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INHIBITORY ACTIVITY OF COMPOUNDS ISOLATED FROM *POLYMNIA SONCHIFOLIA* ON AFLATOXIN PRODUCTION BY *ASPERGILLUS FLAVUS*

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ABSTRACT

Polymnia sonchifolia, commonly known as "yacon", was originally cultivated at Andes moutains in South America. Recently, the specie attracted worldwide attention because of its wide range of uses, for example in the control of diabetes melitus, besides the antifungal and pesticidal compounds were found in the leaves. This study describes the identification of two flavonoids: 3', 5, 7 trihydroxy-3, 4'-dimethoxyflavone (compound 1) and 3', 4', 5- trihydroxy-7-methoxy flavanone (compound 2) and two sesquiterpenes lactones: enhydrin (compound 3) and a mixture of enhydrin and uvedalin (compound 4) isolated from *Polymnia sonchifolia* leaves and their effects on the aflatoxin production by *Aspergillus flavus*. The identification of the compounds were achieved by ^{1}H and ^{13}C NMR. All compounds were tested in different concentration, to evaluate the growth of *Aspergillus flavus* culture and the production of aflatoxin. The compound 1, at the concentration 15 µg/mL, inhibited 25% of the aflatoxin B_1 production ($p<0.01$). The compound 4 inhibited 34% and 76% of the fungal growth and AFB_1 production respectively. These results show that *Polymnia sonchifolia* can be used for the development of agents to control aflatoxin B_1 production by *Aspergillus flavus*.

Key words: *Polymnia sonchifolia*, flavonoids, melampolides, aflatoxin, *Aspergillus flavus*

INTRODUCTION

Aflatoxins are bifuranocumarin mycotoxins produced by *Aspergillus flavus* and *Aspergillus parasiticus*, with aflatoxin B_1 (AFB_1) being the most hepatotoxic, showing mutagenic and carcinogenic and probably teratogenic properties in animals (15,16). According to the International Agency for Research on Cancer, aflatoxins are classified as human carcinogens class 1 (8).

Previous studies have shown that the biosynthesis of aflatoxin B_1 can be inhibited by a number of compounds (4). Extracts of certain plants are toxic to fungi and may be useful in controlling the fungal growth and mycotoxin production (14). Plant extracts, such as those from garlic and onion, effectively retarded growth and aflatoxin production (5). Natural compounds, such as flavonoids, biflavonoids, stilbenes, essential oils and others, were also active in inhibition of aflatoxin production (1,7,10,11,13).

There is increasing interest in antifungical agents for growth control of mycotoxin producing strains, however, some of the agents may pose toxic residue problems (2).

Inoue 1995 (9) reported the isolation of fungicidal compounds against *Pyricularia aryzae* from leaves extracts of *Polymnia sonchifolia*.

Pinto *et al.* 2001 (12) and Gonçalez *et al.* 2003 (6) reported the inhibition of aflatoxin production by aqueous and ethanolic extracts from *Polymnia sonchifolia* when added to *Aspergillus flavus* culture.

This paper reports the chemical structure identification and the activity of compounds isolated from leaves of the *P. sonchifolia* against *Aspergillus flavus*.

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MATERIALS AND METHODS

Preparation of plant extract

P. sonchifolia was collected in Capão Bonito City, São Paulo State, Brazil. Dried and powdered leaves were submitted to extraction with ethanol 98% (three times), at room temperature. The solvent was evaporated under vaccum to yield an ethanolic extract (EE) (6).

Isolation and identification of compounds

The EE was submitted to flash chromatographic column over Silica-gel, eluted with hexane, ethyl acetate and methanol. The ethyl acetate fraction showed higher activity than the other fractions (6), and was submitted to the chromatografic column on silica gel 60H eluted with hexane:acetate (3:7, 2:8, 1:9 and 0:10) for the obtention of the subfractions.

These subfractions were tested in *Aspergillus flavus* culture to verify the inhibitory activity in the production of aflatoxin. The most active subfraction was submitted to the isolation of the constituents by chromatographic column using silica gel 60H and sephadex LH-20, yielding the compounds: 1, 2, 3 and 4. The structures of the compounds were elucidated by ^{13}C and ^{1}H NMR (HMQC, HMBC, DEPT, NOISE, H^{1}-^{1}H COSY) techniques.

