EVALUATION OF ANTIFUNGAL ACTIVITY OF *Pittosporum undulatum* L. ESSENTIAL OIL AGAINST *Aspergillus flavus* AND AFLATOXIN PRODUCTION

Avaliação da atividade antifúngica do óleo essencial de *Pittosporum undulatum* L. em *Aspergillus flavus* e produção de aflatoxina

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ABSTRACT

The presence of mycotoxins as a result of fungal attack can occur before, after and during the harvest and storage operations on agricultural crops and food commodities. Considering the inhibitory property of essential plant oils on the mycelial development of fungi and the importance of *Aspergillus flavus*, the main producer of aflatoxins, this research was designed to evaluate the toxicity of essential oil from *Pittosporum undulatum* against *A. flavus*. The essential oils were obtained from *P. undulatum* leaves, collected in different months and analyzed by GC/MS. The oils were rich in hydrocarbon, monoterpenes and sesquiterpenes and it was observed a significant variation on the chemical composition of the essential oil of leaves at different months. Besides, the essential oils were tested against fungal growth and the results showed different spectrum of inhibition on *A. flavus*. However, the essential oils inhibited the aflatoxin B_1 production.

Index terms: Aflatoxin B_1, toxigenic fungal, volatiles, Pittosporaceae.

RESUMO

A presença de micotoxinas como resultado do ataque fúngico pode ocorrer antes, após e durante a colheita e também no armazenamento de grãos e alimentos. Considerando as propriedades inibitórias dos óleos essenciais de plantas no desenvolvimento do micélio dos fungos e a importâncias do *Aspergillus flavus*, principal produtor de aflatoxinas, relatou-se neste trabalho, a atividade tóxica do óleo essencial do *Pittosporum undulatum* em cultura de *A. flavus*. Os óleos essenciais de *P. undulatum* foram obtidos a partir de folhas coletadas em diferentes meses e analisado por CG/EM. Os óleos se mostraram ricos em hidrocarbonetos, monoterpenos e sesquiterpenos e foi observada uma significante variação na composição química destes óleos nos diferentes meses de coleta. Os óleos essenciais mostraram diferentes espectros de inibição do crescimento de *A. flavus*, porém todos foram capazes de inibir a produção de aflatoxina B_1.

Termos para indexação: Aflatoxina B_1, fungos toxigêncos, voláteis, Pittosporaceae.

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INTRODUCTION

The increase of demand for safe and organic food, without chemical preservatives, provokes many researchers to investigate the antimicrobial effects of natural compounds. Numerous investigations have confirmed the antimicrobial action of essential oils in model food systems and in real food (Rasooli et al., 2008). Essential oils are a rich source of biologically active compounds and they are potential sources of novel antimicrobial compounds (Mitscher et al., 1987; Pereira et al., 2010). It was demonstrated that essential oils have been shown to possess antibacterial, antifungal, antiviral insecticidal and antioxidant properties (Burt, 2004; Kordali et al., 2005).

The essential oils mainly of the species of family Labiateae, Compositeae, Lamiaceae, Apiaceae, Asteraceae, Lauraceae and Umbellifereae were related as antifungal on toxigenic fungi (Mishra & Dubey, 1994; Soliman & Badeaa, 2002; Rasooli & Owlia, 2005; Rasooli et al., 2006; Razzaghi-Abyaneh et al., 2007; Bluma et al., 2008; Bluma & Etcheverry, 2008; Nguefack et al., 2009; Viuda-Martos et al., 2009; Nogueira et al., 2010). Nogueria et al. (2010) related the activity of *Ageratum conyzoides* (Asteraceae) essential oil at 30 µg/mL, to 0.10 µg/mL concentrations inhibited the growth of the *Aspergillus flavus* on average 55% and the aflatoxin production was completely inhibited all the concentrations.

