



## Antifungal Effect of *Thymus vulgaris* Methanolic Extract on Growth of Aflatoxins Producing *Aspergillus parasiticus*

Monga I. Mossa

Botany Department, Faculty of Science, Arish University, N. Sinai, Egypt.



**T**HIS STUDY illustrates a simple method for the detection of the ability of *Aspergillus parasiticus*, isolated from peanut seeds, for the production of aflatoxins. The indicator of aflatoxins production ability is the appearance of colony reverse yellow color of the fungus on the agar media and the natural fluorescence of aflatoxin around the colony when observed under UV (black light 350BL) after three days of incubation at 28°C. The morphological identification of *A. parasiticus* was confirmed by molecular identification rDNA sequencing. The addition of  $\beta$ -Cyclodextrin to media enhanced the natural fluorescence of aflatoxin around the colony under UV. The extract of *Thymus vulgaris* had an inhibitory effect on the growth of *A. parasiticus*. The minimum inhibitory concentration (MIC) of *Thymus* extract against *A. parasiticus* was 80  $\mu$ L of *Thymus* extract /10 ml of YSA medium. The effective concentration of *Thymus* extract causing a 50% inhibition of mycelial growth ( $EC_{50}$ ) was 20  $\mu$ L of *Thymus* extract /10mL of YSA medium. The chemical analysis of *Thymus* extract by Gas Chromatography-Mass Spectroscopy (GC-MS) illustrated that the thymol has a retention time (RT) (min) of 37.538 at the highest percent area (13.8717 Area %).

**Keywords:** Aflatoxin, *Aspergillus parasiticus*, Blue fluorescence, Extract, *Thymus vulgaris*.

### Introduction

Fungi are divided into two distinct groups; a group that invades crops before plant harvesting and the other that invades after harvest so the contamination with aflatoxins can occur before and after harvest (Augusto, 2004). Furthermore, *Aspergillus* spp. section *flavi* is divided into two groups, one that includes the aflatoxigenic species; *A. flavus*, *A. parasiticus* and *A. nomius*; and the second group includes the non-aflatoxigenic species; *A. oryzae*, *A. sojae* and *A. tamari*; traditionally used for the production of fermented food (Lin & Dianese, 1976; Cotty, 1989; Rodrigues et al., 2007). *Aspergillus* section *flavi* includes 22 species grouped into seven clades. *Aspergillus pseudocaelatus*. Nov. and *A. pseudonomius*. Nov. are the two newly discovered aflatoxin-producing species according to Varga et al. (2011). Aflatoxins are probably produced by *Aspergillus* species. The important sources of contamination is during production and handling (Ouf et al., 2019). Aflatoxins are secondary metabolites, highly toxic and the most

carcinogenic are produced by some strains of *A. flavus* and *A. parasiticus*. *Aspergillus flavus* produces aflatoxins B1 and B2 while *A. parasiticus* produces G1 and G2 aflatoxins. The presence of these fungi is enhanced by a number of factors such as inadequate storage conditions (El Koury et al., 2011; Ouattara-Sourabie et al., 2012). Crop rotations influence the quantity of aflatoxins (Jaime-Garcia et al., 2006). Different treatments are used to reduce the quantities of aflatoxins such as double atmospheric pressure cold plasma (DAPCP) (Ouf et al., 2016) and treatment of fruits with ozonized water (Ouf et al., 2019). The main six aflatoxins types are designated as B<sub>1</sub>, B<sub>2</sub>, G<sub>1</sub>, G<sub>2</sub>, M<sub>1</sub> and M<sub>2</sub>. B<sub>1</sub> aflatoxin is the most common and most spread in the world. It accounted for 75% of the contaminated food and feed in the world (Wacoo et al., 2014). Aflatoxin B<sub>1</sub> is one of the most common toxins found in wheat grains (Sadharsan et al., 2017).

Different media were used for the differentiation between aflatoxin-producing or non-aflatoxin-producing *Aspergillus* species.

Lin & Dianese (1976) were the first to indicate the bright yellow pigment production with aflatoxigenic *Aspergillus* species on the reverse side of colonies grown on semitransparent agar media. The degree of yellow pigmentation was proportional to blue fluorescence in media (Davis et al., 1987), but the yellow pigment production was not a reliable predictor of the amount of aflatoxin in media (Cotty, 1989).

The formation of bright blue or blue-green fluorescent area surrounding the colonies when subjected to UV light (365nm), is the simple and fast method to detect the aflatoxin, (Fente et al., 2001; Corry et al., 2003; Augusto, 2004; Ouattara-Sourabie et al., 2012). Agar fluorescence is proportional to the total aflatoxins content in culture of *Aspergillus* spp. (Davis et al., 1987; Cotty, 1989). The blue fluorescence of media under UV is a standard method for the differentiation between groups, which produce aflatoxins and the others non-aflatoxigenic groups. Ouattara-Sourabie et al. (2012) stated that "aflatoxins was detected from isolates of *A. parasiticus* which has fluorescence under UV and not detected from isolates which have no fluorescence" (Okwa et al., 2010). Fluorescence under UV is observed for B<sub>1</sub>, B<sub>2</sub>, G<sub>1</sub> and G<sub>2</sub> (Augusto, 2004).

The inhibitory effect of plant extracts of *Thymus vulgaris* on the growth of fungi was investigated by many authors (Zekovic et al., 2002; Al-Maqtari et al., 2011; Moghtader, 2012; Mousavi & Raftos, 2012).

The aim of this article is the isolation of *Aspergillus parasiticus* from infected peanut seeds and studying the colony fluorescence of the investigated fungus when exposed to UV (320nm). Moreover, to examine the effect of *Thymus vulgaris* extract on the growth of *A. parasiticus*.

