Keywords: Aflatoxin, ELISA, fungi, medicinal plants, ochratoxin, Penicillium spp., spices

INTRODUCTION

Spices and herbs are valued for their distinctive flavors, colors and aromas and are among the most versatile and widely used ingredient in food preparation and processing throughout the world (Ayers et al., 1980). They are widely used as raw materials for pharmaceutical preparations (Galenic products) and as a supplement for dietetic products, especially for “self medications” in public (Weiser et al., 1971). As with many other agricultural products, spices and herbs may be exposed to a wide range of microbial contamination during pre- and post-harvest. Although spices are present in foods in small amounts, they are recognized as important carriers of microbial contamination mainly because of the conditions in which they were grown, harvested and processed. In addition, because of possible neglects during sanitation or processing, foods containing spices are more likely to deteriorate and also could exert harmful effects, having in mind health risks associated with mycotoxins produced by some fungal genera (McKee, 1995; Koci-Tanackov et al., 2007). The potential for spoilage and mycotoxin production depends upon the types of fungi present, the composition of the food and the conditions of handling and storage. For example, dried foods are susceptible to spoilage and toxin production if storage temperature is suitable for fungal growth (Misra, 1981). Moreover, spices are collected in tropical areas by simple methods and are commonly exposed to many contaminants before, being dry enough to prevent microbial growth. They are also stored in conditions favoring contamination by insects, rodents and other vermin (Sharma et al., 1984). The mycological quality of some spices on the market, especially of pepper, is quite poor, bearing many genera and species of fungi. Most fungi are present on pepper of the post-harvest and storage type, which develop after harvest if relative humidity is not controlled during storage (Aziz et al., 1998). Fungi are the predominant contaminants of spices, but most such microbial populations are probably regarded as commensal residents on the plant that survived drying and storage. Soil and air is the main inoculums source for causing contamination in crude spices in field (Kneifel and Berger, 1994). The microbial flora on many spices and related materials is generally dominated by aerobic spore-forming microorganisms. It was found that celery seed, paprika, black and white pepper and Ginger usually show total plate counts in millions per gram (Krishnaswamy et al., 1971). Also, total plate counts above few hundred thousand per gram have been noted in Cassia, Mace and Nutmeg (Guarino, 1974). Spices are commonly heavily contaminated with xerophilic storage moulds and bacteria (Dimic et al., 2000; Romagnoli et al., 2007). There are more and more indications that primary liver carcinoma and other...
Serious diseases may be induced by consuming food or using raw materials for food processing contaminated with fungi or mycotoxins. Aflatoxins, ochratoxin and sterigmatocystin proved resistant to heat and have an ability to accumulate in the organism. In the laboratory, both *A. flavus* and *A. ochraceus* are reported to produce mycotoxins. Other species of molds are frequently isolated from spices, including those of *Penicillium*, *Scopulariopsis* and *Sporendemona* (Galvano et al., 2005; Zinedine et al., 2006).

The present study aimed to throw light on the safety of spices and medicinal plants for direct human use as well as for pharmaceutical purposes. The present investigation reports the association of mycoflora with medicinal plant samples and spices, their screening for mycotoxin producing ability and mycotoxin occurrence in samples under Erbil environmental conditions.

**MATERIALS AND METHODS**

**Sample collection:** A total of sixteen dried samples, representing different types of spices and medicinal plants, were collected randomly from different places of famous (Shekhailla) market in the Erbil city during the months of March and July 2011. These products of spices and medicinal plant were chosen on the basis of their availability in the market and popularly of usage. Spice samples were usually found outside, kept in metal or plastic containers, wooden boxes or gunny bags or on the bare ground. Care was taken to avoid old stocks and visibly contaminated samples. For each spice and medicinal sample, 3 replicates were taken and mixed to prepare one composite sample. A total of 3 composite samples were prepared for each sample. In the laboratory, samples were individually finely ground in a common household blender. The blender’s cup was rinsed in 85% alcohol between samples. The powder kept tightly packed in a new paper bag and stored at 4°C for further analysis. The common names, scientific name and used parts of each sample are presented in Table 1.

