**INTRODUCTION**

Mycotoxins are toxic secondary metabolites produced by various genera of fungi that contaminate crops and processed food and feedstuff all over the World. The most common mycotoxin is Aflatoxin (AF), belongs to family of deketides, is identified as secondary product of mainly the genus Aspergillus (A. flavus, A. parasiticus and A. nomius) often contaminating cereals along the line of their growth, harvest, storage, transport and processing for the production of food or animal feed (Bryden, 2007; Levin 2012; Naseem et al., 2018). Aflatoxin B1 (AFB1) represents a highly toxic, mutagenic, teratogenicity and carcinogenic compound that exhibits an immuno-suppressive activity, acute and chronic toxicity in both humans and animals exposed to this toxic material (Meggs, 2009; Saleemi et al., 2012). Concentrated feeds, highly represented in animal diets particularly for farm animals, might be contaminated by mycotoxin-producing moulds.

The main source of AFB1 exposure, thereby being concentrated feed, cereals and cereal-based products both for human and farm animals. In cattle fed with contaminated feed by AFB1 gets to be converted into AFM1 and subsequently excreted in their milk, and therefore concerns about the entry of mycotoxins into the food chain through meat, milk and particularly into dairy products have been raised in recent decades (Markov et al., 2013). The maximum content of AFB1 in feed materials has been set to 20 mg/kg in the EU regulations (Commission Regulation 574/2011) and lesser amounts are allowed for both AFB1 and AFG1 in food and cereals.

Production of AF in these commodities takes place during the anabolic process of the aflatoxinogenic fungi, involving the expression of aflD (synonym Nor-1) transcript gene (Yu, 2012) which is a determinant gene in the anabolic process of AFs. Molecular methods, along with the conventional microbiological techniques, have been applied in recent years for the identification of fungal aflatoxinogenesis with different levels of success (Sweeney et al., 2000; Degola et al., 2007; Rodrigues et al., 2009). PCR-based methods, including monomeric, quadruples and multiplex
PCR have been used vastly to investigate the genes responsible for AF biosynthesis (Somashkar et al., 2004; Manonmani et al., 2005). Correlating the expression profile of AF biosynthesis genes with aflatoxin production by A. flavus and A. parasiticus strains using RT-PCR and real-time PCR have been previously achieved. There are significant correlation between aflatoxin production by A. parasiticus and expression of two genes, afdR and aflQ, as determined by RT-PCR in AF conductive and non-conductive media (Sweeney et al., 2000). Expression of afdD, aflQ, and aflP genes also provided useful differentiation of aflatoxinogenic and non-aflatoxinogenic strains of A. flavus and A. parasiticus (Rodrigues et al., 2009).

In Syria the compound feed is produced mainly by commercial feed mills and in some extent as homemade mixtures. Manufacturing, distribution and storage of feedstuffs are not regulated by law properly, and their production is not under strict control especially after starting of the interior war. All that truths add new problems to the feed quality by causing mycotoxicosis. The owners of feed mills or manufacturers purchase huge amount of these crude feed materials during the production season and store them for making animal feed throughout the year and it is well known that mold growth is associated with improper storage conditions. Major aims of the present study were to isolate, purify and identify the fungi from different concentrated feeds samples collected from various farms and feed factories in Northern part of Syria and moreover to investigate the aflatoxinogenesis levels of these fungal isolates with the aid of molecular methodologies.

**MATERIALS AND METHODS**

**Samples:** There are five agro-climatic zones in Syria based on the amount of yearly rainfall. Idlib province located in the Northern part of Syria in the border of Turkey with annual rainfall ranged between 250-600 mm. The rainy season in Syria is mainly between December and March and therefore all samples were collected in December 2014 (n=8), January 2015 (n=6) and February 2015 (n=6). For all analysis 1kg of feed samples (for each collection) was collected randomly from factories, farms and stores located geographically in various parts of Idlib province. A total of 20 samples were collected from the concentrated feeds of cattle (n=5), sheep and goat (n=5) and poultry (n=10). All samples were kept in pre-sterilized naylon bags and brought to Biotechnology and Gene Engineering Laboratory of Animal Science Department, Kahramanmaraş Sutsu Imam University and stored at 4°C until further analysis. Nutrient composition of all samples were analyzed according to AOAC (2001).