## Material and Methods

### Isolation of *Aspergillus parasiticus*

After surface sterilization, the peanuts seeds were wetted with sterilized water and maintained in glass Petri dishes at room temperature (25±1°C) in incubator until the appearance of mold on seeds. After one week, the *Aspergillus* sp. were purified on potatoes dextrose agar medium (PDA; gm/L distilled water: 200gm potatoes, 20gm dextrose and 17gm agar) according to Pitt & Hocking

(2009), and maintained until used on slants of Czapek yeast extract agar (CYA; gm/L distilled water: NaNO<sub>3</sub>: 3gm, KH<sub>2</sub>PO<sub>4</sub>: 1gm, KCl<sub>2</sub>: 0.5gm, MgSO<sub>4</sub>: 0.5gm, , FeSO<sub>4</sub>.7H<sub>2</sub>O: 0.1gm, Sucrose: 30gm, Yeast extract: 5gm, agar: 15gm) (Pitt & Hocking, 2009; Ouattara-Sourabie et al., 2012).

### Morphological analysis

For morphological observation the isolates were grown on PDA, Malt extract agar (MEA; gm/L distilled water: 20gm malt extract, 1gm peptone, 20gm sucrose, 15gm agar (Pitt & Hocking, 2009), and yeast extract sucrose agar (YSA; gm/L distilled water: 2gm yeast extract, 20gm sucrose and 17gm agar (Ehrlich et al., 2004). They were incubated at 25±1°C for 7 days and then microscopic study was performed (Varga et al., 2011).

### Molecular identification of *A. parasiticus*

The rDNA sequencing of actively growing mycelium on potatoes dextrose broth (PDB) medium at room temperature (25±1°C) was studied to confirm morphological identification. It was maintained at -80°C until the use of DNA extraction. Polymerase chain reaction (PCR) was performed followed by Gel electrophoresis for the results of PCR, and the internal transcribed spacer of DNA; ITS1 (5'-TCCGTTGGTGAACCAGCGG-3') and ITS4 (5'-TCCTCCGCTTATTGATATGC-3') were compared with the sequencing of other organisms belonging to the genus *Aspergillus*, according to White et al. (1990). Consequently, the trimmed sequences were identified using the database searches of the National Center for Biotechnology Information (NCBI).

### Simple methods to study the ability of *A. parasiticus* for the production of aflatoxins

According to Lin & Dianese (1976), Corry et al. (2003), and Sudini et al. (2015), the fungus was growing on Yersinia Selective Medium (YSA). The appearance of the yellow color on the reverse side of the plates after three days was considered as the primary indicator for the production of aflatoxins. Another method is by growing the fungus in the center of a Petri dish containing potato dextrose agar. The dish was inverted, and 1 or 2 drops of concentrated ammonium hydroxide solution were placed on the lid of the Petri dish then the color on the inverted Petri dish was recorded (Abbas et al., 2004a). According to Davis et al. (1966), the fungus was cultivated on YSA at 30±1°C and was examined under UV (360nm) for the presence

or absence of blue fluorescence surrounding the colonies.

*The effect of the type of media on A. parasiticus biomass*

To study the effect of Potatoes dextrose broth (PDB), Czapek yeast broth (CYB) and yeast sucrose broth (YSB) media on fresh and dry biomass of the studied fungus, 25mL of each medium was dispensed in autoclaved flasks. The flasks were incubated at room temperature on orbital shaker at 125rpm, for 13 days. The fungal growth was filtered through Whatman No. 1 filter paper with a known weight. The fresh and dry weights were measured (Corry et al., 2003; Sudini et al., 2015).

*Effect of the addition of  $\beta$ -cyclodextrin*

For increasing the detection of aflatoxins,  $\beta$ -Cyclodextrin ( $\beta$ -CyD) was added to the media according to the methods used by Fente et al. (2001). The plates were incubated at  $25 \pm 1^\circ\text{C}$  from 1 to 7 days on YSA and PDA media. Fluorescent and non-fluorescent colonies were recorded as +ve or -ve.

*Effect of different concentration of Thymus vulgaris extract on the growth of A. parasiticus*

*Preparation of spore suspensions*

*A. parasiticus* was grown on Czapek yeast extract agar (CYA) slant for 10 days at  $30 \pm 1^\circ\text{C}$ . The harvested spores were kept in sterilized water containing 0.01% (v/v) Tween 80 was added according to Ouattara- Sourabie et al. (2012).

*Preparation of T. vulgaris methanolic extract*

*Thymus vulgaris* (leaves and stems) was extracted by 70% methanol according to Al-Rahmah et al. (2013). The antifungal activity was studied by growing the fungi on YSA plates containing different concentrations of plant extract of *T. vulgaris*, as follows: 0, 10, 20, 30, 40, 50, 60, 70, 80, 100 $\mu\text{L}$ /10mL of medium. Ten microliters of  $10^5$  spore suspension of *A. parasiticus* were prepared from seven days old culture and placed on YSA plates (9cm). Plates were incubated for 7 days at  $30 \pm 1^\circ\text{C}$ . The diameters of treated and control colonies were measured when the fungal growth in the control plates had covered the surface of medium. The experiment was carried out in three replicates. The percentage of relative mycelium inhibition (RMI) by the plant extract was calculated using the following formula:

$$\text{RMI} = (C - T / C) * 100$$

where C represents the mean colony diameter of control plates, and T represents the mean colony diameter of treated plates. The colony diameter was measured in millimeters. Minimum inhibitory concentration (MIC) of *Thymus vulgaris* was measured, which was defined as the lowest concentration of extract that allowed no more than 20% (or 50%) of fungus growth, and the effective concentration of the sample which caused a 50% inhibition of mycelial growth ( $\text{EC}_{50}$ ).