### Table 1: Common, scientific and part used of spices and medicinal plant

| Sample no | Common name       | Plant part used             | Scientific name  |
|-----------|-------------------|----------------------------|------------------|
| 1         | Red tea           | Flower                     | Hibiscus sabdariffa |
| 2         | Saffron           | Flower                     | Crocus sativus   |
| 3         | Peppermint        | Leaves                     | Mentha piperita  |
| 4         | Nutmeg            | Peeled seeds               | Myristica fragrans |
| 5         | Black cumin       | Seeds                      | Nigella sativa   |
| 6         | Garlic            | Clove                      | Allium sativum   |
| 7         | Clove             | Flower buds                | Syzygium aromaticum |
| 8         | Black pepper      | Dried fruits               | Piper nigrum     |
| 9         | Cumin             | Seeds                      | Cuminum cyminum  |
| 10        | Ginger            | Dry rhizomes               | Zingiber officinale |
| 11        | Cardamom          | Seeds                      | Elettaria cardamomum |
| 12        | Cinnamon          | Bark                       | Cinnamomon zeylanicum |
| 13        | Dry lemon         | Fruit                      | Citrus spp       |
| 14        | Sumac             | Dried fruit                | Rhus corioria    |
| 15        | Bay leaf          | Leaves                     | Laurus nobilis   |
| 16        | Thyme             | Leaves plus stems          | Thymus vulgaris  |

Mycological analysis (isolation techniques): In collection of samples, the method described by Neergaard (1973) has been adopted. Accordingly, samples mycoflora was isolated by using Agar Plate Method (APM) and Standard Moi Blotter method (SMB) as recommended by ISTA (1966) and Neergaard (1973).

**Isolation of fungi by direct plating method:**

**Isolation of sample-borne mycoflora:** The method used for isolation of fungi was previously described by Abdullah et al. (2002). Ten gm of each sample were surface sterilized by 6% sodium hypochlorite solution (NaOCl) in a sterile conical flask for 1-2 min, and then washed by distill water 3 times for 1.5-2 min to removing the toxic activity of the chemical agent on the samples. The disinfected samples transferred with sterile forceps into Petri dish contain sterilized Dax Agar (CDA), at the rate of (5-10) pieces per plate, depending on the size of the particles, lager samples cut into small pieces. CDA supplemented with 0.5 mg chloramphenicol/mL to restrict bacterial growth. Three replicates were made and the plates were incubated at 25°C for 5-7 days. Fungi colonies were identified according to morphological and microscopic characteristics.

**Isolation of sample surface mycoflora:** The samples transferred with sterile forceps into Petri dish contain sterilized CDA. Three replicates were made and the plates were incubated at 25°C for 5-7 days. Fungi colonies were identified according to morphological and microscopic characteristics (Pitt et al., 1992).

**Isolation on moist blotting paper (sterile filter plate method):** Plates are sterilized by oven on 80°C over night with 2-3 layers of filter paper of 90 mm size (Whatman No.1), the filter papers saturated with 10-15 mL sterile distilled water.

**Isolation of sample-borne mycoflora:** The samples submerge in the sodium hypochlorite 6% concentration for 1-2 min and then washed by distill water 3 times for 1.5-2 min, then transfer by using sterilized forceps into three layers of moistened 9 cm diameter filter paper in sterilized Petri dishes, sterilized samples were evenly placed at the rate of 10 pieces per Petri plate at equal distance in each Petri plate, samples of each variety were tested by employing standard blotter method in 3 replications. The plates were incubated for 5-7 days at 25°C; fungi developing on samples were examined and transferred to PDA for identification and pathogenicity studies.

**Isolation of sample surface mycoflora:** Non-sterilized samples were evenly placed at the rate of 10 pieces/Petri plate at equal distance in each Petri plate on three layers of moistened 9 cm diameter filter paper in sterilized Petri dishes. The plates were incubated for 5-7 days at 25°C; after incubation the samples were...
examined under microscope for the associated fungi and they were identified based on “habit characters” (Anonymous, 1996).

**Standard dilution plate:** For fungal analysis, dilution method was used to determine total fungal counts in spice and medicinal plant samples. Ten grams of each composite sample (fine powder) were transferred into 250 mL screw-capped medicinal bottle containing 90 mL of sterile distilled water and were mechanically homogenized at constant speed for 15 min. The sample-water suspension was allowed to stand for 10 min with intermittent shaking before being plated. Appropriate tenfold serial dilutions (1:10) were prepared and 1 mL portions of suitable dilutions of the resulting samples suspension were used to inoculate Petri dishes each containing 15 mL Potato Dextrose Agar (PDA). Plates were then incubated for 7 days at 28°C. Three replicates plates per medium were used for each sample and the developing fungi were counted and the number per mg dry sample was determined and identified according to several key processes. Data expressed are average of all these media. After incubation, the results were expressed in Colony-Forming Units (CFU) /g of samples; all plates were examined visually, directly and with a microscope (Aziz et al., 1998).

