

Full Length Research Paper

A new bioassay using *Chlorella vulgaris* cell density for detecting mycotoxins

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Bioassay is an alternative screening technique to evaluate the biotoxicity of the fungal secondary metabolites before any further chemical analyses. In this study, the cell density of *Chlorella vulgaris* cultures were used to detect the biotoxicity of 108 isolates belonging to seven fungal species. The crude extracts of all toxin producing fungal isolates that were tested inhibited the growth of *C. vulgaris* dramatically after 48 and 72 h as compared to the control. The crude extracts of the nontoxin producing fungal isolates that were tested showed that there was no significant inhibition of *C. vulgaris* at the three time intervals as compared to the control. These results corresponded to the *Bacillus subtilis* disk diffusion method. The use of optical density readings of the growth of *C. vulgaris* reflected a rapid, easy and effective tool to detect mycotoxins.

Key words: Bioassay, *Chlorella*, colorimetric, mycotoxins, pathogenicity.

INTRODUCTION

Mycotoxins are an extremely diverse group of biological compounds of secondary fungal metabolites with low molecular weight (mostly below 700 Da). The chemical structure and physical properties of these toxins are widely varied. When these toxins are ingested, inhaled or absorbed through the skin, they cause lowered performance, sickness or death in man or animals and birds (Van Egmond and Speijers, 1999).

Biological assay is the determination of the relative strength of a substance by comparing its effect on a test organism with that of a standard preparation. It has become increasingly useful for mycotoxins detection as a precursor to chemical analysis. Bioassay allows the analyst to make an informed decision when selecting a more detailed chemical analysis procedure. Various short-term *in vitro* biological assays have been employed to screen for the presence of several mycotoxins (Lompe and Milczewski, 1979; Coker, 1997). Problems encountered

with these bioassays include the maintenance of animals or cell lines and cultures, technically complex procedures requiring extensive preparation or assay times or both, expensive materials, and subjective data analyses (Blaise, 1991) also. Recent toxicology, in accordance with recommendations from the European Council, has demanded decrease in the number of vertebrates used in toxicology testing and their partial replacement with invertebrate animals, plants or even organ, tissue, or cell cultures (Petr Dvorak et al., 2012). During the last 50 years, various invertebrate species have been tested for their sensitivity to many chemical or physical agents to prove their possible use for pre-screening tests. Brine shrimp larvae have been used to evaluate fungal toxins (Harwig and Scott, 1971). The lower sensitivity of the *Artemia* species to several chemical or physical agents in comparison with the other invertebrate test organisms, the decreased solubility of some chemical substances in saline or sea

medium, in addition to the various conditions that control the test, such as temperature, pH, chemical composition of the medium, oxygen, photoperiod, nutrients, some population effects, type of growth stage, etc may affect the toxicity results (Nalecz-Jawecki et al., 2003; George-Ares et al., 2003; Mayorga et al., 2010). Algae are especially suitable for biotests because of their sensitivity to environmental pollution and their abundance in aquatic systems. In addition, they have no roots as higher plants and reflect only the properties of the ambient water rather than those of the soil higher plants that are rooted. Also, algal biotests are simple and allow observing multiple generations (Danilov and Ekelund, 2000). The agar diffusion technique of *Chlorella vulgaris*, *Ustilago maydis* and *Trichoderma viride* used by Bean et al. (1992) showed that *Chlorella* was the most sensitive organism toward macrocyclic trichothecens produced by different *Myrothecium* species tested. *Bacillus subtilis* and *C. vulgaris* were proved to be particularly sensitive to mycotoxins (Sukroongreeng et al., 1984).

This study aimed to develop a rapid, easy, sensitive and reliable bioassay technique using optical density readings of liquid cultures of *C. vulgaris* to detect mycotoxic compounds.

