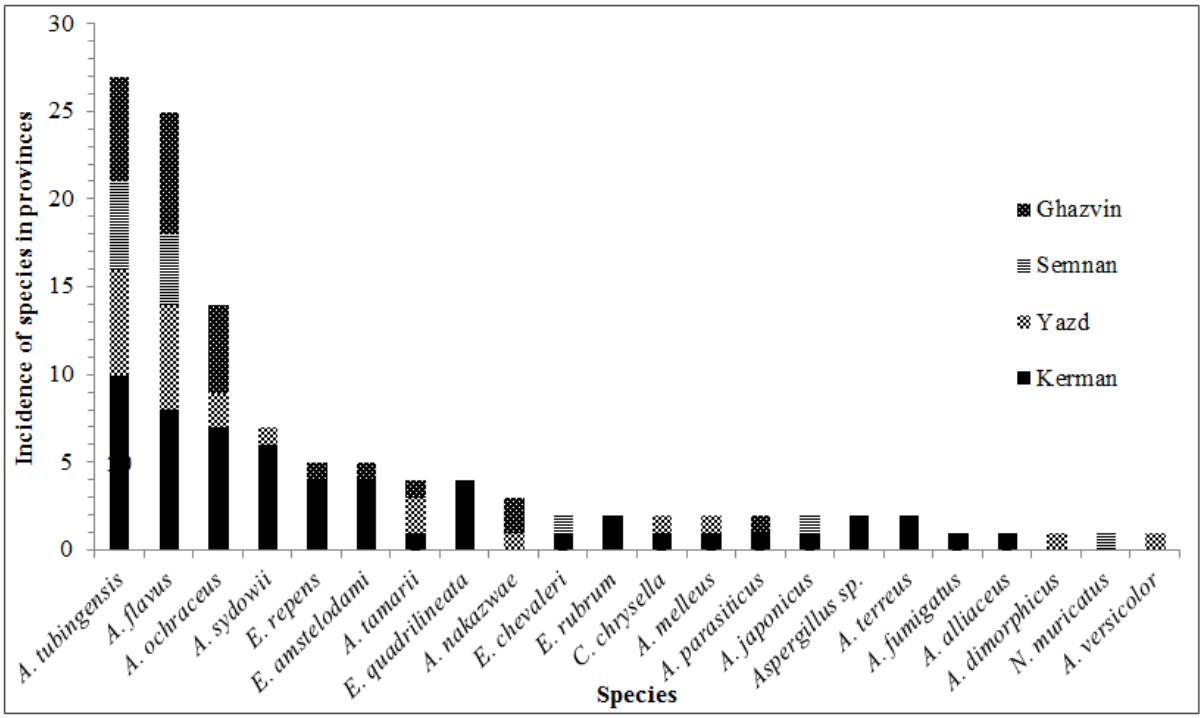

Fig. 3. Distribution of identified species of Aspergillus in main pistachio production area in Iran

Some species such as *A. niger* and *A. carbonarious* (section *Nigri)* have OTA production capability. Fortunately, none of these species were isolated from pistachio orchards. Therefore black *aspergilli* isolates were not analyzed for OTA production. The results showed that 9 of the 10 isolates *of A. ochraceus* and all isolates of *A. melleus* were capable of producing OTA. *A. nak*azaweae and *A. muricatus* isolates are not OTA producers (Fig. 4). Severity of OTA production is different among *A. ochraceous* isolates. Qualitative data of TLC plate showed that most *A. ochraceous* isolates have a high potential for OTA production.

4. Discussion

Our study shows that *A. tubingensis* (formerly identified as *A. niger*) and *A. flavus* were the most prevalent species in all pistachio production aeries of Iran. Many other studies have shown that *A. flavus* and *A. niger* var. *niger* [20] are the most prevalent species. Studies on the incidence of *Aspergillus* in fig (*Ficus carica*) showed that, based on morphological characteristics, *A. niger* var. *niger* is the most common A*spergillus* species (39.6%) in dried fig and fig orchards [21]. *A. niger* and *A. tubingensis* are the most similar morphologic species, and it is often impossible to distinguish them [22]. Varga *et al.* [15] showed that some isolates of *A. ochraaceus* are OTA producers and some others are not. Bayman *et al.* [19] analyzed 41 isolates of *A. ochraceus* and 17 isolates of *A. melleus* recovered from fig, hazelnut, cottonseed, walnut, and soil for OTA production. Their results showed that, except for one isolate of *A. ochraceus*, the others were not OTA producers. Comparing the results of these two studies is difficult. They concluded that some of their isolates had the ability for OTA. 175 production at earlier studies but missed it later. The isolates that were used in our studies were totally fresh. Studies of the *A. ochraceus* group are complicated by difficulties in distinguishing *A. ochraceus* from related species. It is possible that the isolates they used were not a real *A. ochraceus* strain. Iamanaka *et al.* [16] showed that, after black *aspergili*, *A. ochraceus* is the most important OTA producer in dried fruits in Brazil. 87% *A. ochraceus* were ochratoxigenic. El-Shayeb *et al.* [23] investigated that *A. ochraceus* and *A. alliaceus* strains have the maximum quantities of ochratoxins produced on yeast extract-sucrose (YES) medium.

