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Effect of Some Plant Extracts on the Growth and Mycotoxin Production of Three Toxigenic *Aspergillus* Strains Isolated from Raw Meat

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ABSTRACT

The objective aim of this investigation was to compare the inhibitory effects of different concentrations of five plant extracts on three toxigenic *Aspergillus* strains isolated from raw meat samples. These extracts were used in primary screening tests to assess their control ability on growth inhibition of *A. flavus* Fak 268, *A. niger* Zag 28 and *A. terreus* Fak76 growth and production of aflatoxin B1 (AFB1), ochratoxin A (OTA) and citrinin (CTN). The strongest antifungal effect was exerted by cinnamon extract at 200mg ml^{-1} for *A. terreus* and 300 mg ml^{-1} for *A. niger* and *A. flavus*. The second effective plant extract was ginger extract where, complete inhibition of growth and toxin production were achieved at 300 mg ml^{-1} inrelation to the all tested strains. Cinnamon extract was applied as the most effective agent to determine its efficiency on AFB1, OTA and CTN production on meat substrate. The most sensitive strain to cinnamon extract was *A. terreus* with total inhibition of growth and production of toxin at 20 mg Kg^{-1}.The obtained results revealed that all used plant extracts showed positive antifungal activity in a varied concentrations. These extracts can be applied in preservation of meat for longer periods against fungal contamination and food intoxication by mycotoxigenic fungi.

Keywords: Mycotoxins, Meat, Food additives, Aflatoxin B1 (AFB1), Ochratoxin A (OTA), Citrinin (CTN).

1. Introduction

Contamination of meat occurs when raw meat is exposed to pathogenic microbes during slaughtering, handling, transportation and storage (Chambers and Grandin, 2001).Various molds were determined in raw meat (Jay *et al.,* 2005) and are capable of producing mycotoxins which cause food poisoning (Mossel, 1982).These mycotoxins are secondary metabolites produced by filamentous fungi that have deleterious effects on human and animal consumers. They are structurally diverse, deriving from a number of biosynthetic pathways and their effect upon consumers is ranging from acutely toxic to immunosuppressive or carcinogenic (Nicholson and Centre, 2004). Mycotoxins which have possible significance to meats and meat products include aflatoxins, ochratoxins, cyclopiazonic acid, trichothecenes, zearalenone, and fumonisins (Pestka, 1986). Some of these mycotoxins are stable and may resist quite severe processes like roasting, cooking or processing (Marin, 2013). Because mycotoxin contamination is unavoidable, numerous strategies for their detoxification have been proposed by the removal or elimination of the contaminated commodities or by inactivation of mycotoxins present in these commodities (Kabak *et al.,* 2006). In recent years, consumers' preferences are moving towards foods that contain lower levels of chemical preservatives and exhibit more fresh-like and natural characteristics. Numerous studies have demonstrated that plant extracts contain diverse bioactive components that can control mold growth (Cabral *et al.,* 2013). Garlic and onion have been extensively studied for their antimicrobial properties. Aqueous extracts of garlic and various extracts of onion inhibit the growth of many molds such as *Aspergillus flavus* and *Aspergillus parasiticus* (Beuchat, 1994). Aqueous and organic extracts of liquorice were investigated for antioxidant and antifungal

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activities against four pathogens, *A. alternata, A. niger, F. solani, F. oxysporum* (Ahmed *et al.,* 2014). Ginger was reported to have medicinal properties like antimicrobial, antifungal, antiviral, antioxidant, anti-inflammatory, and anticancer (Bartley and Jacobs, 2000). The antifungal activity of cinnamon extract was reported against *A.niger*, *A. flavus, F. moniliforme, F. graminearum, Fusarium spp, P. citrinum and P. viridicatum* (Singh *et al.,* 2007;Velluti *et al.,* 2004).The aim of this work was to investigate the antifungal effects of garlic extract, onion extract, liquorice extract, cinnamon extract and garlic extract on three *Aspergillus* strains. These strains are *A. flavus* Fak268, *A. niger* Zag28 and *A.terreus* Fak76. The plant extracts were tested to determine their potential impact on fungal growth and mycotoxins production of these strains that have been isolated from raw meat either in culture medium or meat substrate as a food model.