The *Aspergillus flavus* can grow on a wide range of agricultural commodities, such as maize grains and is responsible to produce aflatoxins B_1 and B_2 (Bluma et al., 2008; Carvalho et al., 2010). Aflatoxin B_1 is the most carcinogenic, mutagenic and teratogenic substance found naturally in foods and feeds (International Agency for Research on Cancer - IARC, 1993). Therefore the control of *A. flavus* and of aflatoxin biosynthesis is extremely important for agriculture and public health.

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Pittosporum undulatum (Pittosporaceae) (named "pau-incenso" in Brazil) presents a characteristic smell from leaves and fruits. This species has been found as a wild plant in tropical forest from Africa, Asia and New Zealand and have been also planted as ornamental specie in other tropical regions of the world such as Brazilian cities (Lago et al., 2006). Medeiros et al. (2003) related the microbiology activity of the *P. undulatum* against *Staphylococcus aureus* and *S. epidermis*.

The present study report the principal components of the essentials oils obtained from leaves of *P. undulatum* collected in Ribeirão Pires at different months and their antifungal activity on *Aspergillus flavus*.

MATERIAL AND METHODS

Plant materials

Leaves of *P. undulatum* were collected in Ribeirão Pires city, São Paulo State, Brazil in November/2007 (Group I), December/2007 (Group II) and January/2008 (Group III). A voucher specimen (Nº. PMSP 9961) was deposited in the herbarium of the City Hall of São Paulo City.

Oil extraction and analysis

The fresh leaves were cut into small pieces and placed in a distillation Clevenger apparatus for 2 hours. The hydrolyte was extracted with hexane and evaporated at room temperature and the resulting oil was stored in dark glass bottles in a freezer until it was used by test in *A. flavus* and GC/MS analysis.

GC/MS analyses of the main components of essential oil and its fraction were done in a Shimadzu QP-5000 equipped with an OV-5 (30mx0.25mmx0.25 µm, Ohio Valley Specialty Chemical, Inc) capillary column. Operating conditions were undertaken at oven temperature from 60° C to 240° C at 3° C/min, injector and detector temperatures of 240° C and 230° C respectively a 70 eV. Helium as a carrier gas at a constant flow of 1.7 mL/min, split 1/20. The oil components were identified using retention indices with those of authentic compounds or with literature data (Mclafferty & Stauffer, 1989; Adams, 2001).

Culture conditions

The *Aspergillus flavus* strain producer of aflatoxin B_1 was isolated from soil of rice plantation localized at Unidade Laboratorial de Referência de Microbiologia of Instituto de Tecnologia de Alimentos (ITAL)- Campinas – SP. The fungi were plated onto potato dextrose agar (PDA) and incubated for 10 days at 25° C. The spore suspension used as inoculum was prepared washing cultures with sterile 0.01% Tween 80 solution.

Antifungal assay (disk diffusion assay)

Filter paper disk (6mm diameter) containing 5.0 µL of the crude essential oil of *P. undulatum* and the fractions were applied on the Sabouraud dextrose agar in Petri dishes previously inoculated with the fungal inoculum on the surface. The inoculated plates were incubated at 25° C for 5 days. At the end of the period, antifungal activity was evaluated by measuring the zone of inhibition (mm) against the test fungus (Yin et al., 1999). The fungicide Benlate 50 WP was used with positive control. All treatments consisted of three replicates and repeated three times and the averages of the experimental results determined.