**Diagnosis:**

**Identification of the fungal genera:** The fungal isolates were transferred to sterilized plates for purification and identification. The grown fungi were mounted on a slide, Stained with lactophenol-cotton blue to detect fungal structures (Basu, 1980), covered with a cover slip, examined under microscope and identified on the basis of their colony morphology and spore characteristics (Ronhede et al., 2005; Rajankar et al., 2007).

The texts (books) used for identification of fungi, depending on their taxonomic keys are as follows; Moubasher (1993), Larone (1995), Pitt and Hocking (1997), Guarro et al. (1999), Howard (2002), Watanabe (2002), Ulhan et al. (2006) and Pornsuriya et al. (2008).

**Determination of toxigenic potential of fungi in culture media:***

**Dichloran rose bengal chloramphenicol agar test:**

DRBC is a selective medium that supports good growth of fungi. It is formulated as described by King et al. (1979) is a modification of DRBC (Jarvis, 1973). The substances in the DRBC are dichloran (is added to the medium to reduce colony diameters of spreading fungi), rose Bengal (suppresses the growth of bacteria and restricts the size height of colonies of the more rapidly growing moulds) and chloramphenicol (is included in this medium to inhibit the growth of bacteria present in environmental and food samples). The reduced pH of the medium from (7.2 to 5.6) helps inhibition of the spreading fungi (Jarvis, 1973). The isolated fungi were inoculated in the solidified DRBC medium after incubation for 7 days at 25°C search for pigmentation and color change observed in compare to CDA medium control.

**Sample preparation for natural aflatoxin and ochratoxin determination by ELISA method:**

Samples were homogenized and kept in glass bottle and stored at 2-8°C until further analysis. For the quantitative analysis of mycotoxins (aflatoxin and ochratoxin). Enzyme Linked Immunosorbent Assay technique (ELISA) was used. Samples (10 g) were taken in 50 mL of 70% methanol for aflatoxins and ochratoxin analysis. Sample powder was blended individually with high speed blender for three minutes. After blending the material was filtered with Whatman filter paper number 1, the filtrate was used for further analysis. Commercially available immunoassay kit Veratox for quantitative analysis of aflatoxin and ochratoxin test-NEOGEN Crop, Lansing, MI was used. The assay kit was based on Competitive Direct Enzyme Linked Immunosorbent Assay (CD-ELISA) (Stoloff et al., 1991). The antibodies captured the analyze and conjugated to the enzyme (horse reddish peroxidase). Tetra methylbenzidine/hydrogen peroxide was used as a substrate for color development. Finally stopping solution was added to stop the reaction. The color intensity was inversely proportional to the mycotoxin concentration and measured with the ELISA reader. All necessary reagents were present in the kit.

Concentration of mycotoxins was calculated by Log/logit Software Awareness Technology Inc. (Anonymous, 2000; Stoloff et al., 1991).

**RESULTS AND DISCUSSION**

Common and scientific names, plant part used of the spices and herbal drugs were tabulated in Table 1. Totally 16 samples were analyzed for enumeration of fungal isolates.

Fungal genera were isolated by different method on the different media. The results presented in Table 2 show fungi were isolated by using Standard Moist Blotter method (SMB) and Agar Plate Method (APM). Blotter and Agar plate methods were employed for this study and two sets of samples were analyzed i.e., unsterilized and surface sterilized spices and medicinal plant. A total of 10 different fungal genera and 16 species were isolated. All fungi were identified on the basis of their cultural and morphological characteristics. These were identified as Alternaria alternata, Aspergillus aculeatus, A. candidus, A. flavus, A. niger, A. ochraceous, A. tamari, A. terreus, Gliocladium sp.,...
Table 2: The isolated fungi from herbs and spices samples by blotting paper (filter paper) and czapex dox agar