Culture conditions

Aspergillus flavus IMI 190 (International Mycological Institute) was grown on potato dextrose agar (Difco Laboratories, Detroit, Mich) plates for 10 days, at 25ºC, until well sporulated. The spore suspension used as inoculum was prepared washing the cultures with sterile solution of Tween 80 (0.01%). The suspension was submited to spore counting in a Neubauer Chamber.

Aflatoxin production and *Aspergillus flavus* growth evaluation

The semi-synthetic Yes culture medium was used for testing aflatoxin production (3). Suspensions contaning 1.3 x 10^5 spores/mL were inoculated into 50 mL of Yes medium, at different concentration of the subfractions, 0 (control), 25, 50 and 75 µg/mL; 0 (control), 5, 10 and 20 µg/mL for compound 1; 0 (control), 10, 20 and 40 µg/mL for compound 2; 0 (control), 4, 9 and 14 µg/mL for compound 3 and 0 (control), 10, 15 and 20 µg/mL for compound 4. Three replicates were performed for each concentration. The production of aflatoxin B_1 was obtained with cultures incubated at 25ºC for 5 days. The cultures were then filtered and the dry weight of each mycelium was determined after drying at 50ºC, for 4 days. Aflatoxins were extracted with 25 mL of chloroform for three times. The extracts were combined, evaporated and the residue was dissolved and made up to 1 mL in a volumetric flask with chloroform, which was used for analysis.

Aflatoxin analysis

Samples of 5 µL from replicates were spotted on silica gel-G thin layer plate which was developed using chloroform:acetone 9:1 (v/v) as solvent system. Concentrations of the aflatoxin B_1 (AFB_1) were determined by photodensitometry (photodensitimeter Shimadzu, CS 9000), by comparing the area of the spots samples with aflatoxin standards.

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 $q > 4.457$.

RESULTS

Gonçalez *et al.* 2003 related the inhibitory activity of the ethanolic extract of leaves of *P. sonchifolia* and its fractions (hexane, chloroform, ethyl acetate and methanolic). Chemical study was performed in a biomonitoring assay of the ethyl acetate fraction, yielding the isolation of the active principle.

The hexane:acetate (3:7; 2:8; 1:9 and 0:10) subfractions were tested in *Aspergillus flavus* culture. The first fraction showed the highest activity of the tested concentrations (25, 50 and 75 µg/mL) and AFB_1 production was inhibited in 81.3%; 85.4% and 95.3%, respectively. This subfraction was purified by chromatography, yielding four compounds.

The spectral analyses showed the existence of two different groups of natural compounds. Compounds 1 and 2 showed spectrometric characteristics of flavonoids and compound 3 and 4 were sesquiterpene lactones.

The ^{13}C NMR spectra indicated similar A and B- ring oxigenation patterns for the flavonoids 1 and 2 and differences in the C ring.

The comparison of the ^{13}C NMR data of compounds 1 and 2 with the data obtained from literature permitted the identification of flavonoids 1 and 2 as 3', 5, 7 trihydroxy-3, 4'-dimethoxyflavone (Fig. 1) and 3', 4', 5- trihydroxy-7-methoxy flavanone (Fig. 2).

Compounds 3 and 4 showed ^{13}C and ^{1}H NMR spectra (NOISE and DEPT) almost identical to two sesquiterpene lactones described in literature (9). Differences between them were observed in the signals which were indicative of an aditional epoxide group in the carbons C-4 and C-5 of compound 3. Compound 4 was a mixture of compound 3 and another compound with one double bond between C-4 and C-5.

Analysis of these results indicated that compound 3 was as enhydrin (Fig. 3) and 4 as a mixture of enhydrin and uvedalin (Fig. 4).

These compounds were tested in *Aspergillus flavus* culture to evaluate the aflatoxin B_1 (AFB_1) production and the effects on fungus growth. The results are shown in Figs. 1, 2, 3 and 4.