MATERIALS AND METHODES

Fungal isolates

One hundred and eight isolates of filamentous fungi isolated from the air of intensive care units and operation rooms as well as the dust of air conditioning system filters in each of the Assiut University hospitals were collected and identified in Assiut University Mycological Center. Multiple of six isolates of each *Aspergillus flavus*, *Aspergillus fumigatus*, *Aspergillus niger*, *Cladosporium cladosporioides*, *Fusarium solani*, *Fusarium oxysporum* and *Stachybotrys elegans* were tested.

Fungal crude extracts

Each isolate was grown on Czapek's glucose agar medium under aseptic conditions, incubated at 25±2°C for 10 days. After the incubation period, the entire agar media with fungal mycelia were cut into small pieces, transferred to a 250 ml Erlenmeyer flask containing 50 ml 96% methanol. The contents were shaken on a rotary shaker (200 rpm, 24 h) and filtered through filter paper (Whatman No.1). The residue was then washed twice with 96% methanol (25 ml each). The methanol extracts were combined, dried over anhydrous sodium sulphate, and then left to evaporate to near dryness under vacuum. The residues were transferred quantitatively to a dram vial with the minimal amount of methanol and evaporated to near dryness (Bean et al., 1992).

Thin layer chromatographic (TLC) determination of mycotoxins

For qualitative determination of mycotoxin produced by different fungal isolates tested, TLC technique adopted by El-kady and Moubasher (1982) was employed.

Biological assay procedure

Bacillus subtilis

The disc diffusion method (Sleigh and Timburg, 1981) was used to measure the antibacterial activities of different isolates crude extract on *B. subtilis* and the inhibition zone was measured.

Chlorella vulgaris

The strain of *C. vulgaris* used in this investigation was obtained from Laboratory of Microbiology, Department of Botany and Microbiology, University of Assiut. For growth and enrichment, Beijerinck medium was used (Stein, 1966). Algal cultures were grown at a temperature of 28 ±1°C in a light growth chamber (Forma Scientific, USA). The inoculums were maintained to be 0.123 O.D. 750 nm in all the cultures throughout the study period. Three replicates of 10 µl of the clean crude extract tubes and 6 ml of *Chlorella* culture was added to each tube under a septic conditions, optical density (O.D. 750 nm) of cultures was measured at required time intervals (after 24, 48 and 72 h) using spectrophotometer Thermo scientific, evolution 160 UV-Vis, double beam spectrophotometer, USA.

Data analysis

The results were analysed by one way analysis of variance (ANOVA) followed by Newman-Keuls Multiple Comparison Test as a post-test using computer prism program for windows, version 3.0 (Graph pad software, Inc, San Diego CA. USA). The significant difference between the tested groups was accepted at p<0.05, 0.01 or 0.001, the data were expressed as mean ± standard error (SE) and the number of isolates (n) was the multiples of six.

RESULTS AND DISCUSSION

The TLC analysis of the 108 crude extracts showed that only 78 of the fungal isolates that were tested had the ability to produce at least one of these mycotoxic compounds (Aflatoxins B₁, B₂, G₁, G₂ Gliotoxin, fumigillin, T₂, zearalenone, Roridins A and E, verrucarins A and J, Trichoveroids, Satratoxins H and E).

The crude extracts of all toxin producing fungal isolates that were tested according to the TLC analysis inhibited the growth of *C. vulgaris* dramatically after 48 and 72 h of which *F. oxysporum* and *S. elegans* were the fungal isolates that affected the growth of *C. vulgaris* the most as compared to the control (Figure 1B and C). Although, the growth of *C. vulgaris* was significantly affected after 24 h, the most fungal isolates were *A. fumigatus* and *F. oxysporum* (Figure 1A). On the other hand, the crude extracts of the thirty nontoxin producing fungal isolates that were tested showed no significant inhibition of *C. vulgaris* at the three time intervals used when compared with the control (Figure 1D, E and F). These results are in a agreement with those obtained by Bean et al. (1992) in which *C. vulgaris* proved to be the most sensitive organism to macrocyclic trichothecenes produced by *Myrothecium* species. Youssef et al. (2008) found that 14 out of 60 peanut seed samples that were tested inhibited the growth of *C. vulgaris*.

