Evaluation of natural sources for repress cytotoxic Trichothecenes and Zearalenone production with using Enzyme-linked immunosorbent assay

Abdel Ghany TM[1,2], Ganash MA[3], Marwah M. Bakri[4], Aisha M.H. Al-Rajhi[5], Mohamed A, Al Abboud[2]

[1] Botany and Microbiology Department, Faculty of Science, Al-Azhar University, Egypt
[2] Biology Department, Faculty of Science, Jazan University, Jazan, King Saudi Arabia
[3] Department of Biology, Faculty of Science, King Abdulaziz University, Jeddah, King Saudi Arabia
[4]Academic campus for girls, Jazan University, Jazan, King Saudi Arabia
[5] Biology Department, Faculty of Science, Princess Nora Bent Abdularahman University, King Saudi Arabia
tabdelghany@yahoo.com

Abstract: Even though the prevention of fungal growth is still the good practice to prevent contamination by mycotoxins, but there are a number of approaches that can be taken to minimize mycotoxins contamination in the food. The advantage of using-plant produced compounds and bioagent as a source of safer and more effective control substances than synthetically produced antimicrobial agents can be demonstrated both practically and in terms of consumer acceptance. Cinnamon and cyperus oils had antifungal activity against *Alternaria alternata* and *Fusarium culmorum*. Inhibition % of deoxynivalenol (DON) and Trichothecene (T-2) production was 21.89 and 42.83% respectively at 1.5 µL/ml of cinnamon oil. The extracts of the *Aloe vera* inhibit the production of Zearalenone and T-2 mycotoxins ranged from 9.13 to 17.15 and from 33.10 to 36.69% respectively. Strong effective of *Adenium obesum* extract on the inhibition production of ZER, DON and T-2 mycotoxins was reported, inhibition% was 39.15, 43.92 and 45.00 % at 20, 5 and 20 mg/ml respectively. Decrease of ZEA, DON and T-2 mycotoxins levels in comparison with the control (1083.43, 77.03 and 497.67 µg/kg respectively) was observed with *Saccharomyces cerevisiae* interaction (659.90, 42.92 and 294.26µg/kg respectively). *T. harzianum* inhibited the production of ZEA, DON and T-2 mycotoxins with an average inhibition of 25.06, 33.01 and 50.55% respectively. Our results improved that, the tested *T. harzianum* and *S.cerevisiae* had no toxic effects, seems to be a highly promising agent in preventing food contamination with *F.culmorum* and greatly inhibit mycotoxin production *in vitro*.

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1. Introduction

The grains serve as a suitable substrate for mold growth and mycotoxin production (Bryden, 2012). The presence of molds and mycotoxin in food commodities is a potential health threat to humans and livestock. Fungal contamination with poisonous mycotoxins could lead to major health problems while it obviously deteriorates the nutritive value of food (Rapp 2004; Zekavati et al., 2013). At a cellular level, mycotoxins react with nucleic acids and inhibit the biosynthesis of macromolecules DNA and RNA, or act on structures and functions of biological membranes or impair the energy metabolism (Diaz 2005; Wang et al., 1999). Therefore, the presence of toxigenic fungi and mycotoxins in foods and grains stored for long periods of time presents a potential hazard to human and animal health. Considerable interest has developed in the preservation of feeds by the use of essential oils and plant extracts to effectively suppress growth of such fungi and mycotoxin production. Trichothecenes are a group of structurally related compounds with a common tetracyclic sesquiterpenoid 12, 13-epoxytrichothec-9-ene ring system. The trichothecenes are chemically the most diverse of all the mycotoxins with over 200 identified (Grove, 2007; Krska *et al.,* 2007; Marin *et al*., 2013). Examples of trichothecenes are T-2 toxin and deoxynivalenol (DON) (Marin et al., 2013). Trichothecenes are produced by a variety of different Fusarium species (Thrane, 2001). DON a cytotoxic trichothecene, has been associated with liver disease, oestrogenic disorders, oesophageal cancer and immunotoxic effects (Marasas et al. 1984; Lou et al., 1990). Although the prevention of mycotoxin contamination of grain is the main goal of food and agricultural industries throughout the world, under certain environmental conditions the contamination of various cereal grains with Fusarium spp. and their mycotoxins are unavoidable for grain producers. Several strategies have been applied to eliminate mold growth and mycotoxins production or to remove/destroy the preformed toxins from grains and

foodstuffs (Angelo, 2000) Minervini et al. (2004) suggests that human blood cells are sensitive to mycotoxin exposure, that DON is more toxic than other mycotoxins. Palacios et al. (2011) reported that DON considered the important Fusarium mycotoxins that have received considerable attention related to food safety. Zearalenone (ZEN) is a non-steroidal estrogenic mycotoxin biosynthesized through a polyketide pathway by certain Fusarium spp., which are regular contaminants of cereal crops worldwide, it has been known to be hepatotoxic, haematotoxic, genotoxic and immunotoxic. Recently, evidence suggests that ZEA has potential to stimulate the growth of human breast cancer cells (Nesic et al., 2014).

