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INHIBITION OF AFLATOXIN PRODUCTION BY *POLYMNIA SONCHIFOLIA* AND ITS *IN VITRO* CYTOTOXICITY

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ABSTRACT

Polymnia sonchifolia is used popularly in the control of diabetes mellitus and also should contain some antifungical and pesticidal compounds. Thus the ethanolic extract (EE) from *P. sonchifolia* leaves and its hexanic (HE), chloroformic (CL), ethyl acetate (EA) and methanolic (ME) fractions were tested to investigate the inhibitory activity on growth of *Aspergillus flavus* and on production of aflatoxin B1 (AFB1) and B2 (AFB2). The cytotoxicity of EE, EA and ME on Vero cells was also performed. Suspensions of *A. flavus* spores were inoculated into YES medium together with different concentrations of the EE, HE, CL, EA and ME. The aflatoxin B1 and B2 were extracted and analyzed by thin layer chromatography. All the concentrations of EE, EA and ME inhibited the aflatoxin B1 and B2 production and the fungal growth. The HE fraction inhibited the aflatoxin B1 production and the fungal growth at 100µg / mL, and all the concentrations used inhibited the aflatoxin B2 production. The CL fraction did not inhibit the aflatoxin B1 and B2 production and the fungal growth. *Polymnia sonchifolia* leaves have inhibitory activity in aflatoxin B1 and B2 production and that can be an important step for the development of agents to control aflatoxins production.

KEY WORDS: Aflatoxin, cytotoxicity, *Aspergillus flavus*, *Polymnia sonchifolia*.

RESUMO

INIBIÇÃO DA PRODUÇÃO DE AFLATOXINA POR *POLYMNIA SONCHIFOLIA* E SUA CITOTOXICIDADE *IN VITRO*. *Polymnia sonchifolia* é utilizada popularmente no controle da diabetes mellitus e, também, apresenta componentes com atividade antifungica e pesticida. O extrato etanólico (EE) das folhas e suas frações hexânica (HE), clorofórmica (CL), acetato de etila (EA) e metanólica (ME) foram testados para avaliar a atividade inibitória no crescimento e na produção de aflatoxinas B1 (AFB1) e B2 (AFB2) pelo fungo *Aspergillus flavus*. A citotoxicidade de EE, EA e ME foi avaliada em células Vero. Suspensões de esporos de *A. flavus* foram inoculados em meio YES juntamente com diferentes concentrações de EE, HE, CL, EA e ME. As aflatoxinas B1 e B2 foram extraídas e analisadas por cromatografia de camada delgada. Todas as concentrações de EE, EA e ME inibiram a produção de aflatoxinas B1 e B2. A fração HE inibiu a produção de aflatoxina B1 e o crescimento somente na concentração de 100µg / mL, enquanto que a produção de AFB2 foi inibida por todas as concentrações. A fração CL não produziu efeito sobre *A. flavus* nas concentrações testadas. As folhas de *Polymnia sonchifolia* possuem substâncias com capacidade de inibir a produção de aflatoxinas B1 e B2, podendo ser utilizada para o desenvolvimento de métodos de controle da produção de aflatoxinas.

PALAVRAS-CHAVE: Aflatoxinas, citotoxicidade, *Aspergillus flavus*, *Polymnia sonchifolia*.

INTRODUCTION

Aflatoxins are a group of mycotoxins produced by strains of the fungus *Aspergillus flavus* and *A. parasiticus*, and these species have been reported to contaminate a variety of foods and feeds (ANSARI & SHRIVASTAVA, 1991). The interest in aflatoxins has focused on aflatoxin B1, primarily due to its extreme acute and chronic toxicity and its carcinogenic activity in animal, in addition to its potential effects in human (COULOMBE, 1991). Previous studies have shown that the biosynthesis of aflatoxin B1 can be inhibited by a number of compounds (DUTTON & ANDERSON, 1980), and extracts of certain plants are toxic to fungi and may be useful in controlling the fungal growth and mycotoxin production (STEINHART et al., 1996). Plant extracts, such as those from garlic and onion, effectively retard growth and aflatoxin production (FAN & CHEN,

1999). Natural compounds, such as flavonoids, biflavonoids, essential oils and others, are also active in aflatoxin inhibition (BULLERMAN et al., 1977; SOBOLEV et al., 1995; MALLOZZI et al., 1996; NORTON, 1999; GONÇALEZ et al., 2001). There is increasing interest in antifungical agents for growth control of mycotoxin producing strains, however, some of the agents have toxic residue problems (COULOMBE, 1991).