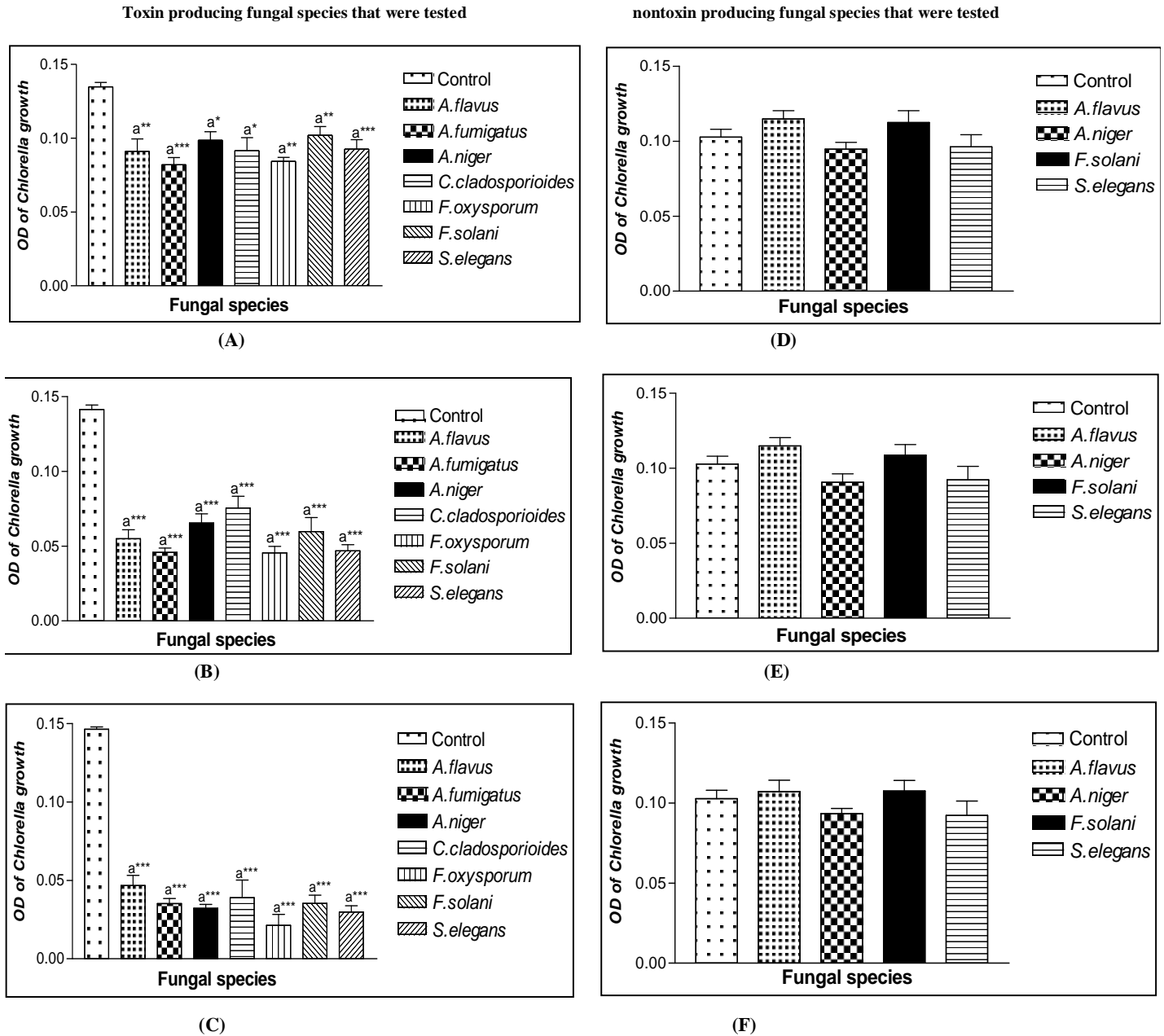


Figure 1. OD of *C. vulgaris* growth inhibited by the crude extracts of fungal isolates that were tested after 24 h (A and D), 48 h (B and E) and 72 h (C and F) of incubation. a= Significant difference between control group and fungal species. * = P< 0.05; ** = P< 0.01; *** = P< 0.001.