2. Materials and Methods

2.1. Effect of plant extracts on fungal growth and mycotoxin production.

2.1.1. Fungal strains

A. flavus Fak268, *A. niger* Zag28 and *A. terreus* Fak76 were locally isolated from fresh meat samples. These fungal strains were selected among other isolated fungi on the basis of their highest producing ability to the mycotoxins AFB1, OTA and CTN. These strains were previously identified according to the macro and microscopic features of the recovered fungal isolates according to (Moubasher, 1993). The strains were molecularly identified based on the sequence of PCR-amplified 18S rRNA–28S rRNA gene analysis. Sequence homologous to that of isolated fungus was identified in the NCBI GenBank database using BLASTP and BLASTX programs (Altschul *et al.,* 1997). Culture was maintained on slants of potato dextrose agar (PDA).

2.1.2. Inoculum preparation

Fungal spores from 7-days old cultures were of *A. flavus* Fak268, *A. niger* Zag28 and *A. terreus* Fak76 growth were harvested separately by flooding of the slants with sterile distilled water containing 0.1 % Tween 20 and gently scrapping off the spores with a sterile glass rod. The spore concentration was adjusted to 2×10^6 spores ml^{-1} using hemocytometer. Two ml of this spore suspension was used as a standard inoculum.

2.2. Extraction procedures:

a) Preparation of aqueous plant extracts.

Five traditional plant species used in this study were garlic (bulbs), onion (bulbs), Liquorice (roots), ginger (rhizome) and cinnamon (bark). These species were chosen based on their reported antifungal activities.

Extracts were prepared from fresh or dried plants parts according to methods described by (Silva *et al.,* 1995). The plant parts of each species were thoroughly washed in running tap water, surface disinfected in 90% ethanol for 2 min, then grinding using sterile blender. Plant samples from each species were extracted by weighing 100g of finely ground plant material and extracting with 100 ml methanol in 250 ml flasks, the mixtures were well shaken for 20 min. then, filtered using preweighted using whatman No1 filter paper. The solvent was removed under a stream of air at room temperature, and the plant material on filter paper was dried at 80 $^{\circ}$C until constant weight produced weight after dryness were measured. Plant extract weight can be calculated by subtracting the initial plant weight used from the plant dry weight as follows:

Plant extract weight= Initial plant weight- plant dry weight.

Each resulted plant extract was dissolved into 100 ml sterile distilled water bottles and different concentrations of plant extracts were prepared for testing their effect on fungal growth and mycotoxins production.

b) Determination of antifungal activity of aqueous plant extracts in culture media

The following concentrations of each aqueous plant extract: 100mg ml^{-1}, 200mg ml^{-1}, 300mg ml^{-1}, 400mg ml^{-1}, 500mg ml^{-1} were added to (YES) broth medium in 100ml flasks. All flasks were sterilized and inoculated with 2ml spore suspension each tested fungus then incubated at 28° C for 10 days. The content of each flask was filtered through Whatman filter paper No. 1 and the filtrate was extracted with

20 ml chloroform in a separating funnel. The chloroform extract was evaporated on water bath at 70 °C and re-dissolved in 0.25 ml chloroform. The efficiency of food preservatives in inhibition of AFB1, OTA and CTN production was determined by TLC method.

2.3. Cultivation conditions

The basal medium used to monitor the growth of the tested strains was YES broth. Fifty ml of YES broth was introduced into a 250 ml Erlenmeyer flask and autoclaved at 121°C for 20 min. After cooling, the flasks were treated with different concentrations of plant extracts as an antifungal agent. The control flasks were prepared using sterilized YES broth and inoculated with 2 ml spore suspension of each tested fungal strain. All flasks were then incubated 28°C for 10 days.

2.4. Analytical methods

2.4.1. Determination of mycelial dry weights

At the end of the incubation period, the fungal culture flasks of *A. flavus* Fak268, *A. niger* Zag28 and *A. terreus* Fak76 were filtered through preweighted Whatman no.1 filter papers and weighed after drying to constant weight at 80 °C.

2.4.2. Extraction of AFB1, OTA and CTN

The filtrate of each flask was extracted with an equal volume chloroform in a separating funnel. The chloroform extract was evaporated on water bath at 70°C and redissolved in 1 ml chloroform. The efficiency of food preservatives in inhibition of AFB1, OTA and CTN production was determined by thin layer chromatography (TLC) in which the concentrated extract and reference standards were loaded on TLC plates with silica gel GF-254.The mycotoxins standards were obtained from SigmaAldrich, Taufkirchen, Germany. For CTN, the TLC plate was impregnated in an 8% solution of oxalic acid in methanol (El-Shanawany *et al.,* 2005). Toluene: 90% ethyl acetate: formic acid (6:3:1, v/v/v) was used as eluent to develop the TLC plates. The mycotoxins (spots and standards) were visualized in UV light at 254 and 366 nm. The detected mycotoxins were then quantified spectrophotometrically, as described below.