*Analysis of Thymus vulgaris extract by GC-MS*

The gas chromatography mass spectroscopy (GC-MS) analysis of *Thymus vulgaris* methanolic extract was carried out at the Central Lab., Faculty of Science, Ain Shams University. GC-MS, Agilent Technologies 7890B GC Systems combined with 5977A Mass Selective Detector, Capillary column was used (HP-5MS Capillary; 30.0m  $\times$  0.25mm ID  $\times$  0.25 $\mu\text{m}$  film) and the carrier gas was helium at a rate of flow of 1.9mL/min with 1 $\mu\text{L}$  injection. The sample was analyzed with the column held initially for 3min. at  $40^\circ\text{C}$  after injection, then the temperature was increased to  $300^\circ\text{C}$  with a  $20^\circ\text{C}/\text{min}$  heating ramp, with a 3.0 min hold. The injection was carried out in split-less mode at  $300^\circ\text{C}$ . MS scan range was (m/z): 50–500 atomic mass units (AMU) under electron impact (EI) ionization (70eV). Phenolic derivatives and fatty acids were determined. Silylation agent (BSA) was N, O- Bis (trimethylsilyl) acetamide, (Klebe et al., 1966). The constituents were determined by mass fragmentations with the NIST mass spectral search program for the NIST/EPA/NIH mass spectral library Version 2.2 Jun 2014.

*Statistical analysis*

All tests were conducted in three replicates for each treatment and the data were reported as mean  $\pm$  standard deviation (SD) using Excel 2010 program.

## Results

*Isolation and characterization of Aspergillus parasiticus from peanut seeds*

Two *Aspergillus* species were isolated from peanut seeds. They were *A. parasiticus* and *A. flavus*. *Aspergillus parasiticus* was used to study its ability for aflatoxin production by simple method (Fig. 1).

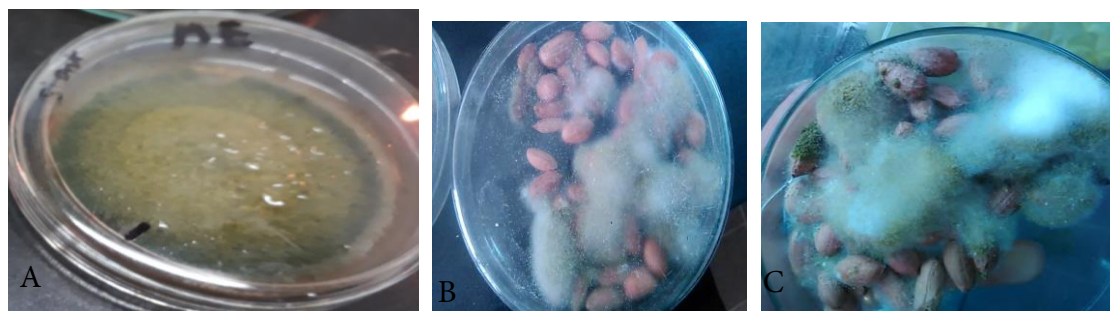


Fig. 1. Colonies of *A. parasiticus* on MEA media (A) and molds on peanut seeds (B) and (C)

*A. parasiticus* was grown on YSA, PDA and CYA media, the inverted colony appeared dark yellowish green with yellow color on MEA medium after three days of incubation at room temperature ( $25\pm 1^\circ\text{C}$ ) (Fig. 1A). The mycelium appears as dark yellowish green on YSA and dark green on PDA medium (Figs. 2, 3).

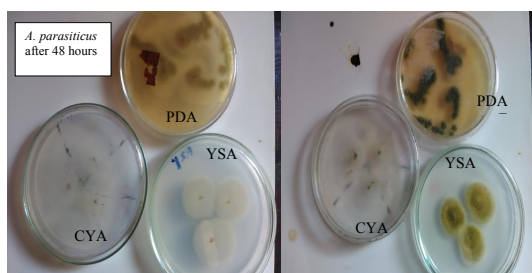


Fig. 2. *Aspergillus parasiticus* growth on YSA, CYA and PDA media

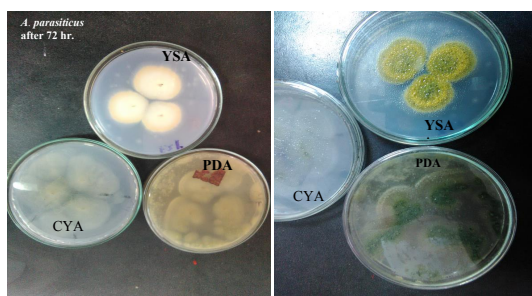


Fig. 3. Yellow color from inverted view of *A. parasiticus* colonies (left) and on YSA medium after three days of incubation

#### Microscopic examination of *Aspergillus parasiticus*

The microscopic examination of *A. parasiticus* showed the presence of uni-seriate phialides, which cover about two thirds of their globose vesicles with dark circular rough conidia. While *A. flavus* showed bi-seriate phialides, which cover all the elliptical vesicle, with slightly rough-oval conidia with noticeable papilla. The morphological identification and microscopic

study of *A. parasiticus* were confirmed by Assiut University Mycological Center (AUMC) and *A. parasiticus* was identified as *A. parasiticus* Speare, No.: 13623.

#### Molecular identification of *Aspergillus parasiticus*

The Comparison of the sequencing of the rDNA internal transcribed spacer of ITS1 (5'-TCCGTTG GTGAACCA G CGG- 3') and ITS4 (5'-TCCTCCG CTTATTGATAT GC-3') with the sequencing of other organisms belonging to the genus *Aspergillus* in NCBI in GenBank, showed that the isolate is a Closest Match to *Aspergillus parasiticus* DTO 046-C2, with accession number: MW485607 (Fig. 4).