The exploration of naturally occurring antimicrobials for food preservation receives increasing attention due to consumer awareness of natural food products and a growing concern of microbial resistance towards conventional preservatives (Schuenzel and Harrison, 2002). Many reports revealed that, plant metabolites and plant based pesticides appear to be one of the better alternatives as they are known to have minimal environmental impact and danger to consumers in contrast to synthetic pesticides (Varma and Dubey, 1999; Gottlieb et al., 2002; Krishnaiah et al., 2011; Abdel Ghany 2014; Abdel Ghany and Othman 2014; Abdel Ghany et al., 2015a&b; Bapat et al., 2016). New researches about biological active secondary compounds present in essential oils (EOs) of plants have been seen as a potential way to control fungal contamination (Burt, 2004; Tajkarimi et al., 2010). Recently, there has been increasing interest in using naturally occurring compounds, especially EOs, to control food spoilage fungi *in vitro* and *in vivo* (Lopez et al., 2004; Marin et al. 2004; Tzortzakis 2009; Choudhary and Kumari, 2010). Varahalarao and Chandrashekar, (2010) tested the antimicrobial activities of of *Calotropis procera* against *Alternaria alternata*, *Aspergillus flavus*, *A. niger* and *Penicillium expansum*. Also, Ennajar *et al.* (2010) evaluated the antimicrobial and antioxidant activities of essential oil from leaves of *Juniperus phoenicea*.

Adenium obesum is a succulent shrub commonly known as desert rose belongs to the family Apocynaceae (Paul et al., 2015),their crude extracts contains alkaloids, steroids, saponins, glycosides, anthraquinones, tannins and flavonoids (Krishnaiah et al., 2011). It is known as a medicinal plant used in different countries around the world as poison on arrows (Barile et al., 2007). Antimicrobial study of different crude extracts of *A. obesum* showed that crude extracts have results against the food borne pathogenic bacteria (Mohammad et al., 2014; Císarová and Dana, 2015). Velluti et al. (2003) screened 37 EOs for their inhibitory activity on growth of *F. verticillioides, F. proliferatum* and *F. graminearum* in maize meal extract agar. They found that cinnamon, clove, lemongrass, palmarose and oregano oils were the best oils tested. The methanolic extract of *Cyperus rotundus* showed best antifungal activity against *Aspergillus candidus* (Parekh and Chanda, 2006). The antimicrobial activity of EOs can be attributed to the presence of an aromatic nucleus and a phenolic OH group that is known to be reactive and to form hydrogen bonds with active sites of target enzymes (Farag et al., 1989). Inhibitory action of natural products on fungal cells involves cytoplasm granulation, cytoplasmic membrane rupturing and inactivation and/or synthesis inhibition of intercellular and extracellular enzymes (Souza *et al*., 2005).similar inhibition mechanisms were reported by Carson et al. (2002), low concentrations of EOs result in changes of the cell structure, inhibiting respiration and changing the permeability of the cell membrane, whereas high concentrations lead to severe membrane damage, loss of homeostasis and cell death. Isolates of Trichoderma, growing on the mycelium of toxigenic *Fusarium* species, are also able to transform or degrade such as DON and ZEA mycotoxins, (Bottalico 2002). Until now, the ability to decompose DON has been found very rarely among microorganisms. Only one mixed culture among 1285 microbial cultures, transformed DON into 3-keto-4-deoxynivalenol (Voelkl et al., 2004). According to Buśko et al. (2008), production of five trichothecene mycotoxins was reduced by over 95% in dual culture bioassay by *Trichoderma* isolates. Previous studies (El-Taher *et al.,* 2012*;* Kapetanakou *et al*., 2012**)** used *S.cerevisiae* to reduced mycotoxins production. Another study (Masoud *et al.,* 2005) found that *Pichia anomala* and *Pichia kluyveri* inhibited the production of mycotoxins by *Aspergillus ochraceus* on malt extract agar medium and on coffee agar medium. The objective of the present study was to evaluate the in vitro activity of plant extracts, essential oils and biological control against fungi and their mycotoxins.

2.Material and Methods

Fungi isolation

Stored wheat grains (50 grains) were surface-disinfected with 1% NaOCl solution for 5 min followed by rinsing twice with sterile water then dried over a filter paper in a sterile laminar flow cabinet. Ten grains were placed on sterile potato dextrose agar (PDA) medium and incubated at 28 ±2°C for 7 days in darkness. The developing fungal colonies were sub-cultured onto PDA and identified based on their macro and microscopic features according to Nelson et al. (1983), Pitt and Hocking (1997) and Simmons

(2000). Isolates description were recorded as *Alternaria alternata* and *Fusarium culmorum*.

Plant extract and essential oils for controlling fungal growth and their mycotoxins

Sample (50 g) of the shade-dried powder of each plant materials (*Alloe vera* and *Adenium obesum*) was extracted separately with methanol using Soxhlet extractor until colorless extract was obtained on the top of the extractor. All the extracts were concentrated separately using rotary flash evaporator and preserved at 5°C in an air tight brown bottle until further use. Two natural essential oils (Cinnamon and Cyperus) were obtained commercially from El-Hawag company for extract and mobilize the natural oils and cosmetics, Badr city, Cairo, Egypt. *F.culmorum* was inoculated in 250ml Erlynmer conical flasks containing sterile 2% wheat natural medium supplemented with different concentrations of plant extract and essential oils. Inoculated flasks were incubated at 28±2°C for 10 days in dark. The filtrates of the culture media were obtained and assayed for the presence of mycotoxins.