2.4.3. Determination of AFB1

AFB1 spots (Rf = 0.5) showed blue in fluorescence under short and long-wave length (254 and 366 nm) and corresponding to the standard were scrapped off and eluted with methanol. AFB1 was then quantified by ultraviolet spectroscopic analyses performed with a Hermle Z230A UV spectrophotometer.AFB1 absorption was monitored at 363 nm, and the concentration was obtained after recording the optical density against a standard curve (Ismaiel and Tharwat, 2014).

2.4.4. Determination of OTA

OTA spots (Rf = 0.61) showed greenish-blue fluorescence under long-wave length. These spots of both sample and standard were scrapped off, eluted with methanol and the absorption was monitored at 356 nm **(Nesheim, 1976)**. The concentration was then obtained from a standard curve.

2.4.5. Determination of CTN

CTN spots (RF=0.42) showed lemon yellow CTN in fluorescence under short and long-wave length, After spots were scraped and eluted in methanol for centrifugation, the optical density of the supernatant determined by UV visible spectrophotometer at wave length of 366 nm. The concentration was then obtained from a standard curve (El-Shanawany *et al.,* 2005).

2.6. Effect of cinnamon extract on fungal growth and mycotoxin production in meat substrate

Ten grams of fresh meat were surface sterilized by hypochlorite for 2 min. and washed 5 times with sterilized distilled water. Each meat sample was transferred into sterile flask containing 8 ml distilled water then, the following concentrations of cinnamon extract were added separately: 5mg ml^{-1}, 10mg ml^{-1}, 15mg ml^{-1}, 20 mg ml^{-1}, 25 mg ml^{-1}.Triplicate flasks were done for each concentration, and each flask was inoculated with the spore inoculum of each tested fungi (*A. flavus* Fak 268, *A. niger* Zag28 and *A. terreus* Fak76) and incubated at 28°c for 7 days. The filtrate was separated from the solid residue using whatman No1 filter paper. The filtrates were extracted with chloroform and separated on

TLC plates (as mentioned earlier). AFB1, OTA and CTN spots revealed under ultraviolet light using (Min, UVIS, DUOUV. Then scrapped off, eluted, and measured as mentioned previously.

2.7. Statistical analysis

Results were expressed as the mean ± standard deviation (SD). Statistical significance was evaluated using analysis of variance (ANOVA, SPSS software version 22) test followed by the least significant difference (LSD) test at 0.01 level.

3. Results and Discussion

3.1. Effect of garlic extract on fungal growth and mycotoxins production.

Different concentrations of garlic extracts were tested for their antifungal activity against *A. flavus, A. niger* and *A. terreus,* AFB1, OTA and CTN production respectively, results revealed that, garlic extract was an effective inhibitor of all tested molds growth and mycotoxins production. Results revealed a great reduction in toxin production by increasing garlic extract concentrations. The reduction of AFB1, OTA and CTN in was increased by increasing garlic extract concentration. The total inhibition of growth and toxin for *A. flavus* and *A. terreus* were achieved at 400 mg ml^{-1} and at 500 mg ml^{-1} for *A. niger*. Significant differences ($P \leq 0.01$) were achieved at 100 mg ml^{-1} concentration of garlic extract for AFB1and CTN concentrations. In case of OTA significant difference ($P \leq 0.01$) was achieved only at 300 and 400 mg ml^{-1} concentrations of garlic extract. The inhibitory activity of *Allium* vegetables extracts against mold have been reported by numerous authors. It was observed that alliicin, thiosulfonates and other compounds show fungistatic activities against *Aspergillus niger*, *Rhodotorula nigricans*, *Penicillium italicum*, *Penicillium cyclopium*, *Aspergillus flavus, Cladosporium macrocarpum, Aspergillus fumigatus, Aspergillus alutaceus, Aspergillus terreus* and *Penicillium chryogenum* (Wei et al., 1967; Graham and Graham, 1986; Topal, 1989; Hafez and Said, 1997; Ankri and Mirelman, 1999; Harris *et al.,* 2001). Yin and Tsao (1999) studied antifungal effects of various *Allium* plants, they observed that garlic shows the highest antifungal activity against *Aspergillus niger*, *A.flavus* and *A. fumigatus*. The antifungal activity of garlic is in agreement with results of who found its extracts very effective in inhibiting the growth of *Aspergillus* species (Darket *et al.,* 1979).