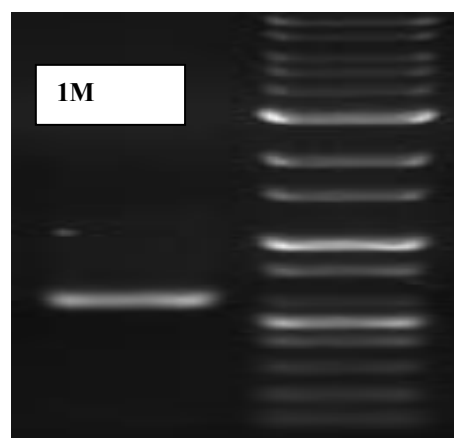
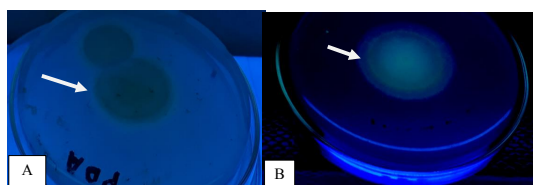


Fig. 4. Compact band of *A. parasiticus* (1M) on gel electrophoresis under UV light

#### Simple and rapid methods to study the ability of *A. parasiticus* for aflatoxins production

When *A. parasiticus* was grown on YSA and *Aspergillus* differentiated medium (ADM), it had a yellow color reverse colony after three days of incubation on YSA, and orange-yellow reverse color colony on ADM. Another simple method was the exposure of isolates growing in the center of the PDA plates to 27% ammonium hydroxide

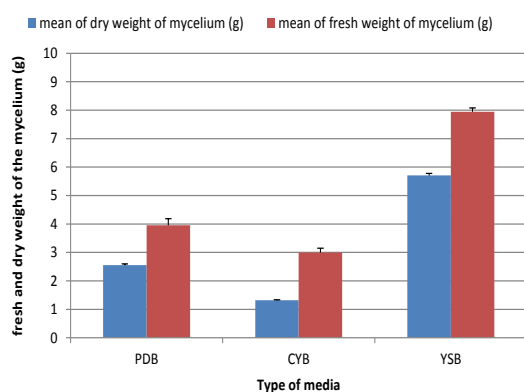
vapor for 30min. The reverse side of the colony of *A. parasiticus* became pink to reddish color after few seconds compared with the *A. flavus* isolate. Conflict of interest when *A. parasiticus* was cultivated on YSA and exposed to UV (black light 350BL), we noted the appearance of blue fluorescence hollow surrounding the colonies (Fig. 5).



**Fig. 5.** Slightly blue hollow around the colony of *A. parasiticus* on PDA medium (A) and blue fluorescence around the colony of the same fungus under UV light in closed chamber

#### The effect of media types on *A. parasiticus* biomass

Mycelial biomass of *A. parasiticus* was different according to the type of medium. Three types of media were used: PDB, CYB and YSB media. The means fresh mycelial biomass were:  $3.97 \pm 0.1$ ,  $2.99 \pm 0.07$  and  $7.91 \pm 0.11$  gm/25mL of medium, and the mean dry weights were:  $2.55 \pm 0.3$ ,  $1.31 \pm 0.25$  and  $5.70 \pm 0.21$  gm/25mL of medium for dry mycelial biomass for MEB, CYB and YSB, respectively. The results indicated that YSA was the most suitable medium for the growth of *A. parasiticus* (Fig. 6).



**Fig. 6.** The mean fresh and dry weight of mycelium of *A. parasiticus* on three types of media; potatoes dextrose broth (PDB), Czapek yeast broth (CYB) and yeast sucrose broth (YSB)

#### Effect of $\beta$ -Cyclodextrin on the degree of fluorescence as an indicator for aflatoxins production

The blue fluorescence area surrounding the

colonies under UV (black light 350BL) was increased by the addition of  $\beta$ -Cyclodextrin ( $\beta$ -Cyd; 0.3gm/100mL of media). The addition of  $\beta$ -Cyd enhanced the fluorescence as an indicator of *A. parasiticus* ability for aflatoxins production. The blue fluorescence started to appear clearly on the fourth day of incubation at room temperature ( $25^\circ\text{C} \pm 1^\circ$ ), but the best appearance of fluorescence was at the sixth day. After three days of incubation, it appeared after one week on YSA only. At the seventh day of incubation, the growth of mycelium, which covered the surface of the agar plates, made the blue hollow not visible. These results indicated that the incubation time is among the factors affecting the appearance of the blue fluorescence hollow around the fungal colony (Table 1).

**TABLE 1.** The effect of addition of  $\beta$ -cyd on fluorescence of colony.

Incubation time	Degree of fluorescence by addition of $\beta$ -cyd	
	YSA	PDA
First day	-	-
Second day	-	-
Third day	+	-
Fourth day	+++	++
Fifth day	+++	+++
Sixth day	++++	+++
Seventh day	++++	+++

#### Effect of *Thymus vulgaris* methanolic extract on *A. parasiticus* growth

When growing *A. parasiticus* on plates containing different concentrations of *T. vulgaris* extract, there were inhibition of fungal growth compared with control plates. The mean diameter of the colony of *A. parasiticus* decreased by increasing the concentration of the plant extract. We noted that the mycelium had white color at 80 and 100  $\mu\text{L}$  of *Thymus* extract /10mL of YSA medium. The relative mycelial inhibition (RMI) increased by increasing the concentration of *Thymus* extract. The means diameter of colony of *A. parasiticus* were;  $9 \pm 0$ ,  $4.7 \pm 0.1$ ,  $4.03 \pm 0.05$ ,  $3.73 \pm 0.11$ ,  $3.33 \pm 0.11$ ,  $2.96 \pm 0.05$ ,  $2.76 \pm 0.11$ ,  $2.16 \pm 0.05$ ,  $1.76 \pm 0.05$ ,  $1.5 \pm 0.14$ ,  $1.3 \pm 0.05$  cm and the RMI values of *A. parasiticus* were; 0,  $47.7 \pm 1.1$ ,  $55.18 \pm 0.6$ ,  $58.51 \pm 0.2$ ,  $62.96 \pm 1.2$ ,  $67.03 \pm 0.6$ ,  $69.25 \pm 1.2$ ,  $75.92 \pm 0.6$ ,  $80.37 \pm 0.6$ ,  $84.07 \pm 1.2$ , and  $85.18 \pm 0.6\%$ , respectively at different concentrations 0, 10, 20, 30, 40, 50, 60, 70, 80, 90 and 100  $\mu\text{L}$  of *Thymus*

extract /10mL of YSA medium respectively. The lowest concentration of extract that allowed no more than 20% growth of the fungus (MIC) was 80  $\mu$ L of *Thymus* extract /10mL of YSA medium. The effective concentration of the *Thymus* extracts which caused a 50% inhibition of mycelial growth ( $EC_{50}$ ) was 20 $\mu$ L of *Thymus* extract /10mL of YSA medium (Fig. 7).