Poisoned food technique for anti-fungal assay

Potato dextrose agar medium (PDA) supplemented with different concentrations of each plant extract and essential oils were prepared separately. About 25 ml of the growth medium was poured into each petri-dish and allowed to solidify. Five mm disc of 5-day old culture of the *A. alternata* and *F. culmorum* was placed at the center of the Petri dish and incubated at 28±2°C for 6 days, the growth was measured in centimeter. For each treatment three replicates were maintained. PDA medium without the methanolic plant extract and essential oils served as control (Grover and Moore 1962). The colony diameter was measured after 6 days and inhibition percentage of the fungal growth in relation to control treatment was calculated according to the given formula: I = C – T/ C x 100.

Where I = percentage inhibition, C = radial growth in control, T = radial growth in treatment.

Biological control of mycotoxins production and cultural conditions

Commercial *Saccharomyces cerevisiae* was prepared in Yeast extract-Peptone-Dextrose broth (5 g yeast extract, 5 g peptone, 40 g dextrose, liter water) and incubation at 25 °C for 24 h. After incubation, cells were collected by centrifugation (5000 rpm for 10 min), washed twice with phosphate-buffered saline (PBS pH 7.2) and once with sterile double-distilled H_2O. Finally, the yeast pellets (Two concentrations 1×10 and 2×10 cells/ml) were suspended in sterile PBS prior use in the control of mycotoxins. *Trichoderma harzianum* was provided from Regional Center for Mycology and Biotechnology (RCMB) Al-Azhar University Cairo, Egypt. Spores of *T. harzianum* was prepared by growing it on PDA medium for 6 days before harvesting and filtering through sterile cotton wool by sterile distilled water plus 0.02 % Tween 80. Two concentrations (1×10 and 2×10 spores/ml) were used in the control of mycotoxins production. The number of spores/cells was determined by using a haemacytometer (Thanaboripat et al., 2009). *S. cerevisiae* and *T. harzianum* were added separately to 2% wheat natural medium inoculated with *F. culmorum* and incubated at 28±2°C for 10 days in dark. The filtrates of the culture media were obtained and assayed for the presence of mycotoxins.

Mycotoxins production conditions, extraction and their assay

The filtrates of the culture media of *F. culmorum* were obtained and assayed for the presence of mycotoxins. Three mycotoxins including Zearalenone (ZER) Deoxynivalenol (DON) and Trichothecene (T-2) (Fig. 1) were detected with using Microtitre plate enzyme-linked immunosorbent assay (ELISA) reader (automated Chem-well) in Saudi Grains Organization (SAGO), Saudi arabia. Fusarium mycotoxins test kit was used to ELISA analyses. The samples were analyzed using the ZER, DON and T-2 test procedure which was described by company(r-biopharm) producer (Enzyme Immunoassay for the quantitative analysis of mycotoxins (Leszczynska et al., 2001).Ten ml of blended fungal broth has been sub-sampled with 20ml of 70% methanol and vortex for 10 min by magnetic stirrer. The extract was filtrated by Whatman number one filter paper and then diluted as 5ml filtered solution, 15ml distilled water and 0.25ml Tween 20. The solution was mixed by magnetic stirrer for 2min. 50 µl toxins (5, 10, 20, 45 ppb) standard solutions and 50 µl prepared test samples were added into separate wells of micro-titer plate. Plates were incubated at room temperature. The liquid was then removed completely from the wells, the each well was washed with 250 µl washing PBS-Tween-Buffer (pH 7.2) and this was repeated two times. Subsequently, enzyme substrate (50 µl) and Chromogen (tetramethyl-benzidine, 50 µl) were added to each well and incubated for 30min at room temperature in the dark. 100 µl of the stop reagent (1M H_2SO_4) was added and the absorbance was measured at 450nm in ELISA reader.

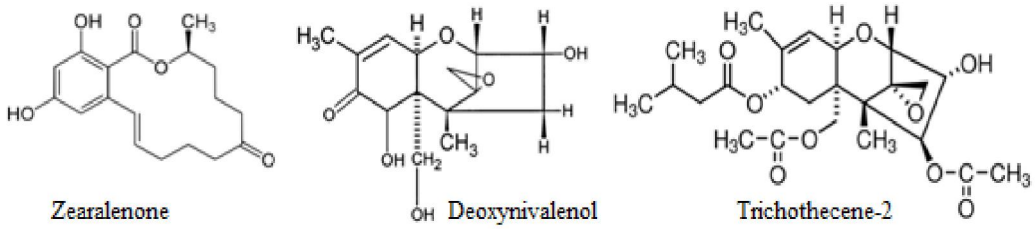

Fig.1 Chemical structure of detected mycotoxins

Diphenyl-picryl-hydrazyl (DPPH) free radical scavenging activity

Diphenylpicrylhydrazyl (DPPH) method (Cheel *et al*., 2007) was employed to investigate the antioxidant activity of plant extract *(Alloe vera* and *Adenium obesum)* and essential oils (Cinnamon and cyperus). Different concentrations of plant extracts and oils (5, 10, 15, 20, 25, 30, 35, 40, 45 and 50 µg/ml) was added to a 0.06 mM DPPH solution in ethanol and the reaction mixture was shaken vigorously. After incubation for 30 min at room temperature, the absorbance at 517nm was recorded spectrophotometrically at RCMB. A control solution without the tested compound was prepared in the same manner as the assay mixture. The degree of decolorization indicates the free-radical scavenging efficiency of the samples. The antioxidant activity was calculated as an inhibitory effect (Scavenging activity %) of the DPPH radical formation using the following formula: Scavenging activity (%) = 100x (A Blank -A sample)/(A Blank). Where were the A Blank and A sample absorbance at 517nm of blank and samples respectively. IC50 (efficient concentration value), is used for the interpretation of the results from the DPPH method and is defined as the concentration of substrate that causes 50% loss of the DPPH activity