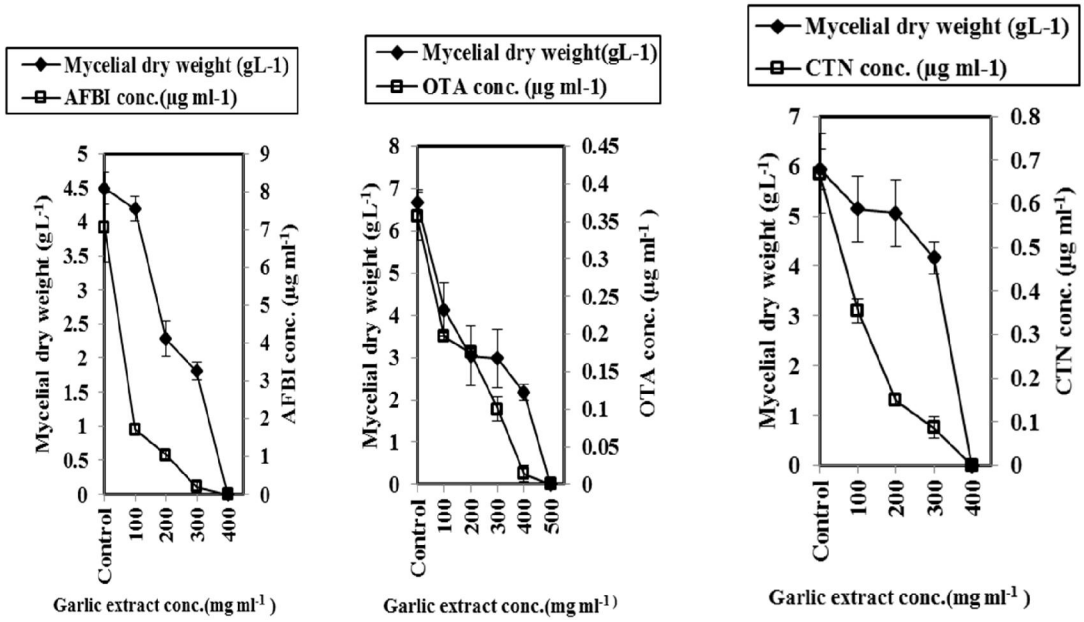

Fig. 1: Effect of garlic extract on fungal growth and mycotoxins production.

3.2. Effect of onion extract on fungal growth and mycotoxins production

Onion extract exhibited a great reduction in AFB1 and OTA production where, initial concentration of onion extract (100 mg ml^{-1}) caused a great reduction in the AFB1, OTA production as compared with control culture, while low concentrations of onion extracts represented weak effect on *A. flavus, A. niger and A. terreus* growth. The total inhibition of growth and toxin was achieved at 500mg ml^{-1} for three tested strains. Significant differences (P≤0.01) were achieved at 300 mg ml^{-1} concentration of onion extract for mycelial dry weight (gL^{-1}) of *A. flavus.* Also significant differences (P≤0.01) were achieved for all CTN concentrations at all concentrations of onion extract. There have been a number of reports citing the inhibitory effects of onion extracts on *A. flavus* growth, with an ether extract of onions, thio-propanol-S-oxide, being demonstrated to inhibit growth. In addition, Fan and Chen (1999) reported that welsh onion ethanol extracts depressed the mycelial growth and aflatoxin production of some strains of aflatoxin-producing fungi (Fan, J.J. and Chen, J.H. 1999). Yin and Tsao (1999) studied the antitoxigenic potential of some spices against OTA-producing strain of *A. ochraceus*. Clove completely inhibited the mycelial growth of the fungi *A. ochraceus*. *A. niger*, *P. italicum*, *Tryptophyton gypseum* and *Microsporon audouini* are inhibited by thiosulfonates compounds in onions. Presence of flavonoids from the onion extract was reported by Palomar *et al.,* (2004), these flavonoids inhibit the growth of microorganisms.