#### GC-MS analysis of *T. vulgaris*

Fifty-five phenolic and carbohydrate derivative compounds were obtained from the analysis of the methanolic extract of *Thymus vulgaris* by Gas-Mass spectroscopy. One sharp peak appeared for Thymol at retention time (RT) (min) 37.538 which covered 3.8717 % area. The RT and area percentages are illustrated in Table 2.

#### Discussion

Mycotoxins produced by different genera of toxic fungi, cause human health problems and economic loss (Ouf et al., 2015). Aflatoxins are produced by some strains of *A. parasiticus*, which are the main aflatoxin-producing *Aspergillus* species, through the contamination of foods, fruits, and vegetables (Rodrigues et al., 2007; El Koury et al., 2011; Rajarajan et al., 2013).

The mycelium of *A. parasiticus* appeared compact with dark yellowish-green color on YSA medium, dark green on PDA medium, and dark green to slightly yellowish on MEA medium. *Aspergillus parasiticus* under a microscope had uniseriate phialides, which covered about two-

thirds of their globose vesicles with dark green circular rough conidia. These characters of *A. parasiticus* are in agreement with those given by Rodrigues et al. (2007).

To confirm the morphological identification, the molecular identification of *Aspergillus* was performed through DNA extraction (Ehrlich et al., 2004; El Koury et al., 2011; Sadharsan et al., 2017).

YSA, PDA and CYA media are used to study the ability of *A. parasiticus* for the production and quantification of aflatoxins according to Fente et al. (2001), Abbas et al. (2004) and Sudini et al. (2015). Therefore, we studied the effect of the type of media on the fungal biomass. The mycelial biomass of *A. parasiticus* varied according to the type of media. YSA medium was the best for the growth of *A. parasiticus*.

Different simple and rapid methods were used for the differentiation between *Aspergillus* species in their ability for the production of aflatoxin. Firstly, by the reversed side color of colony on YSA and ADM media (after three days of incubation); secondly, by the pink-red color of the reversed colony when exposed to 27% ammonium hydroxide vapors. The secretion of yellow pigments into the medium was considered as the first step showing their ability to produce aflatoxin. These results agreed with Lin & Dianese (1976), Saito & Machida (1999), Corry et al. (2003), Abbas et al. (2004), Abbas et al. (2004a), Yazdani et al. (2010) and Abo Dahab et al. (2016).

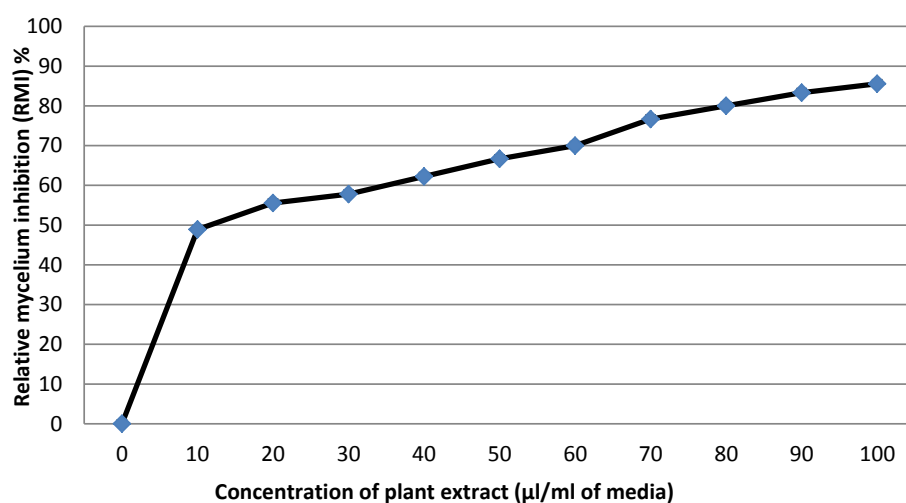


Fig. 7. The percentage of mycelial inhibition of *Aspergillus parasiticus* by different concentrations of *Thymus vulgaris* extract

TABLE 2. GC-MS analysis for *T. vulgaris* extract.

RT	Area %	Library/ID (S2)
8.1415	4.4635	Ethanimidic acid, ethyl ester
8.5087	2.6057	Acetamide
8.771	3.2831	3,7-Diacetamido-7H-s-triazolo[5,1-c]-s-triazole
37.538	13.8717	Thymol
17.9792	8.7767	1-Propanol
9.0858	3.7385	Ethanimidic acid, ethyl ester
9.1441	6.5717	Acetaldoxime
9.2898	0.4561	Phenol, 2-methyl-5-(1-methylethyl)-
9.692	1.228	Phenol, 2,3,5,6-tetramethyl-
10.2049	0.0468	Dibenz[a,h]anthracene, 5,6-dihydro-
10.5429	0.0083	2-Hydroxy-4-methylanthraquinone, O-trimethylsilyl
10.6653	0.0131	3-Isopropoxy-1,1,1,5,5,5-hexamethyl-3-(trimethylsiloxy)trisiloxane
10.8402	0.1498	3-Tetradecen-5-yne, (Z)-
10.9684	0.0357	2-Naphthalenol, 2,3,4,4a,5,6,7-octahydro-1,4a-dimethyl-7-(2-hydroxy-1-methylethyl)
11.1957	0.0088	Naphthalene, 1,2,3,5,6,7,8,8a-octahydro-1,8a-dimethyl-7-(1-methylethenyl)-, [1S-(1.alpha.,7.alpha.,8a.alpha.)]-
12.0351	0.0273	2-Allyl-1,4-dimethoxy-3-methyl-benzene
12.1342	0.0293	2-Hydroxy-3-methylbenzaldehyde
12.3731	0.0756	Benzaldehyde, 2-hydroxy-6-methyl-
12.4664	0.2002	Benzene, (ethenylthio)-
12.5596	0.0367	Ethanone, 1-(4,5-dihydro-2-thiazolyl)-
12.6063	0.0787	Benzaldehyde, 2-methoxy-
12.8744	0.0736	Pentadecanoic acid
12.9385	0.3509	n-Hexadecanoic acid
13.4631	0.0123	10-Methyl-E-11-tridece-1-ol acetate
13.5214	0.0355	2-Isoxazoline, 3-phenyl-
13.6671	0.0317	Succinic acid, di(2,5-difluorobenzyl) ester
13.7312	0.1137	9,12-Octadecadienoic acid (Z,Z)-
13.8128	0.4511	cis,cis,cis-7,10,13-Hexadecatrienal
14.8445	0.0581	9-Octadecenamide, (Z)-
15.3341	0.037	Silane, dimethyl(3-phenylprop-2-enyloxy)isobutoxy-
15.4681	0.0594	Diethyl 4-methylbenzylphosphonate
16.3074	0.0324	Supraene
19.5365	0.1361	Gamma.-sitosterol