Determination of Total Phenolics of plant extract and essential oils

Folin-Ciocalteu assay (Kahkonen et al., 1999) was used for determine the total phenol content (TPC). A volume of 0.2 mL of the extract was amended with 0.5 mL Folin-Ciocalteu's reagent (diluted 10 times with water) and then kept for 5 min in the dark and then one mL sodium carbonate (7.5% w/v) was added. The tubes were covered with parafilm and kept again in the dark for 1 h. Absorption at 765 nm was measured with a spectrophotometer UV-vis and compared to a gallic acid calibration curve. The results were expressed as mg gallic acid/g dried sample. The assay of the total phenol content of the essential oils was done as follows: a volume of 3 mL of the methanol solution of each essential oil was introduced into test tubes followed by 1 mL Folin-Ciocalteu's reagent (diluted 10 times with water) and 1 mL sodium carbonate (7.5% w/v) and measured as plant extract.

3. Results and Discussions

Plant extracts and essential oils have the potential to replace the synthetic fungicides in the management of fungal growth and their mycotoxins. Fungitoxic activity of two different oils and two plant extracts was tested by the poisoned food technique using PDA medium at three different concentrations on radial growth *A. alternata* and *F. culmorum* (Table 1 & Figs.2,3). Data in current study showed that cinnamon and cyperus oils had antifungal activity against *A. alternata* and *F. culmorum*. Inhibition of fungal growth increased with increasing concentration of oils particularly with using cinnamon oil. The efficacy of cinnamon oil as antifungal agents, were reported by many researchers (Barrera-Necha et al., 2009; Hamad et al., 2015). In our study, *A. alternata* was more sensitive (growth inhibition % was 81.70% and 74.39%) than *F. culmorum* (growth inhibition % was 72.09 and 53.49 %) at high concentration 2.5 µL/ml of cinnamon and cyperus oils respectively (Table1). However, Barrera-Necha et al. (2009) studied the antifungal effects of 10 essential oils against *F. oxysporum* f. sp. gladioli. They found that essential oils of cinnamon, clove and thyme inhibited the growth of Fusarium sp. totally. The antifungal activity of cinnamon oil may be due to their components. Bauer and Kirby (2012) stated that antifungal effects of cinnamon essential oil may be related to the ortho-methoxy cinnamaldehyde. It has been reported that fungal spores can't reproduce in medium containing cinnamon. In addition, *F. verticillioides* exposed to Cinnamon oil showed irreversible deleterious morphological and ultrastructural alterations, such as lack of cytoplasmic contents, loss of integrity and rigidity of the cell wall, plasma membrane disruption, mitochondrial destruction, folding of the cell. These modifications induced by cinnamaldehyde may be due to its interference with enzymatic reactions of cell wall synthesis (Fuguo et al., 2014). From the current results, there was a slight decrease in growth of fungal

growth with the same cyperus oil concentrations particularly *F. culmorum*. Our results are in harmony with those reported by Mariana and Camelia, (2012) who stated that inhibition of fungal growth depends on the amount of oil applied, but the relationship is not linear. According to Anupam et al. (2011), Cyperus oil shown good antifungal activity against *Candida parapsilosis* and *Aspergillus fumigates* and inhibit spore formation of *Fusarium oxysporum* and *Aspergillus flavus*. Table (1) illustrates the effect of different concentrations of *Aloe vera* extract on the mean diameter and percent of growth inhibition of *F. culmorum* and *A. alternata*. Colony growth on control treatment was more than other treatments with any concentration of plant extract. The percentage of inhibition at the highest concentration of *Aloe vera* extract (20mg/ml) was 74.42 and 60.98% for *F. culmorum* and *A. alternata* respectively. Our results are in agreement with the previous studies carried out by Jasso et al. (2005) and Oana et al. (2007), antimycotic activity of *Aloe vera* fresh leaves extract was good against the mycelial growth of *Botrytis gladiolorum*, *Fusarium oxysporum*, *Heterosporium pruneti* and *Penicillium gladioli*, *Rhizoctonia solani*, and *Colletotrichum coccodes*. As illustrated in Table (1) *Adenium obesum* extract have demonstrated antifungal activity against *F. culmorum* and *A. alternata*. Similarly, the inhibitory effect on fungal growth by *Aloe vera* extract on *Alternaria alternata*, *A. citri* and *A. tenuissima* was studied (Masood and Ranjan 1991). The good antifungal activity of *Adenium obesum* extract appeared at high concentration (20mg/ml) (Fig.2&3). Few reports on fungitoxic effects of *A. obesum,* however the antibacterial activities of *A. obesum* have been proved against several bacterial strains (Tijjani et al., 2011; Hossain et al., 2014; Sharma et al., 2015).