Polymnia sonchifolia, called "yacon" in Brazil, is used popularly on the control of diabetes mellitus. The hypoglycemic effect of the aqueous extracts of its leaves was demonstrated in rats (AYBAR et al., 2001). Its leaves are highly pest-resistant, because that plant produce antifungical compounds (INOUE et al., 1995). PINTO et al. (2001) showed the inhibitory activity of aflatoxin B1 and B2 production of aqueous extract from *P. sonchifolia* leaves. This paper describes the inhibitory activity of the ethanolic extract from leaves of *P. sonchifolia* and its fractions hexanic (HE), chloroformic (CL), ethyl acetate (EA) and methanolic (ME), against growth of *Aspergillus flavus*, production of aflatoxins B1 and B2, and cytotoxicity in Vero cells.

MATERIALS AND METHODS

Preparation of plant extract and of the fractions

Leaves of *P. sonchifolia* were collected in a crop field located in Capão Bonito city, São Paulo State, Brazil. The dried (40° C) and powdered leaves (725 g) were submitted to three extractions with ethanol (98%) at room temperature. Solvent was filtered and evaporated under vacum to yield 69 g of a dry residue (EE). The EE was separated into 4 fractions by CC on silica gel 60 (Merck, Germany) using first hexane, $CHCl_3$, AcOEt and MeOH respectively yielding 7,8 g of hexanic (HE), 25,5 g of chlroformic (CL), 13,2 g of etyl acetate (EA) and 19,7 g of methanolic (ME) fractions.

Culture conditions

Aspergillus flavus IMI 190 (International Mycology Institute, London) was grown on potato dextrose agar (Difco Laboratories, Detroit, Mich) plates for 10 days at 25° C until well sporulated. The spore suspension used as inoculum was prepared washing culture with sterile 0.01% Tween 80 (Merck, Germany). Spore counts were conducted using a Neubauer Chamber (MALLOZZI et al., 1996).

Growth of *Aspergillus flavus* and aflatoxins production

A. flavus was used to evaluate the fungal growth and the aflatoxins B1 and B2 inhibition. The semi-synthetic YES culture medium was used for aflatoxin production (DAVIS et al., 1966). The method here used was previously described in the literature (PINTO et al., 2001). Briefly, suspensions of *A. flavus* spores (1.3×10^5 spore / mL) were inoculated into 50 mL of YES medium at different concentrations of EE, 0 (control), 50, 100, 150, 200, 250, 300 and 350 µg / mL and 0 (control), 25, 50, 75 and 100 mg / mL for HE, CL, EA and ME. Sterile 0.01% Tween 80 (Merck, Germany) was used like vehicle in the control cultures. Eight replicates were performed for each dilution of the EE, HE, CL, EA and ME. The extraction of aflatoxins were done by adding chloroform to the culture medium, shaking and separating of chloroformic phase. Extracts were analyzed by thin layer chromatography against aflatoxin B1 and B2 standard. Aflatoxins quantification were done by photodensitometry (Shimadzu, CS9000) of the spots.

Cytotoxicity assay

African green monkey cells (VERO) (BIOVET laboratories, Brazil) were grown in minimal essential medium (MEM) supplemented with 10% of fetal calf serum (FCS) for use in cytotoxicity assay (FERNANDES & SIMONI, 1995).

Ethanolic extracts of *P. sonchyfolia* (EE) and its fractions (EA and ME) were dissolved in 1 mL of ethanol, diluted to final concentration of 2 mg / mL in 1:1 distilled water and MEM and filtered through 0.22 µm Millipore membranes.

The method used for the cytotoxicity test was reported in the literature (ITAGAKI et al., 1991). Briefly, using a 96-well tissue culture microplate containing 0.1 mL of MEM into each well (column 1 to 12) was inoculated 0.1 mL of extract into column 1. Extract and fractions were two-fold serial diluted until column 6. The wells from columns 1 to 8 were seeded with 0.1 mL of MEM containing 3.0×10^4 cells. Two-fold serial cell dilutions were made from columns 8 to 11, and the microplate was incubated for 72 h at 37° C in 5% CO_2 atmosphere. The medium was then removed and cells were fixed and stained with 0.1 mL of 0.4% crystal violet in methanol for 30 min. Absorbance at 595nm was measured by an automatic microplate reader (BIO-RAD, 3550 UV). The absorbance of the control wells, which contained no test material, was regarded as 100%, and the percentage absorbance for each well was calculated. The concentration at which the growth of cells was inhibited to 50% of the control (IC_{50}) was obtained from the dose-response curves. The experiment was repeated three times.