The aflatoxin B_1 production was inhibited by compound 1 ($p<0.05$), but the compound 2 did not show the same effect

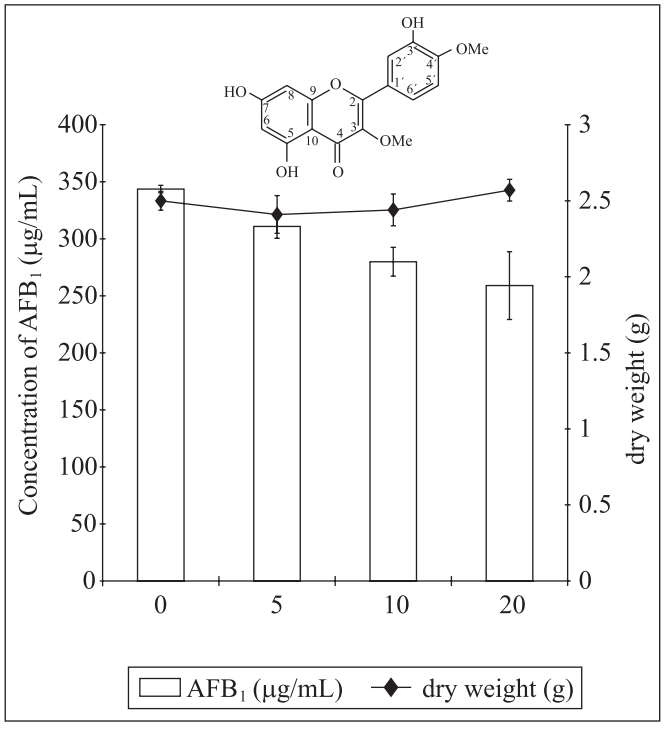

Figure 1. Production of aflatoxin B_1 and dry weight of *Aspergillus flavus* mycelium (IMI 190) in YES medium containing increasing concentrations (control, 5, 10 and 20 µg/mL) of 3', 5, 7 trihydroxy-3, 4'-dimethoxyflavone (compound 1). The results correspond to mean ± S.D.

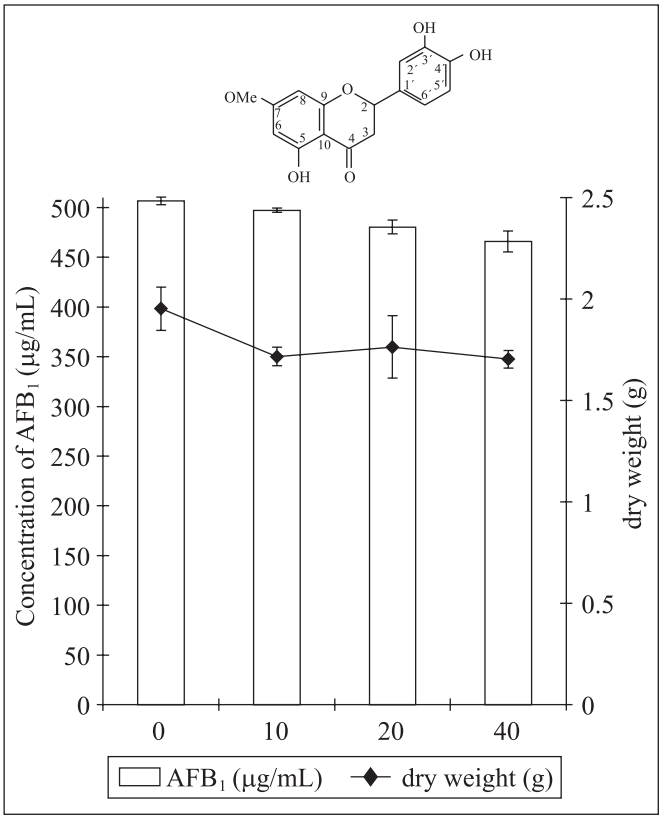

Figure 2. Production of aflatoxin B_1 and dry weight of *Aspergillus flavus* mycelium (IMI 190) in YES medium containing increasing concentrations (control, 10, 20 and 40 µg/mL) of 3',4',5 – trihidroxi-7-metoxi-flavanone (compound 2). The results correspond to mean ± S.D.

(p>0.05), in the concentrations tested. However, neither compounds showed a statisticaly significant level of inhibition of the fungal growth (p>0.05) (Figs. 1 and 2).