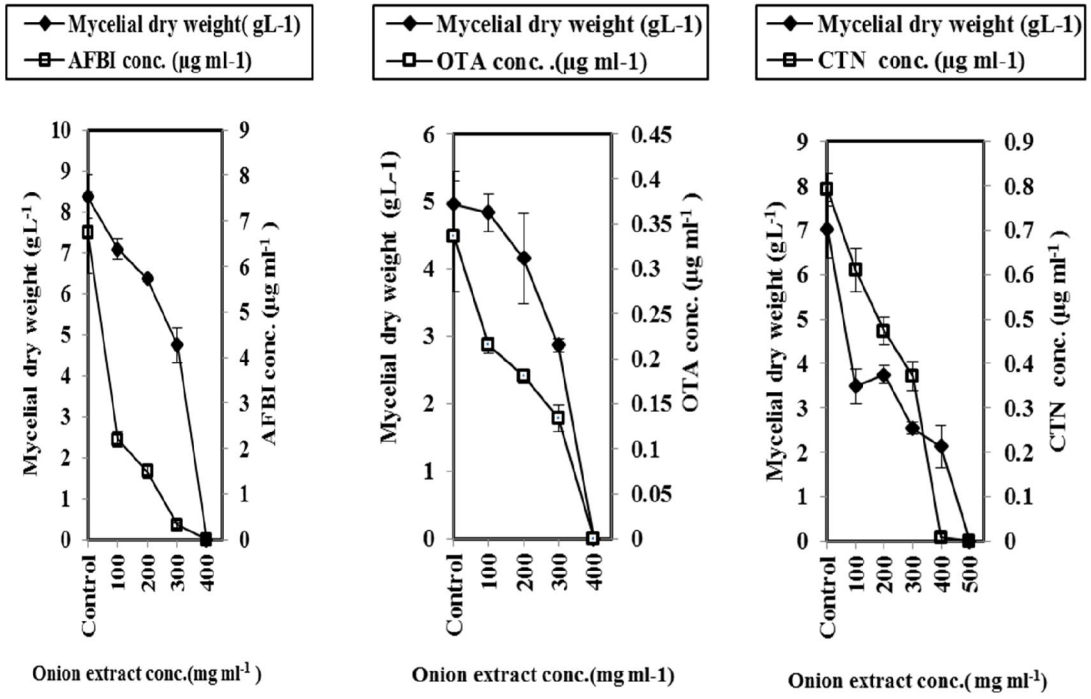

Fig. 2: Effect of onion extract on fungal growth and mycotoxins production.

3.3. Effect of liquorice extract on fungal growth and mycotoxins production

Results illustrated that *A. niger* was the most sensitive mold to liquorice extract concentrations. The total inhibition of *A. niger* growth and OTA production were achieved at 400mg ml^{-1} while *A. flavus* and *Aterreus* growth and toxin production were achieved at 500mg ml^{-1}. Significant differences (P≤0.01) were achieved at 400 mg ml^{-1} concentration of liquorice extract for *A. flavus* mycelial dry weights and AFB1concentrations.Also significant difference (P≤0.01) was achieved for OTA and CTN concentrations. Furthermore, significant difference (P≤0.01) was achieved for *A.flavus* and *A. niger* mycelial dry weights at 100 mg ml^{-1} concentrations of liquorice extrac. Ahmed *et al.,* (2014) studied the effect of aqueous and organic extracts of liquorice for antioxidant and antifungal activities against four pathogens, *A. alternata, A. niger, F. solani, F. oxysporum*. The strongest antifungal activity was

observed using the ethanolic extract against *F. oxysporum* while all the extracts had equal antifungal activity against *A. alternata* and *F. solani*. *A. niger* was more sensitive to methanolic extract than the aqueous and ethanolic extract.