Previous studies (Jouany 2007; Popiel et al., 2008) reported that both *F.culmorum and F. graminearum* isolates produce trichothecenes and ZEA mycotoxins and are considered to be the most important cereal pathogens worldwide. Infection with those fungi leads to accumulation of mycotoxins including DON and ZEA in grains. Therefore the efficacy of selected plants and EOs in the management of *F.culmorum* and their mycotoxins contamination was investigated in this study. The effects of the two EOs and two methanolic plant extracts on growth of *F.culmorum* at three concentrations are shown in Tables (2&3). Inhibition % of ZER production by *F.culmorum* was 35.58, 30.4 and 27.32% with using cinnamon oil at concentration 0.5,15 and 2.5 µL/ml respectively. Decrease in ZER production was proportional to the concentration of cinnamon oil concentrations. Maximum inhibition % of DON and T-2 production was 21.89 and 42.83% respectively at 1.5 µL/ml of cinnamon oil. High concentration (2.5 µL/ml) of cinnamon oil stimulate the production of DON, where their concentration was 79.03 µg/kg compared with control 77.03 µg/kg (Table 2). Cinnamon oil was found to be effective against aflatoxin production by *Aspergillus flavus* under favorable conditions in maize grain (Montes-Belmont and Carvajal, 1998).

Furthermore, Velluti et al. (2003) reported that cinnamon oil was able to control the growth of *Fusarium proliferatum* growth and aflatoxin B_1 production in maize.

On the other hand production of ZER was stimulated or was similar to control with using Cyperus oil at any concentration. In our study, cyperus oil showed highly stimulator of DON production 233. g/kg compared with detected level 77. g/kg at control. T-2 mycotoxin production by the *F. culmorum* was affected by the cyperus oil treatment but their different concentrations had the effect on the inhibition% of T-2 production (Table2). Poor activity was shown by cyperus oil in the current study. These oil have been previously observed to possess good antibacterial activity (Parekh and Chanda, 2006), but they turned out to be poor antifungal agents. Although results (Maswada and Abdallah, 2013) demonstrated that, the methanolic extracts of *Cyperus capitatus* and *Stipagrostis lanata* had potential antifungal activity against *Alternaria solani*, *Aspergillus niger* and *Rhizopus stolonifer*. Recently Kabbashi et al. (2015) reported that *Cyperus rotundus* revealed a zone of growth inhibition for *E. coli* and *Candida albicans* Zeid et al. (2008). The oil of *Cyperus rotundus* was shown a remarkable activity against several bacteri. A previous study evidenced the antifungal activity depends on the amount of oil applied, but the relationship is not linear (Mariana and Camelia, 2012). Generally, the results of the experimental determination of the inhibition of mycotoxin production showed different responses against the presence of the cyperus and cinnamon oils.

Table (3) reveals that two metanolic plant extracts were effective inhibitors of some *F.culmorum* mycotoxins production at three concentrations tested. The extracts of the *Aloe vera* inhibit the production of ZER and T-2 mycotoxins ranged from 9.13 to 17.15 and from 33.10 to 36.69% respectively at tested concentrations. In contrast, *Aloe vera* extract induced the production of DON. Abd El-Aziz et al. (2012) found that aqueous extract of *Aloe vera* could be applied as natural food preservatives against fungal mycotoxin production and that plant extracts can suppress aflatoxin biosynthesis. On the other hand, strong effective of *Adenium obesum* extract on the inhibition production of ZER, DON and T-2 mycotoxins compared with *Aloe vera* extract (Table

3), where maximum inhibition was 39.15, 43.92 and 45.00 % at 20, 5 and 20 mg/ml respectively. Our results reported that oils and plant extracts having capacity for the inhibition of *F.culmorum* did not exert identical effects on the mycotoxins production. This is evident that *Aloe vera* whole plant extract inhibited the growth by 74.42% (Table1) and toxin inhibition by 9.13% of ZER and 0.0 % of DON and 33.01 of T-2 only (Table 3). There was no correlation between the inhibition of growth and mycotoxins production. Our notes were agreement with previous studies (Masood and Ranjan 1991; Abd El-Aziz et al., 2012).

The efficacy of selected *Saccharomyces cerevisiae* and *Trichoderma harzianum* in the management of *F.culmorum* and their mycotoxins contamination was investigated (Table 4). Also, Bejaouii et al. (2004) suggested that Saccharomyces can be used for the decontamination of ockratoxin in synthetic and natural grape juice. Decrease of ZEA, DON and T-2 mycotoxins levels in comparison with the control (1083.43, 77.03 and 497.67 µg/kg respectively) was observed with *S. cerevisiae* interaction (659.90, 42.92 and 294.26 µg/kg respectively) mainly at 10 *S. cerevisiae* cells, whereas the *S. cerevisiae* was not able to decrease these mycotoxins production at 2x10 cells (Table 4). Armando et al. (2013) *S. cerevisiae* was able to inhibit *F.culmorum* growth and reduced ZEA and DON production at different environmental conditions reflecting those found in stored feedstuff. Similar results were reported by Al-Masri *et al.* (2011), where authors found that *S.cerevisiae* potentially inhibited *F. moniliform* growth and fumonisin B_1 production. According to Bata and Lasztity, (1999) **y**east or yeast cell walls can also be used as adsorbents for mycotoxins. Therefore use of yeast cell walls only instead of whole cells, the mycotoxins adsorption can be enhanced. This explanation was confirmed where Yiannikouris *et al.*, (2004) studied the adsorption capacity of yeast cell wall and the role of various B-D-glucan types in the efficacy of ZEA adsorption and thought to elucidate some of adsorption mechanisms *in vitro* studies with ZEA. *S. cerevisiae* therapeutic effects is attributed to its release of a 54-kDa protease that causes cleavage of *Clostridium difficile* toxins A and B (Castagliuolo *et al.*, 1999), this hypothesis may support our results in inhibition of ZEA, DON and T-2 mycotoxins production in the present experimental study.