| Samples no | Samples name | Blotting paper | Czapex dox agar |
|------------|--------------|----------------|----------------|
|            |              | Sterilized surface | Unsterilized surface | Sterilized surface | Unsterilized surface |
| 1          | Red tea      | Negative | Aspergillus niger | Negative | A. niger |
|            |              | Penicillium spp | A. niger | Penicillium spp |
| 2          | Saffron      | Negative | A. niger | Negative | A. niger |
|            |              | Penicillium spp | A. niger | Penicillium spp |
| 3          | Peppermint   | A. niger | A. niger | A. niger | A. niger |
|            |              | Gliocladium sp. | Rhizopus oryzae | Ulocladium botrytis |
| 4          | Nutmeg       | Negative | A. niger | Negative | A. niger |
|            |              | Sterile mycelium | A. aculeatus | Alternaria alternata |
| 5          | Black cumin  | Negative | Penicillium spp | A. aculeatus | Penicillium spp |
|            |              | A. aculeatus | Penicillium spp | A. flavus |
| 6          | Garlic       | Negative | Penicillium spp | Negative | A. aculeatus |
|            |              | A. aculeatus | Penicillium spp | A. flavus |
| 7          | Clove        | Negative | Negative | Negative | A. aculeatus |
|            |              | Alternaria alternata | A. candidus | A. flavus |
| 8          | Black pepper | Negative | Asp. flavus | A. flavus | A. candidus |
|            |              | A. flavus | A. tamarii | A. flavus |
| 9          | Cumin        | Negative | Penicillium spp | Negative | A. aculeatus |
|            |              | A. aculeatus | A. flavus | A. tamarii |
| 10         | Ginger       | Negative | A. flavus | A. flavus | A. flavus |
|            |              | A. niger | A. aculeatus | A. flavus |
|            |              | A. terreus | Penicillium spp | A. aculeatus |
| 11         | Cardamom     | Negative | Rhizopus arrhizus | Negative | Rhizopus arrhizus |
|            |              | A. aculeatus | A. ochraceus | A. aculeatus |
| 12         | Cinnamon     | Negative | Penicillium spp | Negative | Syncephalastrum sp. |
|            |              | A. niger | Memmoniella echinata | A. niger |
| 13         | Dry lemon    | A. niger | Negative | Negative | Memmoniella echinata |
|            |              | A. niger | Penicillium spp | A. niger |
| 14         | Sumac        | A. niger | A. niger | A. niger | A. niger |
|            |              | A. aculeatus | A. aculeatus | A. aculeatus |
| 15         | Bay leaf     | A. niger | A. niger | A. niger | A. aculeatus |
|            |              | A. flavus | A. flavus | A. flavus |
| 16         | Thyme        | Negative | Negative | Negative | Hyalodendron diddeus |
|            |              | Penicillium spp | Penicillium spp | Penicillium spp |

Hyalodendron diddeus, Memmoniella sp., Penicillium spp., Rhizopus arrhizus, R. oryzae, R. stolonifer, sterile mycelium, Syncephalastrum sp. and Ulocladium botrytis. It was observed that treated samples (with sodium hypochlorite) yielded less population of samples-borne fungi than the untreated samples, indicating partial elimination of some contaminating fungi. In this table, it was found that both the agar and blotter paper methods of fungal isolation are effective, routinely and consistently applicable and provide reliable results. A total of 10 fungal genera were isolated by agar plate method and 3 fungi by blotting paper method under unsterilized conditions. Out of 3 samples, Aspergillus spp. was isolated from all sample, showed samples infection in both methods and as such it appeared as the most predominant fungus of spices and medicinal plants. The results agree at large with many of the investigators working on seed pathology. Sumanth et al. (2010), who isolated fungal genera from tested spices, found that the most common fungi isolated were Aspergillus spp. followed by Alternaria alternata, Cladosporium, Curvularia, Fusarium spp., Helminthosporium and Trichoderma show maximum incidence on Agar plate method. Many developing countries have been trying to increase the quality of their seed production. Unfortunately due to the lack of proper post harvest preservation techniques, large portion of annual yield gets damaged by fungal action according to Abou Donia (2008) and Dimic et al. (2008) twenty three different fungi were isolated from the test spices. It indicates the ability of fungi in developing association with broad spectrum of seeds, irrespective of their types. The similar reports regarding the incidence of fungi have been given by Sharma and Sharma (1984) and Regina and Raman (1992) in Ammi and cumin, A. niger, A. flavus and Cladosporium sp., have been recorded as most dominant. Agar plate method is proved to be superior for the highest
incidence of fungi and the results are similar to the reports of Bilgrami and Ghaffar (1993) and Motta et al. (1996). The highest incidence of fungi was observed in ammi and coriander followed by cardamom, caraway and cumin respectively according to Seema and Monica (2003). It is interesting to note that the species of *A. alternata, A. flavus, A. Niger* and *C. cladosporidies* have dominant association, a similar results also reported by Ayres et al. (1980). The overall result reveals that the agar method is a more supporting medium than blotter method used for the isolation of fungi.