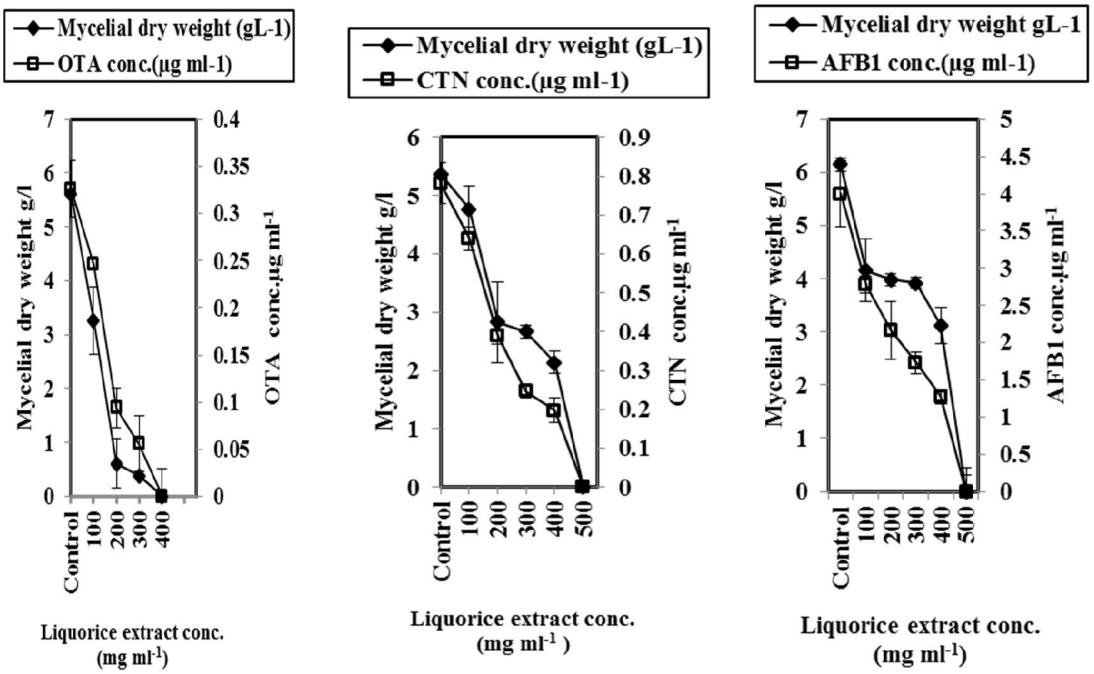

Fig. 3: Effect of liquorice extract on fungal growth and mycotoxins production

3.4. Effect of Cinnamon extract on the growth and mycotoxins production

Cinnamon extract was the most effective plant extract against the three tested strains achieved total inhibition of growth and toxin at 300mg ml^{-1} cinnamon extract for *A. flavus* and *A. niger*, and at 200 mg ml^{-1} cinnamon extract for *A. terreus* and CTN production. Significant differences ($P \leq 0.01$) were achieved at 50 mg ml^{-1} concentration of cinnamon extract for the three toxins. Moreover, Significant differences ($P \leq 0.01$) were achieved at 150 mg ml^{-1} concentration of cinnamon extract for mycelial dry weight of the three tested strains. The antifungal activity of cinnamon extract was reported against *A. niger*, *A. flavus, F. moniliforme, F. graminearum, Fusarium* spp*, P. citrinum and P. viridicatum* (Singh *et al.,* 2007; Velluti *et al.,* 2004). The antimicrobial activity of cinnamon is attributed to eugenol (2-methoxy-4-allyl phenol) and cinnamic aldehyde (Conner, 1993; Ouattara *et al.,* 1997; Zhou *et al.,* 2010). Cinnamic aldehyde (non-phenolic compound) inhibits mold growth and mycotoxin production (Beuchat, 1994; Ouattara *et al.,* 1997). Cinnamon and anis inhibited the synthesis of OTA starting from the concentration of 3% and mint starting from 4% (Pereira *et al.,* 2006).