**Isolation and identification of mould:** All samples taken from various kind of concentrated feed were ground gently and plated on Potato Dextrose Agar (PDA) in duplicates and were incubated at 31°C for 72 h. Fungal colonies on feedstuffs which plated on PDA agar were counted and identified at genus level first according to their basic morphological characters as follow; Fusarium spp. according to (Pitt et al., 2009). In this case the colonies in PDA Broth incubated at 37°C for 2 days, and then the positive samples were transferred and grown in PDA agar to obtain a stock culture. The medium was modified as described by Hepperly et al. (1981). Mixture of chloramphenicol and dichloran were used in all culture media to inhibit possible bacterial contamination and growth.

**DNA Extraction:** Conventional naming system of aflatoxin gene outlined by Yu, (2012) was taken as reference and all samples were tested for the presence of afdD and aflQ genes. The fungal colonies grown in PDA broth for 3 days were transferred into 2 ml eppendorf tubes containing 2 mm diameter glass beads. Then 650 µl of lysis CTAB buffer was added and the mixture was ground in a tissue miller at a frequency for 30 minutes after which samples were incubated at 65°C for 2 hours. Proteins were precipitated by adding 600 µl phenol. The tubes were inverted gently and centrifuged at 13585 g for 20 minutes. The top aqueous layer was pipetted into new tubes, 600 µl phenol: chloroform (25:24 v/v) was added and inverted gently to get a proper mixture and centrifuged at 13585 g for 20 minutes. Cleaned supernatant was transferred into new 2 ml eppendorf tubes and washed in 60 µl sodium acetate buffer (pH 8) then 800 µl isopropanol were added gently and incubated overnight at 4°C for precipitation. The samples were centrifuged at 13585 g for 10 minutes in a refrigerated centrifuge (Selecta, Spain) at 4°C, the supernatant discarded after which the DNA pellet was washed twice using 1ml 70% ethanol and centrifuged again at 13585 g for 5 minutes at 4°C. The supernatant was discarded and the DNA pellet dried in an oven at 55°C for 30 minutes. The pellet was re-suspended in 80 µl low salt TE buffer, 5 µl RNase (1mg/ml) was added to remove any possible RNA contamination and stored at -20°C until further usage (Levin, 2012).

**PCR procedure:** PCR amplification was performed by adding 1 µl dNTP mix, 4 µl Buffer and 0,5 µl Taq DNA polymerase and diluted in 32 µl distilled water. The afdD gene was amplified using the primers of Nor1-F (5'-ACC GCT ACG CCG GCA CTC TCG GCA C-3') and Nor1-R (5’-GGT GCC CGC CAG CCA TCC G-3’) (Rodrigues et al., 2009). The aflQ gene was amplified using the primers Ord-1F (5’-TGA CCG CAG GAT ACA AG-3’) and Ord-1R (5’-GAC GCC CAA AGC GGA ACA CAA A-3’) (Sweeney et al., 2000).

**Aflatoxin analysis:** The fungal isolates which were found to be positive for Aspergillus flavus cultured on YES agar (Rodrigues et al, 2009) were analysed further for aflatoxin production profile. The isolates were incubated on YES agar containing plates at 28°C for three days and examined under UV light to check the positive and negative isolates for aflatoxin production according to Levin (2012) with slight modification as follow. The samples were filtered through Whatman No.4 and 20 ml of clear filtrate was collected. Then 5 ml of the filtrate was diluted using 120 ml of 0,01 M phosphate buffered saline, it was ensured that 125 ml of diluent equivalent to 1g sample was taken into the glass syringe barrel for passage through the immune affinity column. All extracts were passed through an immune affinity column at a flow rate of 2-3 ml min⁻¹. Sample containing vials were then placed beneath the column and 1 ml of HPLC grade methanol was transferred to the glass barrel. Aflatoxins
were eluted from the column at a flow rate of 1 drop per second. After completion of the election, 1 ml of distilled water was pipetted into the glass syringe barrel and passed through the column to get a final volume of 2 ml (Levin, 2012).