When the disk diffusion method (Sleigh and Timburg, 1981) was used to evaluate the antibacterial activity of the 78 fungal crude extracts of the toxic fungal, isolates on *B. subtilis* showed highly significant inhibition zone of which *S. elegans*, *A. fumigatus* and *A. flavus* were the most toxic isolates used (Figure 2). Several *Bacillus* species have been shown to be sensitive to other mycotoxins (Madhyastha et al., 1994). There was a great diversity in the response of the bacterial strains to fusaric acid; however, as a group, *Bacillus* species and *Paenibacillus macerans* were much more sensitive to fusaric acid than

Pseudomonas species (Landa et al., 2002). Relative insensitivity of aflatoxin B towards *B. subtilis* has been reported by Eka and Zo (1972). Reiss (1975) reported that the bioassay of patulin with *B. subtilis* spores can be recommended as a sensitive technique to supplement TLC identification. In addition, Aboul-Nasr and Abdul-Rahman (2013) found that Gram positive bacteria (*Bacillus cereus* and *B. subtilis*) especially *B. subtilis* showed the highest sensitivity towards 76 *Fusarium* isolates producing FB₁ and FB₁ plus FB₂ tested.

The results of *C. vulgaris* were confirmed by those of

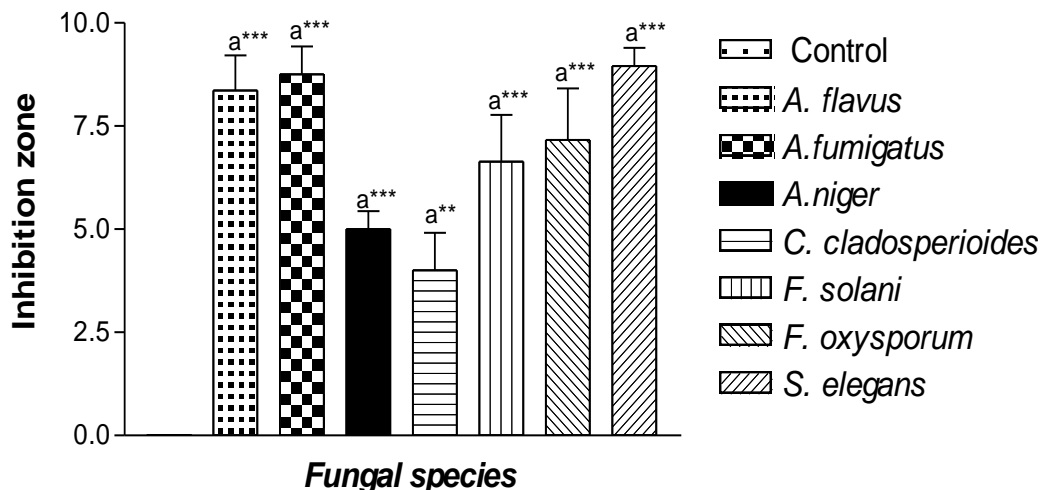


Figure 2. The inhibition zone of *B. subtilis* growth by toxin producing fungal isolates that were tested. A = Significant difference between control group and each fungal species group. * = $P < 0.05$; ** = $P < 0.01$; *** = $P < 0.001$.

bacteria by the proportional resemblance according to the method used either by the increase of inhibition zone in bacteria or the decrease of growth in *C. vulgaris*.

Conclusion

The results obtained by *C. vulgaris* procedure demonstrated a reliable short-term method for assessing the toxicity of mycotoxins and it can be performed easily.

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