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In vitro anti-fungal activity of a new compound isolated from *P. pubescens* Benth

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ABSTRACT

This study investigated the antifungal activity of some compounds chromatographically isolated from *Pterodon pubescens* oil and identified by mass spectrometry: geranylgeraniol, 6α-hydroxy-17β-7β-dihydroxivouacapan-oate, ester isomers 6α-hydroxy-7β-acetoxy-17β-vouacapan-oate and 7β-acetoxy-17β-hydroxyvouacapan-oate and an unknown compound (m/z 355). Minimal inhibitory concentration (MIC) assessment showed antifungal activity at 2000 µg/ml only for the unknown compound (m/z 355) against *Candida albicans, C. krusei*, *C. glabrata* and *C. tropicalis*. Exposure to this compound caused morphological changes observed by scanning and transmission electron microscopy (SEM, TEM).

Keywords: *Pterodon pubescens*, Candida spp., vouacapan, antimicrobial.

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INTRODUCTION

Interest in the discovery of new antimicrobial drugs has increased due to the spread of resistant microorganisms that trigger untreatable infections, causing public health problems (Lewis, 2013). Studies show that *Candida* species, including *C. krusei* have innate resistance to drugs based on azoles, such as fluconazole, voriconazole, miconazole, itraconazole, ketoconazole, ravuconazole (Pfaller et al., 2012). *C. albicans* has a relevant role in triggering oral infections associated with biofilms, which form highly structured and resilient communities that withstand exposure to antifungal azoles as well as immune response (Ansari et al., 2013). Thus, interest has increased in the identification and characterization of new classes of antimicrobials, especially plant-based secondary metabolites.

Plants of the genus *Pterodon* contain a number of secondary metabolites that have action against a broad range of diseases such as sore throat (Corrêa, 1978), gram-positive bacterial infections (Bustamante et al., 2010), the parasite *Trypanosoma cruzi* (Menna-Barreto et al., 2008), larvae of *Aedes aegypti* (Pimenta et al., 2006), and prostate cancer (Spindola et al., 2009). Additionally, *P. emarginatus* oil has been shown to have activity against gram-positive bacteria with a MIC (Minimum Inhibitory Concentration) between 0.72 and 50 mg/ml (Santos et al., 2010). Based on studies with bacteria and the constituent chemical in the seeds of *P. pubescens* suggesting its pharmacological potential, this study aimed to evaluate the action of the components of *P. pubescens* in fungal cell.

P. pubescens is a species distributed throughout the central region of Brazil (Dutra et al., 2008). The literature reports a variety of chemical compounds have been obtained from *Pterodon* spp., such as alkaloids (Torrenegra et al., 1989), isoflavones and triterpenes (Marques et al., 1998), diterpenes (Coelho et al., 2005; Euzébio et al., 2009 Spindola et al., 2009), steroids (Santos et al., 2010) and further evaluated pharmacological potential against several diseases. The variety of compounds is due to environmental factors such as abiotic stress, fauna and microorganisms that live in the environment (Hansen et al., 2010).

Herein the isolation of geranylgeraniol (m/z 388), 6α-acetoxy-17β-7β- dihydroxivouacapan oate (m/z 362), 6α-

Table 1. Proportions of Hexan and Ethil acetate used in the first (proportion I), second and third (proportion II) column chromatography.

Proportion I		Proportion II	
Hexan (ml)	**Ethil acetate (ml)**	**Hexan (ml)**	**Ethil acetate (ml)**
500	0	400	0
400	100	360	40
300	200	320	80
275	225	280	120
250	250	260	140
225	275	240	160
200	300	220	180
175	325	200	200
150	350	180	220
125	375	160	240
100	400	140	260
25	475	120	280
0	500	100	300
		80	320
		60	340
		40	360
		20	380
		0	400

hydroxy-7β-acetoxy-17β-vouacapan oate and 7β-acetoxy-17β-hydroxy-vouacapan-oate (404) and unknown compound with (m/z 355) from the fruit of *P. pubescens* Benth is reported. Furthermore the antifungal activity of these compounds in *Candida* spp. - based MIC assay and the morphological effects of exposure to one of these compounds using SEM and TEM were evaluated.