The *T. harzianum* at 10 spore suspension inhibited the production of ZEA, DON and T-2 mycotoxins with an average inhibition of 25.06, 33.01 and 50.55% respectively (Table 4). A previous study evidenced the antifungal activity of *T. harzianum* (Bahareh et al., 2014), but mechanisms leading to suppression of mycotoxin production are yet to be better understood. Recently, production of enzymes by *Trichoderma* as part of their mycoparasitic process has been reported (Parmar et al., 2015; Osorio-Hernández et al., 2016). Suhaida and NurAinIzzati (2013) suggest that *Trichoderma* sp. released metabolites, which are toxic and fungistatic to *F. proliferatum.* Detoxification of mycotoxin contaminated food and feed by *T. harzianum* has been amply documented in the literature Haggag et al. (2014). Same antagonist with *Trichoderma* sp reduced the amount of moniliformin from 100 µg/g to 6.5 µg/g when inoculated to rice culture contaminated with MON (Popiel et al., 2008). Such difference in the inhibition % of myctoxins productions between our study and others may be related to varying culturing conditions.

Our results improved that, the tested *T. harzianum* and *S.cerevisiae* had no toxic effects, seems to be a highly promising agent in preventing food contamination with *F.culmorum* and greatly inhibit mycotoxin production *in vitro*. Surprisingly, in our study, *S.cerevisiae* not failed only to inhibit the mycotoxin production but induced their production at 2x10 cells (Table 4). At the same time, cultivation of *F.culmorum* in the presence of 2x10 spores of *T. harzianum* resulted in lowest inhibition of ZEA, DON and T-2 production in comparison at 10 spores (Table 4).This results may be due to the faster nutrient depletion and therefore the yeast cell died and used as nutrients for growth *F.culmorum* and their mycotoxins production. Other explanation of our results, the mycotoxins production increased as a biotic stress increased.

Determination of Antioxidant Activity Using DPPH Radical Scavenging Method

The DPPH method is widely used to test the ability of compounds to act as free radical scavengers or hydrogen donors, and to evaluate antioxidant capacity. As shows in Figure (4), all tested samples exhibited good radical scavenging activity with varied degrees. Cinnamon EO demonstrated the highest DPPH Scavenging % compared to other antioxidants studied, reaching as high as 77.872 % at 25 µg/ml. Recently, Ervina et al. (2016) reported that cinnamon EO is potential antioxidant compounds with the ability to scavenge free radicals. Our present study showed that different concentration of *Adenium obesum* exhibited strong free radical scavenging activity (Fig.4). The strong free radical scavenging activity in different stems crude extracts might be due to high quantity of phenolic compounds (42.54 mg /g dry weight of plant) (Fig. 5). Alseini (2014) reported high antioxidant activity of methanolic extract *A. obesum* in Saudi Arabia. Also, AL-Ghudani and

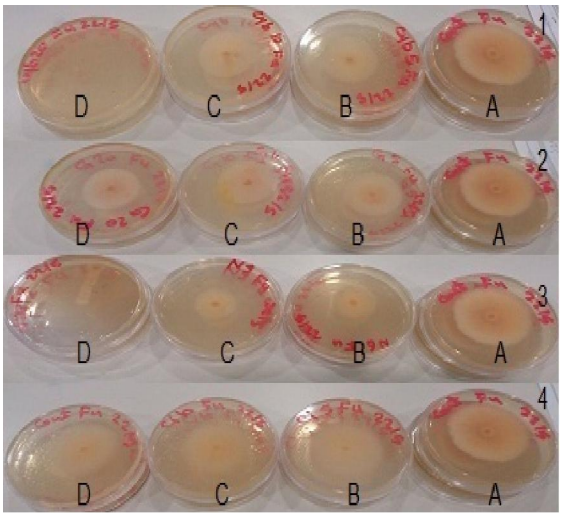

Fig. 2 Effect of 1, cinnamon oil; 2, cyperus oil; 3, *Alloe vera* extract; 4, *Adenium obesum* extract on *F. culmorum.* A, control; B, 0.5 µL/ml oil or 5 mg/ml plant extract; C, 1.5 µL/ml oil or 10 mg/ml plant extract; D, 2.5 µL/ml oil or 20 mg/ml plant extract

Hossain (2015) demonstrated that different crude extracts of roots of *A. obesum* exhibited significant antioxidant activity by DPPH method. *Aloe vera* extract showed appreciable free radical scavenging activities at the highest concentrations. The antioxidant activity of *A. vera* was 43.65 to 45.76% at high concentration ranged from 30 to 40 µg/ml of plant extract, which is similar to the one reported by Rakesh et al. (2016). Lowest total phenolic content (28.34 mg/g dry weight of plant) was recorded (Fig.5) compared with the other treatments of *A. obesum* extract or EOs. Free radical scavenging activity was sharply proportional to the concentration of the cyperus oil up to 35 µg/ml but then neglected increased was recorded (Fig. 4).Cinnamon EO have shown more total phenolic content than other cyperus EO and plant extracts (Fig. 4). This finding clearly suggests that the total phenolic content and the DPPH free radical scavenging activity are in direct proportion Hsiao-Fen (2008) demonstrated similar results. Some authors relate an antioxidant activity of the phenolic compounds to the inhibition of mycotoxin biosynthesis (Jayashree and Subramanyam, 1999).