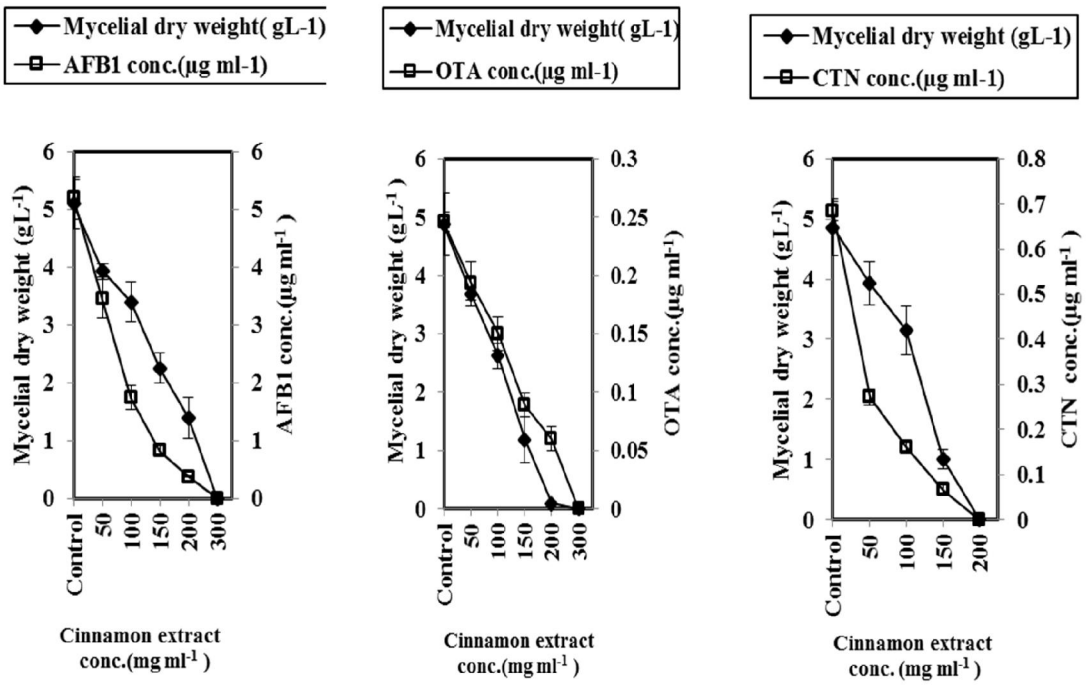

Fig. 4: Effect of Cinnamon extract on the growth and mycotoxins production

5-Effect of ginger extract on the growth and mycotoxins production.

Ginger extract was the second most effective plant extract. The total inhibition of fungal growth and toxins production for the three tested strains were achieved at 300 mg ml^{-1} ginger extract. Significant differences ($P \leq 0.01$) were achieved at 50 mg ml^{-1} ginger extract for the three toxins. Furthermore, significant differences ($p \leq 0.01$) were achieved at 200 mg ml^{-1} concentration of ginger extract for mycelial dry weight of the three tested strains represented. Ginger contains the compound caprylic acid, which has potent antifungal properties (Ernst and Pittler, 2000).The strong inhibition potential of ginger is attributed to fact that it contains over 400 different compounds a mixture of both volatile and non-volatile chemical constituents such zingerone, shogaols and gingerols, sesquiterpenoids (β-sesquiphellandrene, bisabolene and farnesene) and a small monoterpenoid fraction (β-phelladrene, cineol, and citral) (Chrubasik *et al.,* 2005). The efficacy of some local plants in the management of *A. flavus* and aflatoxin contamination was investigated. Aqueous extracts of rhizome of *ginger* could suppress both the growth and aflatoxin production by *A. flavus.* No positive correlation could be observed between mycelial growth and aflatoxin production by *A. flavus* (Reddy *et al.,* 2011). Gingerols were identified as the major active component in the fresh ginger rhizome. Ali *et al.,* (2008) indicating that it potential as an antifungal agent. The methanol fraction of *Zingiber officinalis* caused total inhibition of growth and aflatoxin production by *A. flavus* (Reddy *et al.,* 2011).

6-Effect of cinnamon extract on mycotoxins production on meat substrate by A.flavus, *A. niger* and *A. terreus*.

The efficiency cinnamon extract as the best plant extract on controlling growth and mycotoxins production of the three tested strains, was applied for determination its ability also to control mold growth and toxin production in meat substrate. The moisture content were adjusted at 8%. the total inhibition of *A.flavus* and *A. niger* growth and toxins were achieved at 0.25 g Kg^{-1} cinnamon extract while, the total inhibition for *A. terreus* growth and CTN production was achieved at 0.20 g Kg^{-1} cinnamon extract Significant differences ($P \leq 0.01$) were achieved at all concentrations of cinnamon extract for OTA and CTN concentrations. Moreover, significant differences in the three toxins concentrations were achieved at 0.15 mg ml^{-1} of cinnamon extract.