The Compound 4 (enhydrin + uvedalin) has inhibited both the AFB_1 production and fungus growth in a concentration-dependent manner (Fig. 4). On the other hand, in the concentrations tested (Fig. 3), enhydrin (compound 3) did not show a statistically significative inhibition activity (p>0.05) on AFB_1 production and fungal growth.

DISCUSSION

In previous investigations (1,7,10,11,13), very encouraging results were obtained on inhibition of aflatoxin production or fungal growth by flavonoids and sesquiterpene lactones. Compounds 3 and 4 were already isolated from leaves of *P. sonchifolia* by Inoue *et al.*, 1995, but compounds 1 and 2 are described for the first time as constituents of this plant.

Inoue *et al.*, 1995, reported that enhydrin showed lower fungicidal activity against *Pyricularia oryzae* than uvedalin. This result was attributed to the 4,5 epoxide group in enhydrin. The authors observed that activity of enhydrin against *A. flavus*, was low inhibiting only 8% of aflatoxin B_1 production and 20% of fungal growth at 14 µg/mL concentration. This effect was not statistically significant (p>0.05), but it was concentration dependent (Fig. 3). However, compound 4, a mixture of enhydrin and uvedalin, showed the highest inhibitory action on aflatoxin B_1 production in the tested concentrations. These results could caused by uvedalin only or by the synergic effect of the lactones mixture. The percentages of fungal growth inhibition and AFB1 production were 34% and 76%, respectively, and showed statistical significance (p<0.05).

Some flavonoids and biflavonoids are biologically active against *A. flavus* and *A. parasiticus* (7,10,11). In this work, two active flavonoids were isolated from *P. sonchifolia* leaves. Compound 1 inhibited 25% of the aflatoxin B_1 production at the concentration of 15 µg/mL (p<0.01) (Fig. 1), and the compound 2 inhibited 8% of the aflatoxin B_1 production (p>0.05) (Fig. 2). The chemical analyses of compound 1 showed a higher oxidation pattern than compound 2, and the oxidation pattern of the flavonoids and their position could be responsible by AFB_1

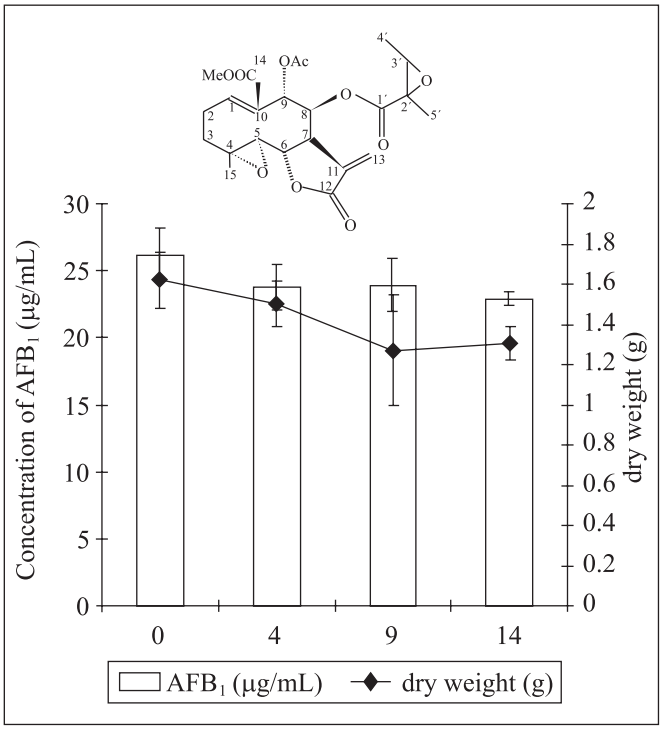

Figure 3. Production of aflatoxin B_1 and dry weight of mycelium *Aspergillus flavus* (IMI 190) in YES medium containing increasing concentrations (control, 4, 9 and 14 μg/mL) of enhydrin (compound 3). The results correspond to mean ± S.D.