Aflatoxin quantification: Aflatoxin contents were measured using High-Performance Liquid Chromatography (HPLC-F). All the samples were derivatized using trifluoroacetic acid (TFA) according to the standard method reported by Anastassiades et al. (2003). An HPLC system (Waters 600E) with a fluorescence detector (Waters 470) and an auto-sampler (Waters 712 WISP) were used for aflatoxin quantification and detection. Reference standards (Sigma-Aldrich, USA) were prepared in 50 µl of each of Aflatoxin B1, G1, B2 and G2 containing 10, 20, 30 and 50 ng ml⁻¹ of B1 and G1 and 3, 6, 9, 12 and 15 ng ml⁻¹ of AFB2 and AFG2 were run through HPLC system and calibration curves were calculated (Levin, 2012).

RESULTS AND DISCUSSION

Chemical analysis of feedstuff: Dry matter, ash and crude protein content of all samples, collected during December 2014, January 2015 and February 2015 were determined. Crude protein levels of concentrated feed samples produced for cow (dairy), cow (beef), sheep and goat (dairy), sheep and goat (meat), broiler stage 1, broiler stage 2, layers stage 1 and layer stage 2 were found as 10.98, 10.10, 11.12, 10.60, 19.66, 18.71, 16.47 and 13.45% respectively. Crude ash contents of the samples were also determined and ash levels were recorded as 21.49, 19.16, 19.57, 21.17, 25.06, 19.42, 20.18 and 22.72% respectively for the samples mentioned above. For same samples, dry matter contents were found as 88.5, 89.84, 89.43, 88.12, 90.97 90.43 and 92.06% respectively. There was different nutrient composition of concentrated feeds in comparison with the standard of NRC (2002) values. Its mean that the factories in these areas added high amount of calcium carbonate. The lowness of protein due to high temperatures and drier conditions are known for the ability of infection by A. flavus but in this study the situation is not similar to the results observed in Nigeria (Atehnkeng et al., 2008). The study may be affected by the number of samples and short period of the collection of samples. Toxicogenic strains of A. flavus were more prevalent than non-toxigenic strains through Northern part of Syria. The widespread occurrence of the fungus indicates the extent of pre-harvest infection; so, field management strategies stand out as an indispensable intervention strategy towards the fight against aflatoxin contamination of compound feed in exemplified region of Syria. Moreover, presence of these fungi may refer to the fact that mycotoxins are important issues because of lack of authority and consequently no strategies to control the system against contamination by aflatoxin in Syria after interior war started. The results are intersected with other studies in referring that a lot of strain of A. flavus isolated from corn and other widely varying materials have a high potential for aflatoxin production (Reddy et al., 2009a & 2009b).

Aflatoxin production on YES: All isolates were positive for either one or both gene and observed under UV light in YES media after 3 days of incubation at 28°C to test their aflatoxin production profiles. It was found that all the isolates were positive (aflatoxinogenic). That means all isolates had an aflatoxin production profile (Fig. 1). These findings were in parallel of the results of Konietzny and Greiner, (2003) who reported that the samples, they analyzed, had 50% aflatoxin positive (aflatoxinogenic) on YES agar under UV light. Due to fact that mycotoxins are important issues because of health and economic considerations, more sensitive and rapid techniques to detect them are desired. Strains were cultivated in Yeast Extract Sucrose (YES) medium and photographed on the third day of incubation at 28°C.

The genes controlling Aflatoxin production: All fungal isolates were examined weather they have the genes responsible for aflatoxin formation, and 6 of them were found to be positive for aflD gene which responsible for aflatoxin biosynthesis from averantin (Fig. 1).