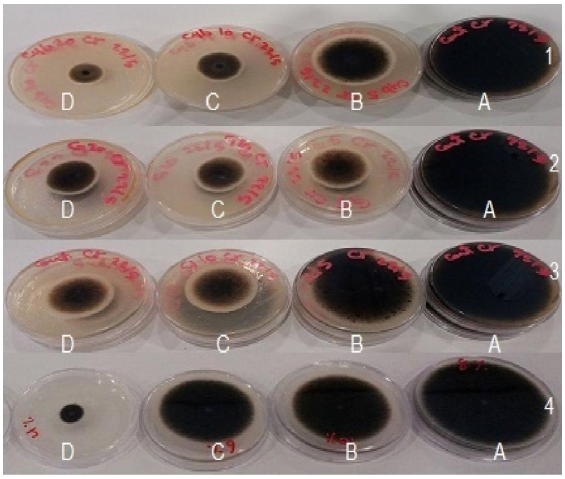

Fig. 3 Effect of 1, cinnamon oil; 2, cyperus oil; 3, *Alloe vera* extract; 4, *Adenium obesum* extract on *A. alternata.* A, control; B, 0.5 µL/ml oil or 5 mg/ml plant extract; C, 1.5 µL/ml oil or 10 mg/ml plant extract; D, 2.5 µL/ml oil or 20 mg/ml plant extract

Table 1. Effect of different concentrations of essential oils and plant extracts on fungal growth

Treatment	Concentration	*Fusarium culmorum*		*Alternaria alternata*	
		Colony radius (cm)	Growth inhibition%	Colony radius (cm)	Growth inhibition%
Control	0.0	4.3±0.08	0.00	8.2±0.12	0.00
Cinnamon oil	0.5 µL/ml	2.5±0.14	41.86	4.0±0.15	51.21
	1.5 µL/ml	2.4±0.15	44.19	3.2±0.09	60.97
	2.5 µL/ml	1.2±0.17	72.09	1.5±0.13	81.70
Cyperus oil	0.5 µL/ml	2.2±0.19	48.84	3.2±0.20	60.97
	1.5 µL/ml	2.2±0.25	48.84	2.3±0.15	71.95
	2.5 µL/ml	2.0±0.12	53.49	2.1±0.26	74.39
Aloe vera	5.0 mg/ml	2.2±0.50	48.84	6.0±0.17	26.83
	10.0 mg/ml	2.2±0.15	48.84	4.2±0.19	48.78
	20.0 mg/ml	1.1±0.20	74.42	3.2±0.22	60.98
Adenium obesum	5.0 mg/ml	3.0±0.19	30.23	5.8±0.12	29.26
	10.0 mg/ml	2.8±0.29	34.88	5.7±0.50	30.48
	20.0 mg/ml	2.6±0.21	39.53	1.3±0.21	84.15

Table 2. Effect of different concentrations of cinnamon and cyperus oils on mycotoxins production

Oil treatment	Conc. µL/ml	ZER		DON		T-2	
		Concentration µg/kg	Inhibition %	Concentration µg/kg	Inhibition %	Concentration µg/kg	Inhibition %
Control	0.0	1083.43±1.53	0.00	77.03±0.06	0.00	497.67±0.58	0.00
Cinnamon oil	0.5	698.03±1.15	35.58	73.57±0.40	4.50	308.33±1.15	38.05
	1.5	753.90±1.73	30.42	60.17±0.58	21.89	284.50±1.73	42.83
	2.5	787.40±0.35	27.32	79.03±1.15	0.00	315.83±0.64	36.54
Cyperus oil	0.5	1100.43±0.58	0.00	76.93±0.81	0.13	386.27±0.58	22.38
	1.5	1058.63±0.46	2.29	221.33±1.15	0.00	371.53±1.15	25.35
	2.5	1050.73±0.58	3.01	233.90±1.73	0.00	370.43±0.58	25.57

Table 3. Effect of different concentrations of *Alloe vera* and *Adenium obesum* extracts on mycotoxins production

Plant extract Treatment		ZER		DON		T-2	
		Concentration µg/kg	Inhibition %	Concentration µg/kg	Inhibition %	Concentration µg/kg	Inhibition %
Control	Conc. µL/ml	1083.43±1.53	0.00	77.03±0.06	0.00	497.67±0.58	0.00
Alloe vera	5.0 mg/ml	897.60±0.90	17.15	236.30±0.04	0.00	315.05±1.15	36.69
	10.0 mg/ml	907.20±0.72	16.23	302.32±0.03	0.00	320.04±0.04	35.70
	20.0 mg/ml	984.51±1.20	9.13	270.20±1.15	0.00	333.41±0.58	33.01
Adenium obesum	5.0 mg/ml	811.63±0.50	25.05	43.92±0.90	42.98	325.92±1.15	34.51
	10.0 mg/ml	800.01±0.04	26.13	55.30±0.12	28.20	315.70±0.15	36.56
	20.0 mg/ml	659.02±0.92	39.15	50.61±0.40	34.30	273.70±0.58	45.00