Aspergillus flavus growth and aflatoxin production

The semi-synthetic YES culture medium was used for aflatoxin production (Davis et al., 1966). Suspensions of *A. flavus* containing 1.3×10^5 spores/mL were transferred to 50 mL of YES medium with different concentrations of essential oil (0.1 µg/mL, 0.2 µg/mL, 0.3 µg/mL). Four replicates were performed for each concentration, and the experiment was repeated three times. For production of aflatoxin B_1, cultures were incubated at 25º C for 5 days. The cultures were filtered and submitted to drying at 50º C for 4 days. The weight of each mycelium was determined. To aflatoxin extraction, the filtrate was treated three times with 25 mL of chloroform. The extracts were combined, evaporated and the residue was dissolved in chloroform and made up to 1 mL in a volumetric flask. An aliquot (40 µL) of each sample was spotted on silica gel-G thin layer plate (Merck, Germany) and then developed with chloroform: acetone 9:1 (v/v) as the solvent system. The concentration of aflatoxin B_1 in each area was determined by photodensitometry (Shimadzu, CS 9000) comparing the area of the spots samples with aflatoxin B_1 standards (Sigma Aldrich, USA) (Gonçalez et al., 2001). The AFB_1 quantification and detection limit were 0.08 ng and 0.04 ng, respectively.

Statistical analysis

Antifungal experiments were performed in triplicate and data analyzed are mean ± SD subjected to one way ANOVA. Means are separated by Tukey's multiple range tests when ANOVA was signiûcant ($P < 0.05$).

RESULTS AND DISCUSSION

The essential oil extracted from *P. undulatum* leaves yielded 0.02%. Chemical analysis by GC/MS of the essential oil from leaves of *P. undulatum* collected in Group I and Group III led to identiûcation of 11 and 14 main compounds, respectively and the Group II was constituted by 16 main compounds (Table 1).

Preliminary experiments were carried out *in vitro* using the disc diûusion methods to investigate antifungal activity of the essential oils from leaves of *P. undulatum*, using 5 µl per disc. The Group II showed no inhibitory effect on *A. flavus* growth. Otherwise the Group I inhibited completely the fungal growth, and Group III demonstrated an inhibitory zone against *A. flavus* that was measure at 5.0 mm (average n=3), comparing with the fungicide (Benlate) that was measure at 2.5 mm.

According to the results obtained in the disc diffusion test, the Group III was submitted to the Yes test to evaluate the fungal growth and aflatoxin production. The results showed that both fungal growth and aflatoxin biosynthesis were suppressed by *P. undulatum* L. The inhibitory effect on fungal growth of Group III increased according to the proportion of their concentrations. All concentrations of the essential oil reduced signiûcantly the mycelium dry weight *A. flavus* ($P < 0.05$) at concentrations of 0.1 µL/mL, 0.2 µL/mL, 0.3 µL/mL at 50.5%, 70.46%, 97.4%, respectively. All concentrations tested in this study inhibited completely the aflatoxin production.

An important characteristic of essential oils and their components is their hydrophobicity, which enable them to partition the lipids of the bacterial and fungal cells membrane and mitochondria, disturbing the cells structures and rendering them more permeable (Sikkema et al., 1994; Nogueira et al., 2010).

Aspergillus flavus is a spoiling mould and aûatoxins producer that reduces the nutritive values of foods becoming them unût for human consumption. Some essential oils of ethno medicinally important higher plant species have been tested for their antifungal activity (Kumar et al., 2010). The inhibition of *A. flavus* growth by essential oils has already been reported (Dikbas et al., 2008; Kumar et al., 2008, 2010; Viuda-Martos et al., 2008; Nogueira et al., 2010). However there are no reports that describe the effect of *P. undulatum* essential oil as an inhibition of *A. flavus* growth and aflatoxin synthesis.

The results of the present study revealed that essential oil of leaves of *P. undulatum* demonstrated an inhibitory activity against *A. flavus* fungi and aflatoxin production in lower concentrations 0.3 µL/mL. The total inhibition of aûatoxin production was observed using the essential oils at 0.1 µL/mL, 0.2 µL/mL, 0.3 µL/mL. A similar result was reported by Kumar et al. (2010) that observed a complete inhibition of aûatoxin B_1 production by *Ocimum sanctum* essential oil and eugenol at 0.2 and 0.1 µL/mL.