After four days of the experiment, slightly blue fluorescence appeared around the colony, these results were following Lin & Dianese (1976). The production of blue fluorescence around the colony of *A. parasiticus*, when exposed to UV, was an indicator of the ability of this isolate to produce aflatoxins. These results concurred with Hara et al. (1974), Cotty (1988), Fente et al. (2001), Abbas et al. (2004a), Augusto (2004), Ouattara-Sourabie et al. (2012), and Abo Dahab et al. (2016). On the other hand, they disagreed with Yazdani et al. (2010) who stated that “this method was not sensitive to all *Aspergillus* species”.

The natural blue fluorescence area surrounding the colony on the exposure to UV was enhanced by the addition of  $\beta$ -Cyclodextrin. These results were supported by many authors: Davis et al. (1987), Fente et al. (2001), Abbas et al. (2004) and Augusto (2004). The diameter of colony was the same with and without the addition of  $\beta$ -Cyclodextrin. Hence, it has no effect on fungal growth. This result agreed with Fente et al. (2001). After three days of incubation, a blue fluorescence appeared around the colony on YSA medium. These results are matching with the study of Davis et al. (1987), which illustrated that; “for the appearance of blue fluorescence the colony must be incubated from three to five days” and this was also supported by Ouattara- Sourabie et al. (2012). The blue fluorescence hollow increased by the increase of the incubation time. Davis et al. (1987) claimed that after seven days of *A. parasiticus* incubation, the growth of the colony covered all the surface of the agar, this is why it was not easy to observe the blue fluorescence.

The addition of different concentrations of *Thymus vulgaris* extract to the medium inhibited *A. parasiticus* growth compared with the control plates. The inhibitory effect of *Thymus* against the studied fungi was investigated by many authors: Ewais et al. (2014), Ibrahim & Al-Ebady (2014), and Mossa & Abdul Wahid (2014). In addition, the analysis of *Thymus* extract by GC-Mass spectroscopy was illustrated by many authors; Pinto et al. (2006) and Ewais et al. (2014). Thymol is the main constituent of *T. vulgaris* extract and its antifungal activity may be of major importance as confirmed earlier by Miladi et al. (2013) and Kohiyama et al. (2015).

## Conclusion

*Aspergillus parasiticus* is one of the aflatoxin-producing fungi. A simple and rapid method for the detection of aflatoxin-producing species is by fluorescence at UV (320 - 360nm). The methanolic extract of *Thymus vulgaris* has antifungal effect on the growth of aflatoxigenic fungi.

*Ethical approval:* Not applicable.

## References

- Abbas, K.H., Shier, W.T., Horn, B.W., Weaver, A.M. (2004) Cultural methods for aflatoxin detection. *Toxin Reviews*, **23**(2, 3), 295-315.
- Abbas, H.K., Zablotowicz, R.M., Weaver, M.A., Horn, B.W., Xie, W., Shier, W.T. (2004a) Comparison of cultural analytical methods for determination of aflatoxin production by Mississippi Delta *Aspergillus* isolates. *Canadian Journal of Microbiology*, **50**, 193-199.
- Abo Dahab, N.F., Abdel-Hadi, A.M., Abdul-Raouf, U.M., El-Shanawany, A.A., Hassane, A.M.A. (2016) Qualitative detection of aflatoxins and aflatoxigenic fungi in wheat flour from different regions of Egypt. *Journal of Environmental Science, Toxicology and Food Technology*, **10**(7) (Ver. II), 20-26.
- Al-Maqtari, M.A.A., Alghalibi, S.M., Alhamzy, E.H. (2011) Chemical composition and biochemical activity of essential oil of *Thymus vulgaris* from Yemen. *Turkish Journal of Biochemistry*, **36**(4), 342-349.
- Al-Rahmah, A.N., Mostafa, A.A., Abdel-Megeed, A., Yakout, S.M., Hussein, S.A. (2013) Fungicidal activities of certain methanolic plant extract against tomato phytopathogenic fungi. *African Journal of Microbiology Research*, **7**(6), 517-524.
- Augusto, J. (2004) Development of a screening method for determination of aflatoxins, Chapter 2; Fluorescence enhancement of aflatoxin B<sub>1</sub> with cyclodextrin. *Msc Thesis*, Athens, Georgia, pp. 34-60.
- Corry, J.E.L., Curtis, G.D.W., Baird, R.M. (2003) *Aspergillus flavus* and *parasiticus* agar (AFPA), Progress in industrial Microbiology. In: “*Handbook of Culture Media for Food Microbiology*”, 37, pp. 397-399.