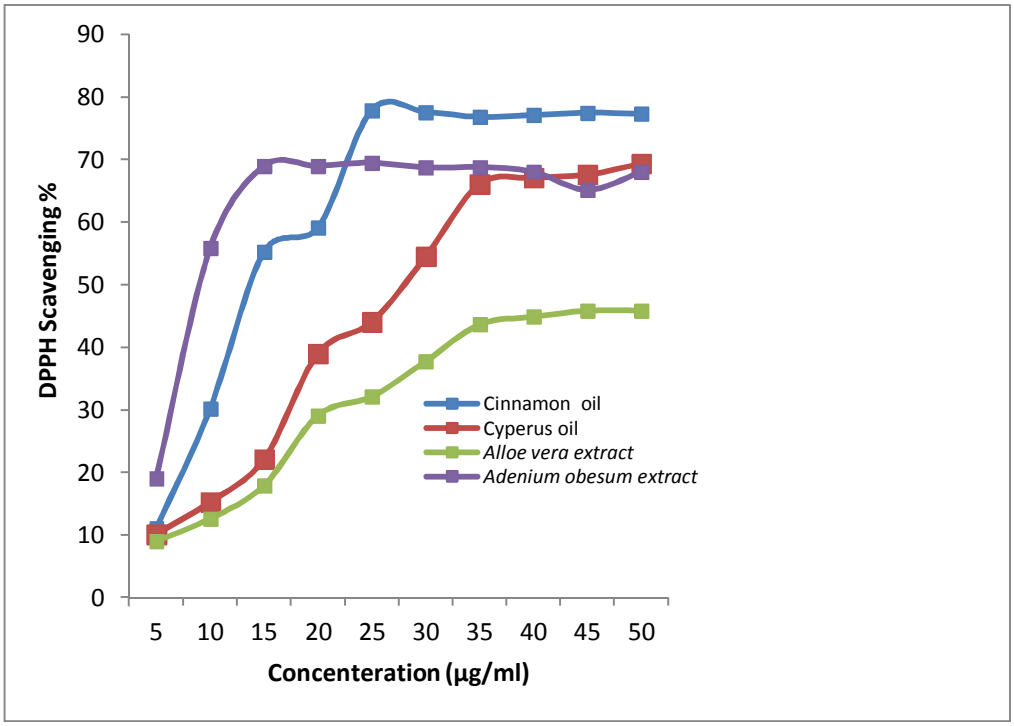

Fig.4 **Antioxidant activity *plant* extracts and essential oils using DPPH Scavenging**

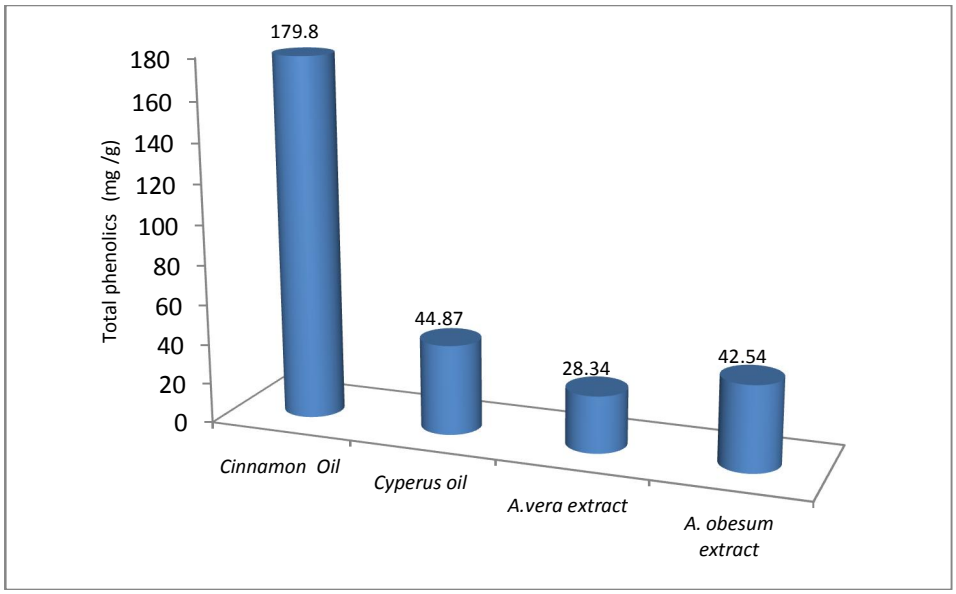

Fig.5 Total phenolics of plant extract and essential oils

Table 4. Biological control of mycotoxins production with using *Saccharomyces cerevisiae* and *Trichoderma harzianum*

Treatment		ZER		DON		T-2	
		Concentration µg/kg	Inhibition %	Concentration µg/kg	Inhibition %	Concentration µg/kg	Inhibition %
Control		1083.43±1.53	0.00	77.03±0.06	0.0	497.67±0.58	0.0
S. cerevisiae	cell1x10	659.90±0.54	39.09	42.92±1.15	44.28	294.26±1.15	40.87
	cell2x10	1126.72±1.15	0.00	144.23±0.08	0.00	498.06±0.09	0.00
T. harzianum	spore 1x10	811.91±0.50	25.06	51.60±0.52	33.01	246.05±0.06	50.55
	spore2x10	1002.90±1.20	7.43	54.40±0.50	29.38	302.62±1.53	39.28

Conclusion

Research data indicates that essential oils and plant extracts inhibit the growth of mycotoxin-producing fungi. Further studies of the extraction, isolation, identification of bioactive compounds and further in vivo studies are needed for the better understanding of the mechanisms of action.

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To Regional Center of Mycology and Biotechnology, Al Azhar University, Cairo, Egypt.

Corresponding Author:
Prof. Tarek M. Abdelghany
E-mail: tabdelghany@yahoo.com

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