Statistical analysis

The statistical analysis was performed using one way analysis of variance (ANOVA) and Tukey-Kramer multiple comparisons test with significance level $p < 0.05$ and $q > 4.457$. The correlation coefficient was also calculated.

RESULTS AND DISCUSSION

Effects on aflatoxin B1 and B2 production and fungus growth after using the EE, HE, EA and ME compared with the control are shown in Figures 1, 2, 3, 4 and 5.

The concentrations of EE 50, 100, 150, 200, 250, 300 and 350 μg / mL reduced the aflatoxins B1 and B2 at 64%, 83%, 68%, 79%, 84%, 62%, 61% and 87%, 98%, 98%, 96%, 98%, 90%, 83% respectively. The EE did not inhibited the AFB1 and AFB2 production in a concentration-dependent manner (Figs. 1 and 2). The inhibition of aflatoxin B1 and B2 production by EA and ME fractions were concentration-dependent (Figs. 4 and 5). The EA and ME concentrations 25, 50 75 and 100 μg / mL reduced AFB1 prodution at 20%, 35%, 60%, 78% and 33%, 49%, 75%, 87% respectively. AFB2 prodution was also reduced for EA and ME at 55%, 70%, 83%, 95% and 74%, 75%, 88%, 97% respectively. These inhibition were significant as measured by the ANOVA test, showing significance at $p < 0.05$. The Tukey-Kramer test, comparing the control with all the concentrations, showed $p < 0.05$ and $q > 4.457$, proving the inhibitory effect caused by EE, EA and ME.

Mycelial dry weight was used to compare fungal growth in treated and untreated cultures. EE inhibited the fungus growth only at 350 μg / mL concentration ($p < 0.05$) (Fig. 1). The percentage of inhibition was calculated in 32%.

EA and ME inhibited fungus growth in all concentration tested ($p < 0.05$) (Figs. 4 and 5), and the highest inhibition was obtained in 100 μg / mL, 34% and 57% respectively.

HE fraction inhibited AFB1 and fungus growth in all concentration tested, but only 100 mg / mL showed statistically significant inhibition ($p < 0.05$), where the inhibition were 97% and 38% respectively. All concentration inhibited the AFB2 production ($p < 0.05$), but 100 μg / mL inhibited 99% of the production (Fig. 3).

CL fraction did not inhibit the fungus growth either AFB1 and AFB2 production ($p > 0.05$) in the concentration tested.

The correlation between the fungus biomass and AFB1 and AFB2 production were calculated for EE, EA, ME and HE. EE showed low positive correlation between fungus growth and AFB1 ($r = 0.28$) and $r = 0.33$ for the fungus growth and AFB2. This was because the EE inhibited the aflatoxin B1 and B2 but the fungal growth was inhibited only in 350 μg / mL. EA, ME and HE fractions showed high positive correlation between fungus growth and AFB1 production ($r = 0.91$; $r = 0.95$ and $r = 0.95$ respectively), and the same results were obtained to AFB2 ($r = 0.98$; $r = 0.98$ and $r = 0.92$ respectively). These results were obtained because all the concentrations of EA, ME and HE inhibited the fungus growth and the AFB1 and AFB2 production. *P. sonchifolia* leaves probably have more than one substance able to inhibit AFB1, AFB2 production and the fungal growth, because fractions with different polarity are active.

HE fraction is composite for apolar substances, and relates in the literature showed that apolar substances like essential oils and terpenes are active against *A. flavus* or *A. parasiticus* (Ansari & Shrivastava, 1991; Bullerman et al., 1977).

Polar substances like flavonoids, biflavonoids, isoflavonoids, estilbenes and tanines were related with inhibited the fungus growth and the aflatoxin B1 and B2 production (Mallozzi et al., 1996; Norton, 1999; Gonçalez et al., 2001; Weidenborner et al., 1989; Azazeh & Pettit, 1990; Weidenborner et al., 1990). The ME and EA fractions are composite for polar substances. The CL fraction is composite for substances with polarity lower than EA and ME.

The literature did not relate inhibition for AFB2 production but all the extract and its fractions decreased the production when compare with the control.