MATERIALS AND METHODS

Extraction of oil from *Pterodon pubescens* and isolation of chemical constituents

Dried and ground *P. pubescens* Benth fruit (210.11 g) were extracted in a Soxhlet apparatus for 2 h using dichloromethane (Merck®). After removal of the solvent under vacuum, 101.16 g of crude extract was obtained and further purified by column chromatography using silica gel 60® (Merck, 0.063 to 0.200 mm) as the stationary phase and hexane and ethyl acetate (Table 1) as mobile phases. Fractions were collected, monitored by thin layer chromatography and divided in eight final fractions according to the chemical profile observed. The fifth fraction (FR5,35,91 g) contained the major vouacapan components, therefore was further purified using silica gel as the stationary phase and hexane and ethyl acetate (Table 1) as the mobile phases that provided fraction 1E crystallized that was further purified under the previous conditions described. The resulting compound called FRB was analyzed by gas chromatography coupled to mass spectrometry; it was verified m/z 355.

GC-MS

Chromatographic separations were performed on HP 6890 gas chromatograph coupled with a HP 5975 mass spectrometer Helium gas at a flow rate of (0.7 bar, 1 ml min^{-1}) was used. Column oven temperature program was 110°C for 5 min followed by a 5°C/min ramp until attaining 240°C temperature, maintaining that temperature for further 5 min. The temperature of the detector and injector was 250°C.

Dilution of the compound FrB

The compound FrB was diluted to a concentration of 5 mg/ml in DMSO (dimethyl sulfoxide) at 1% in distilled water in appropriate proportions and stored in freezer. Sterile distilled water was added to dilute the extract to a concentration end of 4 mg/ml, yielding then the other dilutions.

Minimal inhibitory concentration

Preparation of inoculum for susceptibility tests was carried out by microdilution as set forth by the CLSI's M27-A2 recommendation protocol with modifications. The yeasts were grown overnight at 37°C in Sabouraud Dextrose Agar (Merck) plates, and inocula for the assays were prepared in 0.85% NaCl solution, adjusted to 0.5 Mc Farland scale and confirmed by spectrophotometric reading at 530 nm. After that, the samples were incubated at 37°C for 24 to 48 h, in triplicate. Nistatin was used as control standard in concentrations ranging from 1.9 to 250 µg/ml. The microplates were incubated at 37°C for 48 h. Minimal fungicidal concentrations were determined by subculturing in plate of 10 µl of sample and control on Sabouraud Dextrose Agar (SDA) after 24 h of incubation. MIC was defined as the lowest concentration that resulted in visually evident growth inhibition. Analyses were performed in triplicate.

Scanning electron microscopy (SEM)

Samples were prepared in 96-well plate (CLSI M27-A2, 2002). After

24 h of incubation, the samples of yeast were centrifuged to form pellets, which were washed twice with phosphate buffer (pH 7.4) and then added to 2% glutaraldehyde for 30 min. Samples were then dehydrated using sequential rinses of ethanol at concentrations of 50, 70, 90% and absolute ethanol. Each rinse concentration was used twice. The samples were sputtered with gold for 120 s (BAL-TEC SCD 050, Balzers Liechtenstein). The analysis of samples was performed using an scanning electron microscope operating at 15 KV acceleration (JSM-5600 Lv; JEOL, Tokyo, Japan).

Transmission electron microscopy (TEM)

Samples were prepared in 96-well plate as described previously [CLSI M27-A2, 2002]. After 24 hours of incubation, the samples were centrifuged for 10 min and the supernatant was discarded. The precipitate was rinsed 5x with phosphate buffered saline (PBS). The cell precipitate was fixed with 2.5% glutaraldehyde in PBS (0.1 M, pH 7.2 for 4 h at 4°C) rinsed with PBS and then fixed with osmium tetroxide in PBS (0.1 M pH 7.2) for 2 h at room temperature. Samples were rinsed with PBS and dehydrated using a sequence of acetone: water rinses (50, 70, 90% acetone). The samples were then embedded in Spurr resin using a sequence of washes of increasing resin: acetone ratio (1:3, 1:2, 1:1, 3:1, 9:1; for 4 h each) followed by incubation with and 100% resin for 48 h. Embedded samples were cut into the ultrathin sections using an ultra-microtome (MT2B; Sorvall Porter Blum). Finally, the ultrathin sections were double stained with uranyl acetate and lead citrate, and then examined in a TEM (JEOL JEM-1400; Zeiss).