- Cotty, P.J. (1988) Simple fluorescence method for rapid estimation of aflatoxin levels in a solid culture medium. *Appl. and Environ. Microbiology*, **54**(1), 274-276.
- Cotty, P.J. (1989) Virulence and cultural characteristics of two *Aspergillus flavus* strains pathogenic on cotton. *Phytopathology*, **79**(7), 808-814.
- Davis, N.D., Diener, U.L., Eldridge, W. (1966) Production of Aflatoxins B1 and G1 by *Aspergillus flavus* in a semisynthetic medium. American Society for Microbiology, *Applied Microbiology*, **14**(3), 1-3.
- Davis, N.D., Iyea, S.K., Diener, U.L. (1987) Improved method of screening for aflatoxin with a coconut agar medium. *Applied Environmental Microbiology*, **53**(7), 1593-1595.
- Ehrlich, K.C., Chang, P., Yul, J., Cotty, P.J. (2004) Aflatoxin biosynthesis cluster gene cypA is required for G aflatoxin formation. *Applied Environmental Microbiology*, **70**(11), 6518-6524.
- El Koury, A., Atoui, A., Rizk, T., Lteif, R., Kalassy, M., Leberhi, A. (2011) Differentiation between *Aspergillus flavus* and *Aspergillus parasiticus* from pure culture and aflatoxin- concentrated grapes using PCR-RFLP analysis of aflR-aflJ intergenic spacer. *Journal of Food Science*, **76**(4), 247-253.
- Ewais, E. A., Aly, M.M., Ismail, M.A., Abdel Shakour, E.H., Hassanin, M.F. (2014) Antibacterial, antifungal, antitumor and toxicity of essential oils of *Salvia officinales*, *Thymus vulgaris*, *Eugenia caryophyllata* and *Artemisia absinthium*. *Scientific Journal of Flowers and Ornamental Plants*, **1**(3), 265-274.
- Fente, C.A., Jaimez O.J., Vazquez, B.I., Franco, C.M., Cepeda, A. (2001) New additive for culture media for rapid identification of aflatoxins-producing *Aspergillus* strains. *Applied Environmental Microbiology*, **67**(10), 4858- 4862.
- Hara, S., Fennell, I.D.I., Hesseltine, C.W. (1974) Aflatoxin- producing Strains of *Aspergillus flavus* detected by fluorescence of agar medium under ultraviolet light. American Society for Microbiology, *Applied Environmental Microbiology*, **27**(6), 4858-4862.
- Ibrahim, F.A.A., Al-Ebady, N. (2014) Evaluation of antifungal activity of some plant extracts and their applicability in extending the shelf life of stored tomato fruits. *Journal of Food Process Technology*, **5**(6), 1-6.
- Jaime-Garcia, R., Tucson, A.Z., Cotty, P.J., Tucson, A.Z. (2006) Crop potential influences aflatoxin potential of *Aspergillus* communities in south Texas, 2006. *Beltwide Cotton Conferences*, San Antonio, Texas, pp. 60-64.
- Klebe, J.F., Finkbeiner, H., White, D.M. (1966) Silylations with Bis (trimethylsilyl) acetamide, a highly reactive silyl donor. *Journal of the American Chemical Society*, **88**(14), 3390-3395.
- Kohiyama, C.Y., Ribeiro, M.M.Y., Mossini, S.A.G., Bando, E., Bomfim, N.S., Nerilo, S.B., Rocha, G.H.O., Grespan, R., Mikcha, J.M.G., Machinski, J.M. (2015) Antifungal properties and inhibitory effects upon aflatoxin of *Thymus vulgaris* L. by *Aspergillus flavus* link. *Food Chemistry*, **173**, 1006-1010.
- Lin, M.T., Dianese, J.C. (1976) A coconut- agar medium for rapid detection of aflatoxin production by *Aspergillus* spp., *Phytopathology*, **66**, 1466-1469.
- Miladi, H., Slama, R.B., Mili, D., Zouari, S., Bakhrouf, A., Ammar, E. (2013) Essential oil of *Thymus vulgaris* L. and *Rosmarinus officinalis* L.: Gas Chromatography- mass spectrometry analysis, cytotoxicity and antioxidant properties and antibacterial activities against foodborne pathogens. *Natural Science*, **5**(6), 729-639.
- Moghtader, M. (2012) Antifungal effects of the essential oil from *Thymus vulgaris* L. and comparison with synthetic thymol on *Aspergillus niger*. *Journal of Yeast and Fungal Research*, **3**(6), 83- 88.
- Mossa, M.I., Abdul Wahid, O.A. (2014) The effect of *Thymus vulgaris*, *Salvia* and *Artemisia* plant extracts on four pathogenic fungi in North Sinai. *Egypt J. Botany*, 6<sup>th</sup>. International Con. 11-12 May, 2016, Menoufia Univ. *Egyptian Journal of Botany*, (Special Issue), 301-316.
- Mousavi, M.S., Raftos, D. (2012) *In vitro* antifungal activity of a new combination of essential oils against some filamentous fungi. *Middle-East Journal of Scientific Research*, **11**(2), 156-161.