Trace production of ochratoxin A in *A. melleus*, *A. ostianus*, *A. pers*ii and *A. petrakii* was reported by Frisvad *et al.* [24]. They also concluded that *A. muricatus* is ochratoxigenic, which is different from the results we obtained. We analyzed just one isolate and it is not enough to make a decision about their ability for OTA production.

5. Conclusion

Higher Prevalence of *A. tubingensis* than *A. flavus*, in pistachio production area can be a hopeful finding. Since the competition of these fungi for colonizing niches and substrate can lead to suppression of *A. flavus*, the most common aflatoxins producer species in many agricultural products is OTA production assay just performed *in vitro*, and it should not be determined as contamination of pistachio to this mycotoxin. So far, there has not been any study on the contamination of Iranian pistachio to OTA.

Conflicts of interest

The authors declare no conflicts of interest.

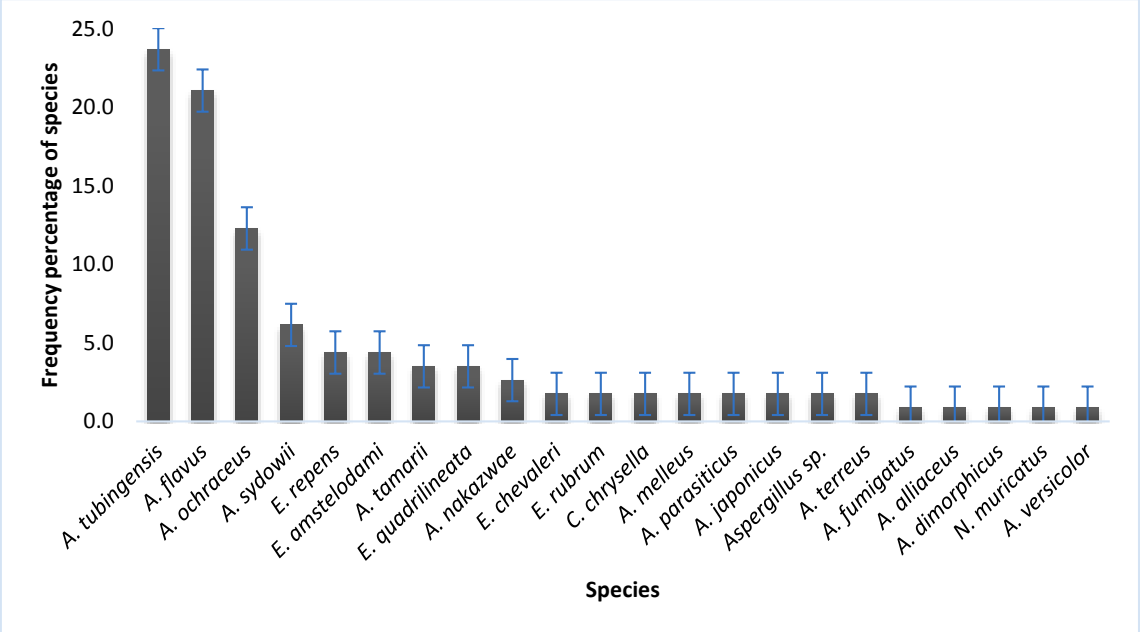

Fig. 4. Incidence of *Aspergillus* species in main pistachio production area of Iran