Cell culture systems provide rapid and inexpensive information for toxicity studies (Segner et al., 1994). Figure 6 shows representative dose-response curves obtained for EE, EA and ME from *P. sonchifolia* by the crystal violet staining method using VERO cells. As much as lower the concentration, greater was the absorbance, indicating that the number of viable cells decreases with increasing concentrations of the extract or fractions. EE and ME at the concentration below 125 μg / mL did not inhibit cell growth, and exposure to 1,00 μg / mL caused complete suppression of cell growth after 72h, and EA showed suppression at 125 μg / mL, thus showing that inhibition of cell growth were dose-dependent. The IC_{50} and the standard deviation of cell growth were 460 μg / mL ± 62,22; 130 μg / mL ± 96,72 and 592 μg / mL ± 115,12 for EE, EA and ME respectively, indicating that the EE from *P. sonchifolia* leaves may be safely used at the concentrations tested, although additional toxicity tests are necessary.

Natural substances may be important for the aflatoxin control, mainly if these do not leave toxic residues, and the cytotoxicity assay showed that EE, EA and ME had low cytotoxicity for Vero cells.

The HE fraction was not tested in Vero cells because it was only soluble in solvents no toxic for the cells. The CL was not tested in Vero cells because it was not active against *A. flavus*.

P. sonchifolia leaves may have more than one substances with inhibitory activity towards production of aflatoxins B1 and B2 by *Aspergillus flavus* under conditions *in vitro*. All the tested concentrations caused inhibition of aflatoxin production, and these did not cause cytotoxicity. Therefore, *Polymnia sonchifolia* could be a promising plant to reduce aflatoxin B1 and B2 production.

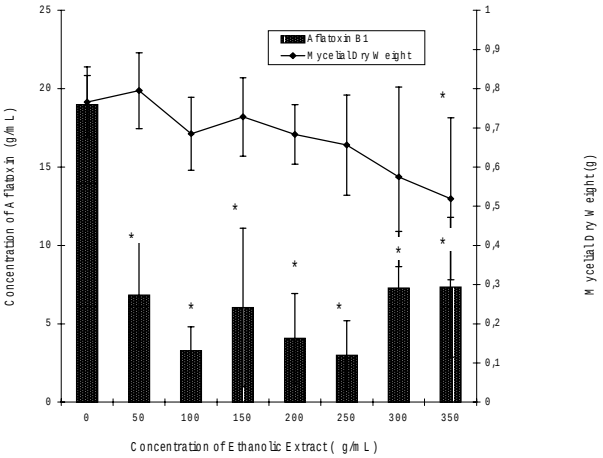

Fig. 1 - Production of aflatoxin B1 and dry weight of mycelium by *Aspergillus flavus* cultures after treatment with different concnetrations of the ethanolic extract (EE) from *P. sonchifolia*. The results correspond to mean ± S.D. * $p < 0.05$.

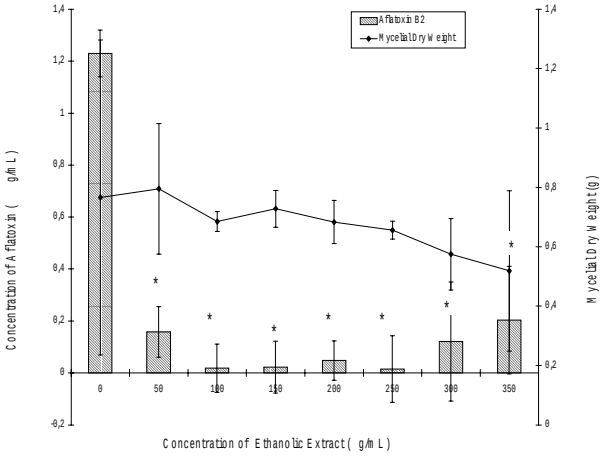

Fig. 2 - Production of aflatoxin B2 and dry weight of mycelium by *Aspergillus flavus* cultures after treatment with different concentrations of the ethanolic extract (EE) from *P. sonchifolia*. The results correspond to mean ± S.D. * $p < 0.05$.