Molecular characterization: The fungi were isolated from a concentrated feed by culturing them on PDA agar and they were purified on selective media (AFPA) to identify the A. flavus and A. parasiticus according to their basic morphological characters. A. flavus produced yellow green colonies and they were predominantly biseriate or uniseriate with smooth to finely rough conidia. A. parasiticus had dark green colonies with a diameter of between 24-36 mm, was predominantly uniseriate with 20% biseriate and had rough conidia. The samples investigated here were mainly contaminated by Aspergillus flavus and this incidence was recorded regardless of temperature variation (15-20°C) and the remarkable level of the rainfall (over 400 mm/year) during sampling period which had not recorded in that area for over a decade. These results are in agreement with Abbas et al. (2004) who observed greater variation in aflatoxin formed by A. flavus different aflatoxin production capabilities of A. flavus strain would be influenced by different sources of strains and environmental conditions. relatively higher temperatures and drier conditions are known for the ability of infection by A. flavus but in this study the situation is not similar to the results observed in Nigeria (Atehnkeng et al., 2008). The study may be affected by the number of samples and short period of the collection of samples. Toxicogenic strains of A. flavus were more prevalent than non-toxigenic strains through Northern part of Syria. The widespread occurrence of the fungus indicates the extent of pre-harvest infection; so, field management strategies stand out as an indispensable intervention strategy towards the fight against aflatoxin contamination of compound feed in exemplified region of Syria. Moreover, presence of these fungi may refer to the extent of pre-harvest infection because of lack of authority and consequently no strategies to control the system against contamination by aflatoxin in Syria after interior war started. The results are intersected with other studies in referring that a lot of strain of A. flavus isolated from corn and other widely varying materials have a high potential for aflatoxin production (Reddy et al., 2009a & 2009b).

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Fig. 1: *An exemplified culture of Colonies of a Aflatoxinogenic strains of Aspergillus grown in YES agar containing petri. visualised under ultra violet lightened.* PCR amplification results of Aspergillus flavus run on 1% agarose Electrophoresis gel electrophoresis and photographed under 312nm UV light. of Aspergillus flavus DNA extracted in lab and isolated in AFPA; *Media M: 100 bp Marker (Favorgen, Taiwan); aflD and aflQ: 400 bp positive samples for aflD and 719 bp positive samples for aflQ.
Total of 18 positive samples for aflQ which responsible for biosynthesis o-methylsterigmatocystin to aflatoxin (Fig. 2) were found and expression of it had been reported as showing a high correlation of aflatoxinogenic ability.

The aflQ gene was specifically chosen because it is considered to be the only gene involved in the final step of transforming o-methylsterigmatocystin (OMST) into aflatoxin, crucial step of aflatoxin pathway that seems to be unique to aflatoxigenic species (Levin, 2012; Scherm et al. 2005). As expected the presence of these two genes could not be correlated with aflatoxin producing ability.

Isolates were found 21, 30 (70%) positive and distinct colour characterization for selective media. All isolates were confirmed as Aspergillus section flavi by the bright orange colour of the colony reverse on AFPA media and remarked that all isolates in YES media under ultra violet lights had aflatoxin produced. It is mean that 100% of isolates had aflatoxinogenic characterization. There were 6 isolates were positive for aflD (nor-1>). That means these isolates have the step from aflatoxin biosynthesis series, which responsible for aflatoxin biosynthesis from averantin (Table 1).

We detected 18 samples are positive for aflQ (ord-1) (Table 1). These samples have a step from aflatoxin biosynthesis series, which responsible for transition of methylsterigmatocystin to aflatoxin. There was 6 (20%) common positive both aflQ and aflD observed them in concentrated feed from Northern Syria. In general, climatic condition in this area was not favorable for aflatoxin contamination. However, reduced rainfall and increased temperature during manufacturing can occur with associated levels of aflatoxin B1 in the excess legal limit (Piva and Pietri, 2006).