The inherent activity of essential oil can be expected to relate to the chemical conûguration of the components, the proportion in which they are present and the interactions between them (Delaquis et al., 2002). Comparing the two groups with antifungal activity (Group I and III) it was observed that the chemical constitution are similar, just varying in the amount of the constituents. The chemical profile of Group II was very different. The composition of groups I and II showed more hidrocarbon compounds that group II. The 4-terpeniol and spatulenol were main compounds of the Group II and 2-heptanone and 3-methyl-4-heptanone and n-heptanol (Table 1) were the main constituents of the essential oil of Group I and III. Although the collection has been performed in the same plant and these data have been attributed to the influence of abiotic factors that occurred in different months.

The literature data report the predominance of the same class (sesquiterpenes) according to the chemical profile of essential oils obtained in this present study but no report cited substances with internal fragmentation of masses. This difference in chemical composition justifies the obtained results on the antifungal activity of plant essential oils (Medeiros et al., 2003; Lee et al., 2004). Lago et al. (2006) identified the monoterpene: β-pinene, β-myrcene, limonene, δ-elemene and sesquiterpenes: α-copaene, β-elemene, β-caryophyllene, aromadendrene, bicyclogermacrene, δ-cadinene, being limonene the main constituent of essential oil *P. undulatum*, on the other hand, Medeiros et al. (2003) related that *P. undulatum* oil essential contain monoterpenes, sesquiterpenes, diterpenes and alkanes, such as: calamenene (41.4%), farnesol (10.9%), spathulenol (5.6%) and β-selinene (5.2%) and the diterpene (8 β, 13β)-kaur-16-ene (10.7%).

The same author described the antimicrobial activities tested against *Staphylococcus aureus*, *S. epidermis* and *Pseudomonas aeruginosa*, and those oils with the highest activities against *S. aureus* and *S. epidermis* were obtained from *H. gardnerianum*; none had activity against *P. aeruginosa* (Medeiros et al., 2003).

The composition of essential oil plants varies significantly in different genera, species and environmental conditions which the plant is subjected. The variation in fungical toxicity of tested essential oil against toxigenic strain of *A. flavus* may be due to considerable diversity in essential oil constituents (Kumar et al., 2008).

Table 1 – Identified compounds, retention time and percentage composition from essential oil of the leaves of *Pittosporum undulatum* collected in November/2007 (Group I), December/2007 (Group II) and January/2008.

Compound	Retention time (min)	Group I (%)	Group II (%)	Group III (%)
2-heptanone	4.95	30.96	2.08	25.64
3-methyl-4-heptanone	5.36	19.78	2.55	17.47
Sabinene	5.74	1.15	nd	0.93
ß-pinene	5.85	1.03	nd	0.82
α-mircene	5.91	7.34	nd	7.42
n-heptanol	6.52	18.75	1.95	19.92
α-decene	7.23	1.54	nd	1.45
limonene	7.35	1.23	nd	1.32
Trans-3-hexenil acetate	7.67	1.17	nd	0.57
Cis-3-hexenil acetate	7.82	0.62	nd	5.44
4-terpineol	14.10	nd	25.58	5.44
α-terpineol	14.62	nd	2.53	1.40
n-dodecane	15.25	0.34	1.34	1.36
n-tridecane	19.47	nd	3.39	0.52
n-tetradecane	23.65	nd	7.88	nd
n-pentadecane	27.69	nd	6.04	nd
γ-cadinene	28.08	nd	2.63	nd
cubenene	28.79	nd	4.13	nd
spatulenol	30.46	nd	13.52	nd
cariophyllene oxid	30.72	nd	3.02	nd
n-hexadecane	31.54	nd	2.19	nd
n-heptadecane	35.21	nd	1.46	nd
EE-farnesol	35.80	nd	4.00	nd

CONCLUSION

The results of this study showed the efficiency of the essential oil from leaves of P. undulatum on growth inhibition and aflatoxin B1 production by A. flavus. However, his efficacy as fungicide is depends of environmental conditions and the period of year.

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