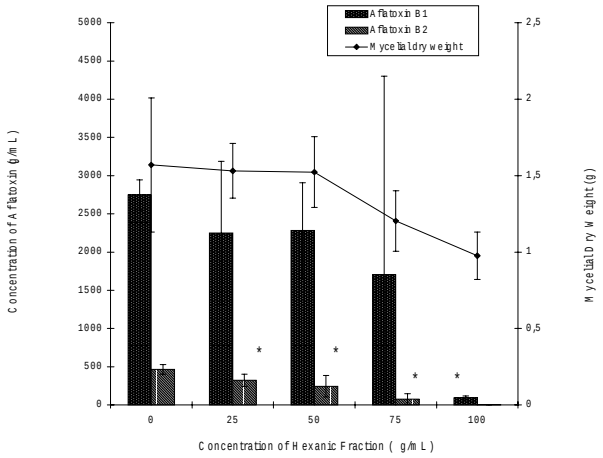

Fig. 3 - Production of aflatoxin B1 and B2 and dry weight of mycelium by *Aspergillus flavus* cultures after treatment with different of the hexanic fraction (HE) of *P. sonchifolia*. The results correspond to mean ± S.D. * $p < 0.05$.

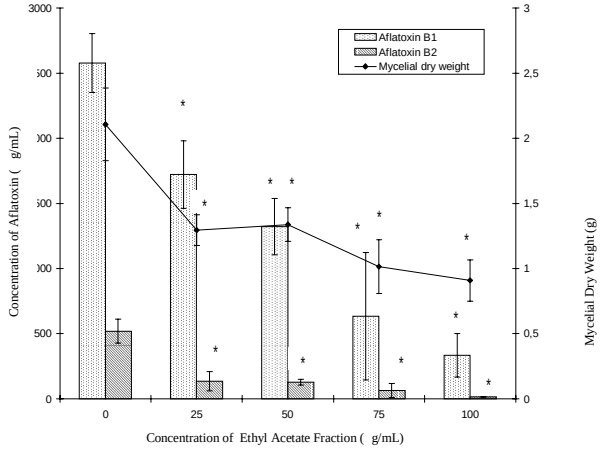

Fig. 4 - Production of aflatoxin B1 and B2 and dry weight of mycelium by *Aspergillus flavus* cultures after treatment with different of the ethyl acetate fraction (EA) of *P. sonchifolia*. The results correspond to mean ± S.D. * $p < 0.05$.

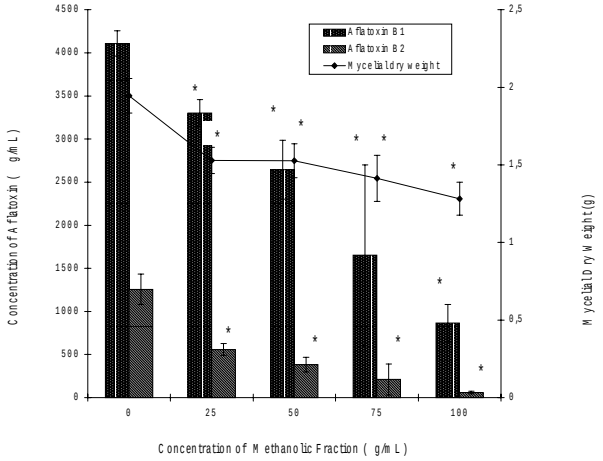

Fig. 5 - Production of aflatoxin B1 and B2 and dry weight of mycelium by *Aspergillus flavus* cultures after treatment with different of the methanolic fraction (ME) of *P. sonchifolia*. The results correspond to mean ± S.D. * $p < 0.05$.

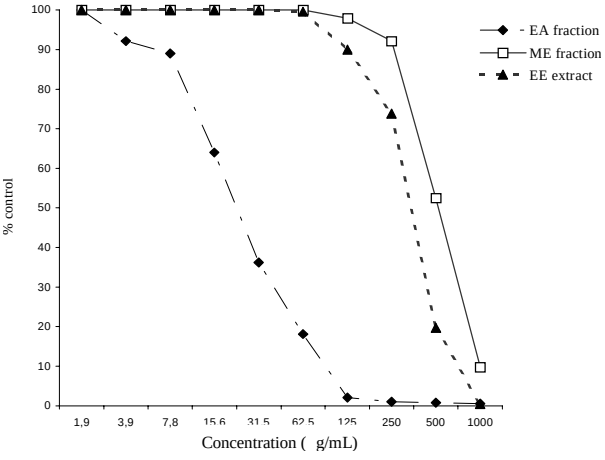

Fig. 6. - Dose-response curve of EE, EA and ME from *P. sonchifolia* on Vero cells growth. Data correspond to percentages of the control (100%).

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