The results presented in Table 3 show the identity and the total Colony Forming Units (CFU) of fungi were found in all of the collected samples, they were serially diluted and plated on PDA medium. The total number of isolated fungi from the all sixteen selected samples was \((203 \times 10^3)\) cfu/g. samples. The minimum number of fungi was detected in Thyme (1×10⁰) compared to blotter agar method is a more supporting medium than blotter method. The results presented in Table 3 show the identity and the total Colony Forming Units (CFU) of fungi were found in all of the collected samples, they were serially diluted and plated on PDA medium. The total number of isolated fungi from the all sixteen selected samples was \((203 \times 10^3)\) cfu/g. samples. The minimum number of fungi was detected in Thyme (1×10⁰) compared to blotter agar method.

Incidence of fungi was found by Bokhari (2007) were isolated fungi from spices samples during the investigation. *Aspergillus* was the most common genus in the different spices tested. *Aspergillus flavus* and *A. Niger* were the most prevalent. The result less agreement to this finding by Srivastava and Chandra (1985) recorded that *Aspergillus* followed by *Fusarium* were the most frequent members of the mycobiota of coriander, cumin, fenel and fenugreek. Our results were in well agreement with those found by Hassan (1984) reported that members of *Fusarium* was completely absent in 12 kind of spices tested. Hashem and Alamri (2010) the most predominant fungal genera encountered were *Aspergillus, Penicillium and Rhizopus*. Samples obtained from sumac encountered very rare colony counts indicating its antifungal prosperities. Alternaria was represented by *Alternaria alternata*. Ath-Har et al. (1988) reported that *A. flavus, A. Niger, Aspergillus nidulans, A. sydowii, A. ochraceus, Penicillium* and *Rhizopus* spp. were most frequently isolated from spices and drug plants. Bugno et al. (2006) show that the predominant mycoflora obtained was distributed in 10 genera. The genus *Aspergillus* was the most dominant genus recovered (179 isolates) followed by *Penicillium* (44 isolates). The presence of a wide range of storage fungi indicates that considerable improvements could be made during post-harvest storage. The dominant of *Aspergillus* and *Penicillium* spp. in all examined medicinal plant samples and spices was in accord with the results of Takatori et al. (1977) and Ayres et al. (1980), who stated that *Aspergillus* and *Penicillium* spp. were the main components of cardamon, cinnamon, fenel, coriander, cumin, black cumin and white pepper, all of which are common in the food industry. They found a high degree of contamination in all samples.

Data represented in Table 4 show the result of mycotoxin detection on DRBC media for fungi isolated from spices and medicinal plant samples, *A. flavus, A. Niger* and *A. ochraceous*, show Positive results on...
Table 4: Determination of aflatoxin and ochratoxin content in fungal culture by dichloran rosebengal chloramphenicol agar media.

| No | Fungi                        | Dichloran rosebengal chloramphenicol agar media |
|----|------------------------------|-----------------------------------------------|
| 1  | Aspergillus flavus           | Positive                                      |
| 2  | Aspergillus niger            | Positive                                      |
| 3  | Aspergillus ochraceus        | Positive                                      |
| 4  | Penicillium sp.              | Negative                                      |
| 5  | Rhizopus sp.                 | Negative                                      |

Table 5: Total aflatoxins and ochratoxin content in herbs and spices samples by ELISA method.

| Sample         | Natural aflatoxin content (ppb) | Natural ochratoxin content (ppb) |
|----------------|---------------------------------|----------------------------------|
| Sumac          | 0.210                           | 0.120                            |
| Ginger         | 1.039                           | 0.669                            |
| Garlic         | 1.406                           | 1.133                            |
| Black pepper   | 1.055                           | 0.551                            |
| Thyme          | 0.913                           | 0.470                            |
| Dry lemon      | 0.221                           | 0.105                            |
| Cinnamon       | 0.489                           | 0.086                            |
| Red tea        | 0.085                           | 0.061                            |
| Bay leaf       | 0.677                           | 0.363                            |
| Peppermint     | 0.598                           | 0.689                            |
| Nutmeg         | 0.887                           | 0.157                            |
| Cardamon       | 1.085                           | 0.627                            |

Aspergillus species are common fungal contaminants of maize and also produce mycotoxins.

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