- Okwa, G.M.L., Achar, P.N., Sharma, S.K. (2010) Quantification of aflatoxins B1 in ready-to-use food thickeners in south-east geo-political zone in Nigeria. *African Journal of Microbiology*, **4**(16), 1788-1793.
- Ouattara-Sourabie, P.B., Nikiema, P.A., Barro, N., Savadogo, A., Traore, A.S. (2012) Aflatoxigenic potential of *Aspergillus* spp. isolated from groundnut seeds, in Burkina Faso, West Africa. *African Journal of Microbiology Research*, **6**(11), 2603-2609.
- Ouf, S.A., Basher, A.H., Mohamed, A-A.H. (2015) Inhibitory effect of double atmospheric pressure argon cold plasma on spores and mycotoxin production of *Aspergillus niger* contaminated date palm fruits. *Journal of Science of Food and Agriculture*, **95**(15), 3204-3210.
- Ouf, S.A., Mohamed, A-A.H., Sayed, W.S. (2016) Fungal decontamination of fleshy fruit water washes by double atmospheric pressure cold plasma (DAPCP). *Clean, Soil, Air, Water*, **44**(2), 134-142.
- Ouf, S.A., El-Yasergy, K.F., Abd El-Ghany, M.N. (2019) Efficacy of ozonized water for fungal decontamination of fresh fruit pieces decorating dessert cakes. *Egyptian Journal of Botany*, **59**(3), 845-855.
- Pinto, E.N., Pina-Vaz, C.L., Salgueiro, L.G., Goncalves, M.J., Costa-de-Oliveira, S., Cavleriro, C., Palmeira, A., Rodrigues, A.C., Martinez-de-Oliveira, J. (2006) Antifungal activity of the essential oil at *Thymus pulegioides* on *Candida*, *Aspergillus* and dermatophyte species. *Journal of Medical Microbiology*, **55**, 1367-1373.
- Pitt, J.T., Hocking, A.D. (2009) "*Fungi and Food Spoilage*", 3<sup>rd</sup> ed., New York, Springer.
- Rajarajan, P.N., Rajasekaran, K.M., Asha Devis, N.K. (2013) Isolation and quantification of aflatoxin from *Aspergillus flavus* infected stored peanuts. *Indian Journal of Pharmaceutical and Biological Research (IJPBR)*, **1**(4), 76-80.
- Rodrigues, P., Soares, C., Kozakiewicz, Z., Paterson, R.R.M., Lima, N., Venancio, A. (2007) Identification and characterization of *Aspergillus flavus* and aflatoxins. *Communication Current Research and Educational Topics and Trends in Applied Microbiology*, **2007**, 527-534.
- Sadharsan, S., Britzi, M., Zakin, V., Kostyukovsky, M., Trostanetsky, A., Quinn, E., Sionov, E. (2017) Rapid detection and identification of mycotoxins in stored wheat grains. *Toxins*, **9**(10), 302, 1-17.
- Saito, M., Machida, S. (2009) A rapid identification method for aflatoxin-producing strains of *Aspergillus flavus* and *A. parasiticus* by ammonia vapor. *Mycoscience*, **40**(2), 205-208.
- Sudini, H., Srilakshmi, P., Vijay, K.K.K., Samuel, M.C., Njoroge, O.M., Seetha, A. (2015) Detection of aflatoxigenic *Aspergillus* strains by cultural and molecular methods. *African Journal of Microbiology Research*, **9**(8), 484-491.
- Varga, J., Frisvad, J.C., Sanson, R.A. (2011) Two new aflatoxins producing species and an overview of *Asp. Section Flavi*. *Studies in Mycology*, **69**(1), 57-80.
- White, T.J., Bruns, T., Lee, S., Taylor, J., Innis, A., Gelfand, D.H., Sninsky, J.J. (1990) PCR Protocols. *Academic Press, San Diego, USA*, 315-322.
- Wacoo, A.P., Wendi, D., Vuzi, P.C., Hawumba, J.F. (2014) Methods for detection of aflatoxins in agricultural food crops. *Journal Applied Chemistry*, **2014**, Article ID 706291. 15p.
- Yazdani, D., Abidin, Z.M.A., Tan, Y.H., Kamaruzaman, S. (2010) Evaluation of the detection techniques of toxigenic *Aspergillus* isolates. Full Length Research Paper. *African Journal of Biotechnology*, **9**(45), 7654-7659.
- Zekovic, Z.P., Lepojevic, Z.P., Markov, S.L., Milosevic, S.G. (2002) Tablet with thyme (*Thymus vulgaris* L.) extract. . Original scientific paper, APTEFF, **33**, 1-174, *BIBLID*, 1450-7188, **33**, 159-165.

## دراسة التأثير الضد فطري لمستخلص الميثانول لنبات الزعتر على نمو الاسبرجيلس بارازيتيكس المنتج للسموم الفطرية

منجه ابراهيم موسى

قسم النبات - كلية العلوم بالعريش - جامعة العريش - شمال سيناء - مصر.

توضح هذه الدراسة إحدى الطرق البسيطة المستخدمة لقياس مدى مقدرة فطر الاسبرجيلس بارازيتيكس على إنتاج الافلاتوكسينات. فأحد المؤشرات لمقدرة الفطر على إنتاج التوكسين هو تلون ظهر المستعمرة باللون الأصفر وكذلك توهج الفطرة عند تعرضه للأشعة فوق البنفسجية وإحاطتها بهالة زرقاء اللون. تم تعريف الفطر اعتماداً على الشكل الظاهري ثم تأكيد ذلك بدراسة الحامض النووي الديوكسي ريبوزي لتعريفه والمقارنة بالعزلات المتاحة بقواعد البيانات. وجد إن إضافة بعض المواد مثل بيتا سيكلو دكسترين (0.3%) إلى الوسط الغذائي قد أدت إلى زيادة الهالة الزرقاء حول النمو الفطري عند تعرضها للأشعة فوق البنفسجية. تم تأكيد تعريف الفطرة اعتماداً على الشكل الظاهري بالتعريف عن طريق دراسة التتابع النيوكليوتيدي للحامض النووي للفطر. عند دراسة تأثير المستخلص الميثانولي لنبات الزعتر (النبات كاملاً ما عدا الجذر) وجد أن أقل تركيز أدى إلى تقليل النمو إلى أقل من 20% مقارنة بالكنترول هو 80 ميكروليتر من المستخلص 10 ملليتر من الوسط الغذائي. وكذلك تم قياس تركيز المستخلص النباتي الذي أدى إلى تقليل نمو الفطرة إلى النصف مقارنة بالكنترول هو 20 ميكروليتر من المستخلص النباتي/10 ملليتر من الوسط الغذائي. عند التحليل الكيميائي (بجهاز GC-MS) للمستخلص النباتي للزعتر وجد أن أعلى تركيز للمركبات التي تم الحصول عليها كانت لمركب الثايمول وهو من المركبات الفينولية المعروف بخواصه التثبيطية للفطريات. ومن ذلك يتضح تأثير المستخلص الميثانولي لنبات الزعتر على نمو فطرة الاسبرجيلس بارازيتيكس الذي له الدور الكبير في تلوث العديد من الاطعمة بالسموم الفطرية.