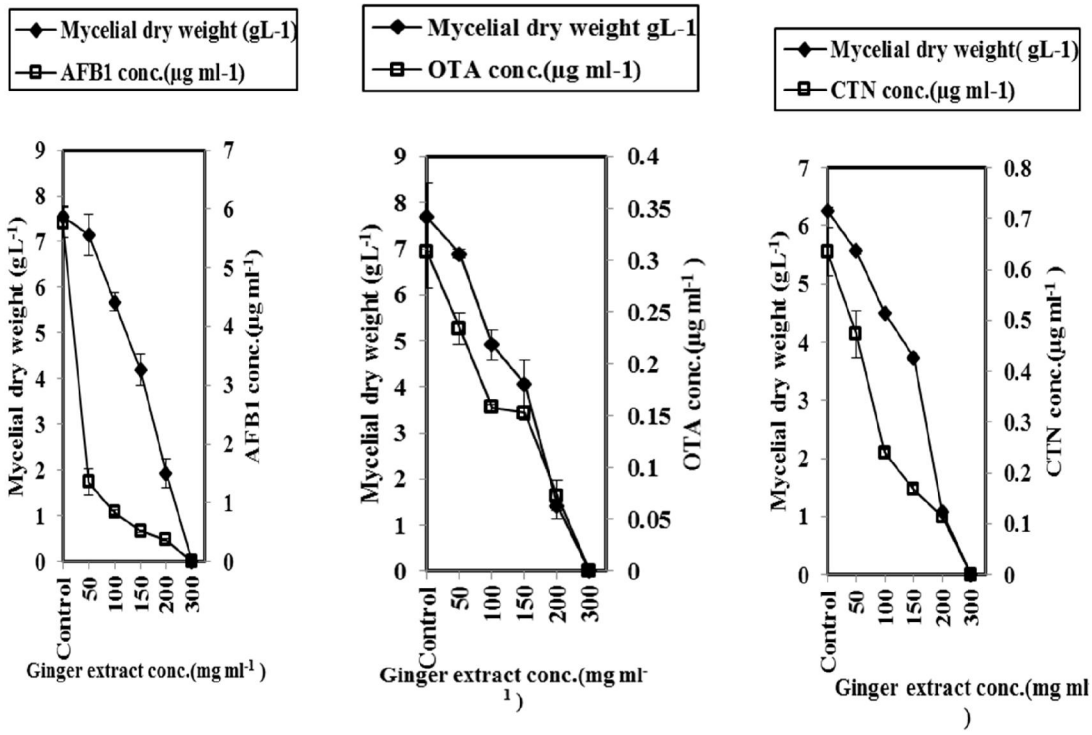

Fig. 5: Effect of ginger extract on the growth and AFB1 production by *A. flavus.*

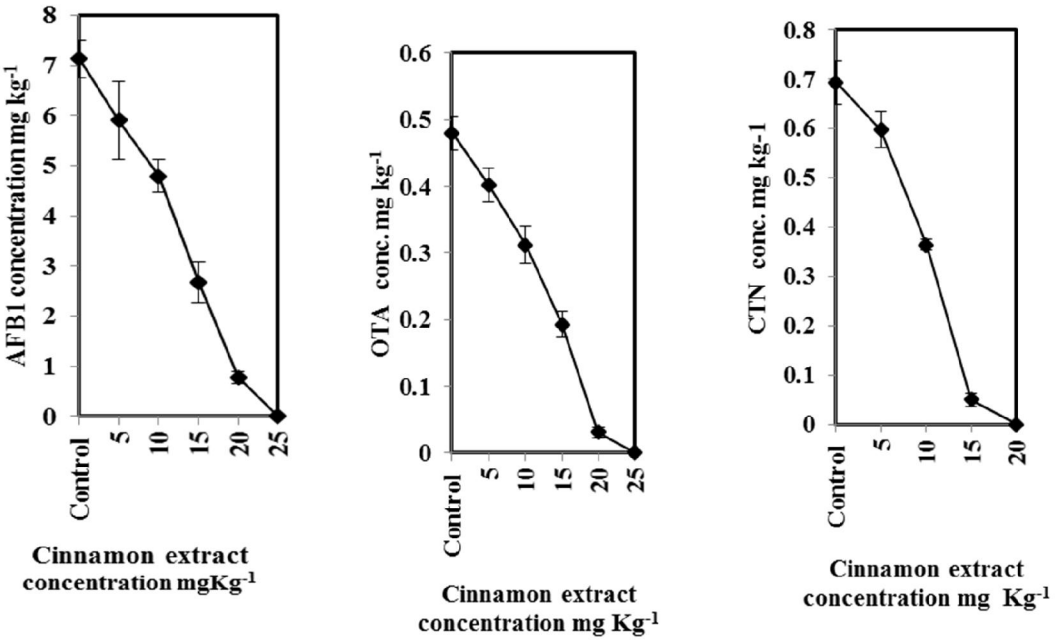

Fig. 6: Effect of cinnamon extract on mycotoxins production on meat substrate by *A. flavus*, *A. niger* and *A. terreus*

4. Conclusion

Results revealed that the five plant extracts possessed antifungal activities against the three tested strains, but at different concentrations, depending on the nature of the chemical used and the sensitivity of the tested strains to these chemicals. These extracts can be used in controlling infection, preventing cold meat spoilage and preserve meat for longer periods against fungal contamination.

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