Measuring of Aflatoxin levels by (HPLC-F): Aflatoxin levels of all feed samples collected from Northern Syria during December 2014, January 2015 and February 2015, were also determined (Table 1). These samples were analyzed by HPLC-F to detected levels of B1. The levels ranged from high to standard levels in comparison with acceptable limit of aflatoxin B1 in animal feed material in Syria (20 ppm), (0.354 ppm, 8.247 ppm, 4.45 ppm, 1.10 ppm, 0.134 ppm, 0.133 ppm, 0.360 ppm, 0.025 ppm, 18.13 ppm, 32.936 ppm, 10.326 ppm, 0.324 ppm, 9.977 ppm and 0.427 ppm). Same results were observed in Nigeria (Atehnkeng et al., 2008) but high temperature and drier condition are known infection by Aspergillus flavus, consequently raising in aflatoxin B1 levels.
Similar results were reported from India, where aflatoxin B1 levels exceeded the permissible Indian regulatory limit of 30 ppm in 21% of ground nut samples and 26% of maize samples collected from 11 different states. Therefore, it is so important particularly for underdeveloped or developing countries (such as Syria) that aflatoxin B1 levels had to be considered along with total aflatoxin before accepting or choosing a feed or feed ingredient for dietary use in animals (Bhat et al., 1997; Bagheri and Mojtaba, 2014).

**Conclusions:** This study has displayed that *A. flavus* is the main fungal species infecting compound feed in Northern part of Syria and that toxicogenic strains are widespread. The influence of Aflatoxin on human population's in Syria over the past decade demonstrates a clear need to improve novel pathways to manage contamination of locally produced animal feed stuff. Given the extended nature of toxicogenic strains, any control strategy will have to include field management. Fungal infection of compound feed varies with households and this reflects different farm management practices, some of which adversely contribute to contamination of the feed value chain. The compound feed in Syria was contaminated mainly with *A. flavus* and *A. parasiticus* whose occurrence frequencies varied according to different months during rainy winter season. Feeds and their ingredients were all contaminated with Aflatoxin B1 and B2. Aflatoxin levels of majority of samples were over the safe limit of FDA (20 ppm).

The results of this study showed that 100% of the tested *A. flavus* strains produced aflatoxins, and most of the aflatoxinogenic. *A. flavus* strains showed a high capacity for aflatoxins production in compound feed.

**Table 1:** Molecular characterization in AFPA media and the content of Aflatoxin B1 in concentrated feeds

| Sample          | Month/year | Aflatoxin B1/ppm | AFPA Agar | YES | Nor-1 | Ord-1 |
|-----------------|------------|------------------|-----------|-----|-------|-------|
| Cow/dairy       | December 2014 | 0.354           | -         | +   | -     | -     |
| Cow/beef        | December 2014 | 8.247            | -         | +   | -     | -     |
| Broiler stage 1 | December 2014 | 1.10             | -         | +   | -     | -     |
| Broiler stage 2 | December 2014 | 0.134            | -         | +   | -     | -     |
| Layers stage 2  | December 2014 | 0.133            | -         | +   | -     | -     |
| Layers stage 3  | December 2014 | 0.360            | -         | +   | -     | -     |
| Cow/dairy       | January 2015  | 18.133           | -         | +   | -     | -     |
| Cow/beef        | January 2015  | 32.936           | -         | +   | -     | -     |
| Sheep and goat/dairy | January 2015 | 10.346        | -         | +   | -     | -     |
| Sheep and goat/meal | January 2015 | 0.324            | -         | +   | -     | -     |
| Broiler stage 1 | January 2015  | 0.126            | -         | +   | -     | -     |
| Broiler stage 2 | January 2015  | 16.648           | -         | +   | -     | -     |
| Cow/dairy       | February 2015 | 15.75            | -         | +   | -     | -     |
| Cow/beef        | February 2015 | 19.367           | -         | +   | -     | -     |
| Sheep and goat/dairy | February 2015 | 9.977          | -         | +   | -     | -     |
| Sheep and goat/meal | February 2015 | 1.100            | -         | +   | -     | -     |
| Broiler stage 1 | February 2015 | 0.427            | -         | +   | -     | -     |

(+) Aflatoxin-producing Aspergillus sp when grown on YES media; (−) Aflatoxin formation has not been determined on that samples; NOR 1(−): The sample of *Aspergillus* positive for Nor-1 (ARQ); ORD 1(−): The sample of *Aspergillus* positive for Ord-1 (AFID); ORD 1(+) The sample of *Aspergillus* negative for Ord-1 (AFID).

Acknowledgements: This study was supported by Kahramanmaras Sutcu Imam University, Faculty of Agriculture and data of Ahmed Hussein’s MSc thesis were partially used.

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