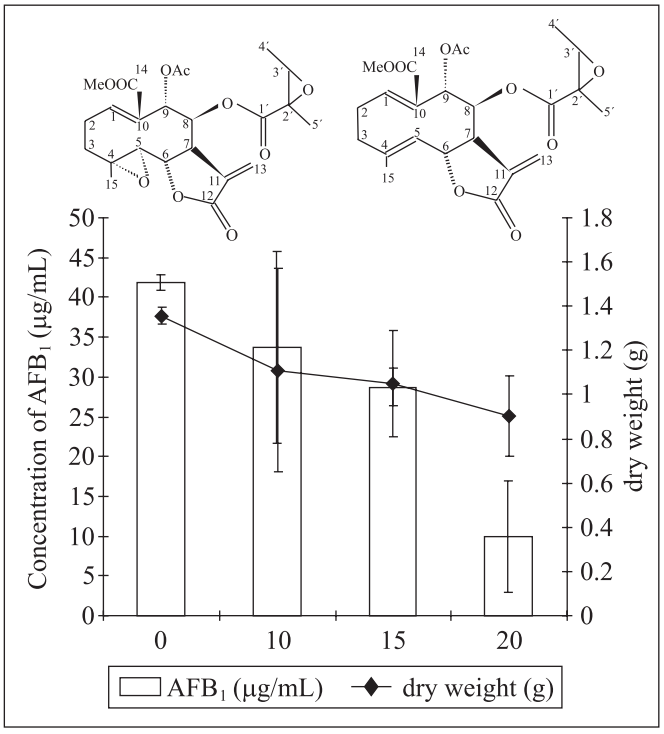

Figure 4. Production of aflatoxin B_1 and dry weight of mycelium *Aspergillus flavus* (IMI 190) in YES medium containing increasing concentrations (control, 10, 15 and 20 μg/mL) of mixture enhydrin and uvedalin (compound 4). The results correspond to mean ± S.D.

production inhibition (7,10). Norton, 1999, reported that highest AFB_1 inhibition was obtained with 3-OH compounds, which were three times more active than the related 3-deoxy compounds. Likewise, the 3-methoxyl in the ring C of the compound 1 can explain its higher activity than compound 2 (Figs. 1 and 2).

Compound 1 was not able to inhibit the fungal growth, while compound 2 inhibited 13% of the fungal growth at concentration 40 μg/mL (Figs. 1 and 2). Weidenborner *et al*, 1989, (17) observed that hydroxyl groups contribute to the higher polarity of the molecule which minimize the fungal membrane permeability of the substance in the same *Aspergillus* species. Therefore, several hydroxyl groups in the molecule are not conducive to antifungical activity. Our results agreed with literature, because compound 1 has higher oxidation pattern than compound 2.

In conclusion, *Polymnia sonchifolia* is a promising plant that can be used in the control of aflatoxin B_1 production by *Aspergillus flavus*.

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RESUMO

Produção de aflatoxina por *Aspergillus flavus* é inibida por compostos isolados de *Polymnia sonchifolia*

Polymnia sonchifolia, conhecida como "Yacon", é originária da cordilheira dos Andes, sendo muito conhecida devido ao uso de seu tubérculo no controle da Diabetes melitus. Suas folhas podem conter compostos com atividade antifúngica e pesticida, pois não é necessário o uso destes produtos no seu cultivo. Neste trabalho descrevemos a identificação da estrutura química de dois flavonóides isolados das folhas de *Polymnia sonchifolia*: 3', 5, 7 trihydroxy-3, 4'-dimethoxyflavone (composto 1) e 3´,4´,5-trihidroxi-7-metoxiflavanona (composto 2) e de duas lactonas sesquiterpenicas: enidrina (composto 3) e uma mistura de enidrina e uvedalina (composto 4), bem como seus efeitos na produção de aflatoxinas por *Aspergillus flavus*. A identificação dos compostos foi realizada por RMN ^{13}C e ^{1}H.

Todos os compostos foram testados em diversas concentrações em cultura de *Aspergillus flavus* para avaliar o crescimento e a produção de aflatoxina. O composto 1 na concentração de 15 µg/mL inibiu 25% da produção de aflatoxina B_1 ($p<0,01$). O composto 4 inibiu o crescimento do fungo e a produção da aflatoxina B_1 em 34% e 76%, respectivamente. Os resultados mostraram a possibilidade do uso de *Polymnia sonchifolia* no controle alternativo da produção de aflatoxina B_1 pelo fungo *Aspergillus flavus*.

Palavras-chave: *Polymnia sonchifolia*, flavonóides, melampolideos, aflatoxinas, *Aspergillus flavus*

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