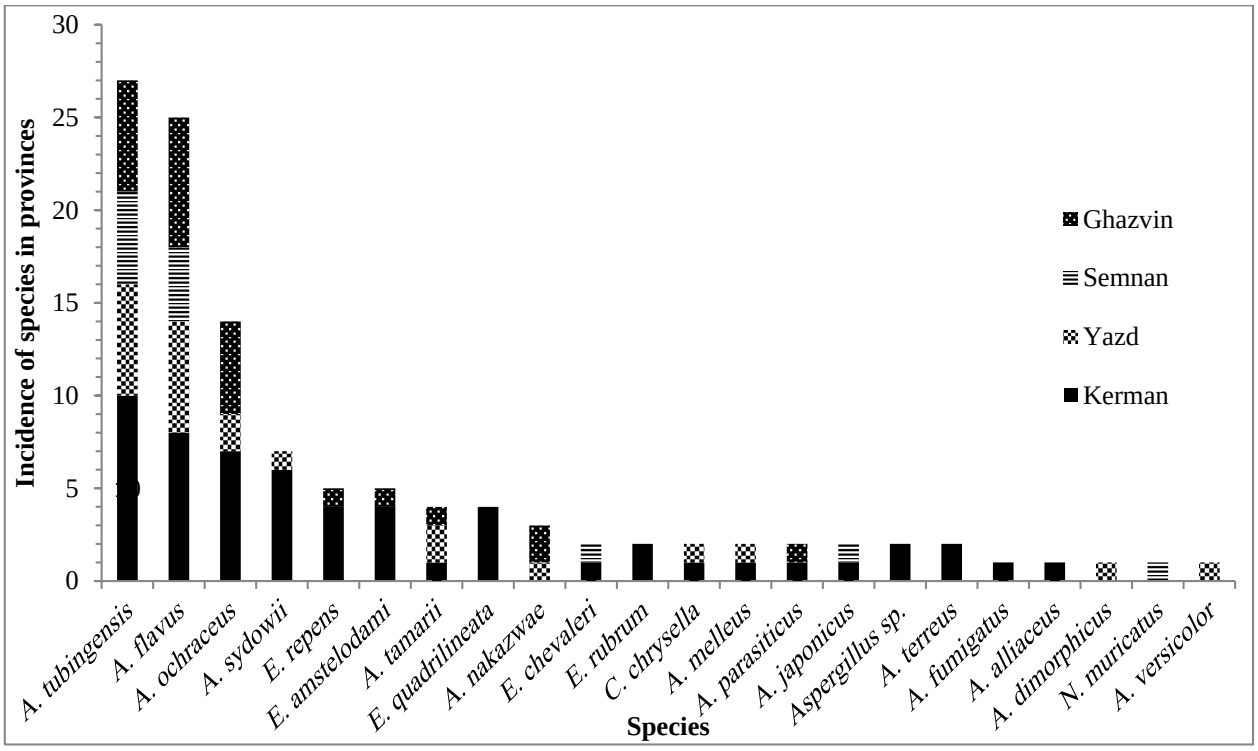

Fig. 5. Distribution of identified species of Aspergillus in main pistachio production area in Iran

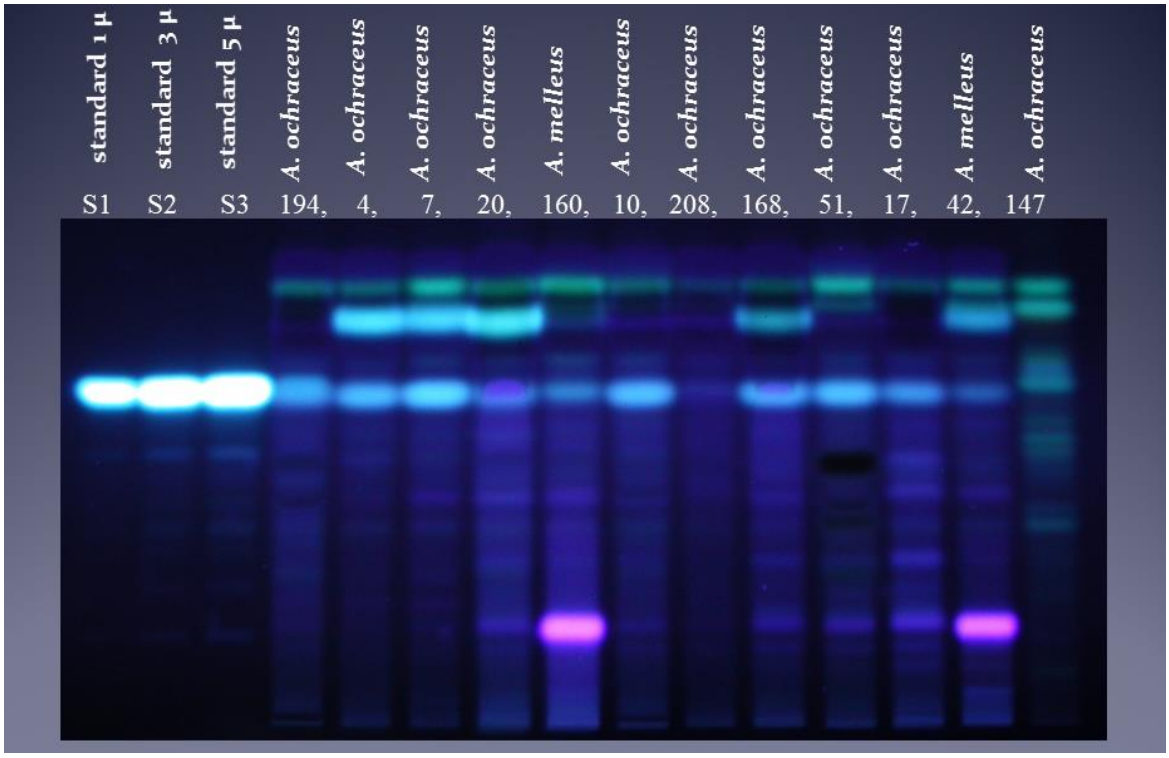

Fig. 6. High performance TLC of ochratoxin A extracted from *Aspergillus* section *Circumdati* isolates recovered from Pistachio orchards

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