RESULTS

Analysis of FrB by CG/MS provided a chromatographic profile with four peaks were identified components: geranylgeraniol (m/z 288; Figure 1), 6α-acetoxy-17β-7β-dihydroxivouacapan oate (m/z 362), 6α-hydroxy-7β-acetoxy-17β-vouacapan-oate and 7β-acetoxy-17β-hydroxy-vouacapan-oate (m/z 404; Figure 2) that were identified by comparison with authentic standards previously isolated and identified (Spindola et al., 2009). Another unknown compound with (m/z 355; Figure 3) was detected that will be reported elsewhere. The unknown compound was isolated and further evaluated against *Candida* spp. (Table 2).

Effect of unknown compound m/z 355 on the morphology and structure of *Candida* spp.

We evaluated the changes in cell wall structure of *Candida* spp. in the presence of unknown compound m/z 355 by SEM and TEM. Comparison with control (Figure 4), SEM images obtained of *C. albicans* cells exposed to the unknown compound (m/z 355) exhibited altered cell wall morphology. In Figure 4, the cells are oval with a smooth surface, but in Figure 5 we find elongated cells that have surface irregularities. TEM images of *C. albicans* cells in the absence (Figure 6) and presence (Figures 7 and 8) of the compound (m/z 355) also indicate altered cell wall morphology, where cell walls

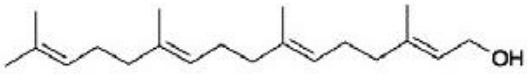

Figure 1. Chemical structure geranylgeraniol.

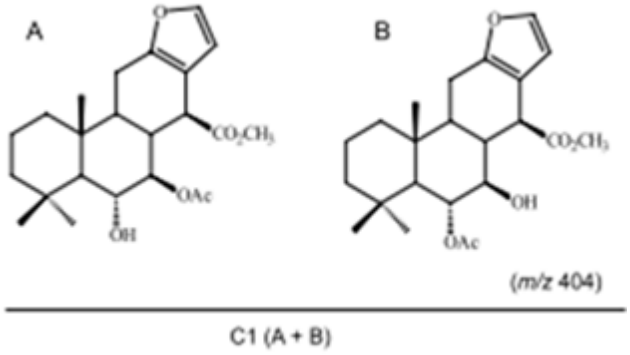

Figure 2. Chemical structure of ester isomers 6α-hydroxy-7β-acetoxy-17β-vouacapan-oate and 7β- acetoxy-17β-hydroxy-vouacapan-oate.

become thicker and cells exhibit elongated morphology.

Similar changes were observed in *C. krusei* cells. SEM images of cells exposed the unknown compound (m/z 355) are electrodense and irregular (Figure 9) compared to the control (Figure 10). TEM images of *C. krusei* cell wall irregularities and thickening (Figures 12 and 13) compared with control (Figure 11) were also observed.

DISCUSSION

According to MIC tests (Table 2), the unknown compound (m/z 355) exhibited similar antifungal action against all four *Candida* spp. tested. The other compounds tested did not exhibit this property. We verified a new compound extracted with hexane and ethyl acetate that was active (2.000 µg/ml) on all the strains of *Candida* spp. tested. We visualized the morphological effects of compound (m/z 355) on *Candida* spp. cells using SEM and TEM and found that exposure to the chemical caused cell wall thickening (Figures 7, 8 and 13), and irregularities of the cell surface (Figures 5, 9 and 12). These changes support a mechanism by which the unknown compound (m/z 355) disrupts cell wall homeostasis. Indeed, it has been reported that fungal cell wall damage caused by monoterpenes obtained from essential oils and the oil itself may affect the biosynthesis of ergosterol, the absorption of steroids, lipid metabolism, and the structure of the fungal cell wall (Parveen et al., 2004).

It has been shown that crude extracts of *Ptedoron* spp. have anti-*Candida* spp. activities (Bustamante et al., 2010). For instance, Bustamante et al. (2010) reported bactericidal and fungicidal activity of the crude ethanol extract of the bark of *Pterodon* spp. against *C. albicans*

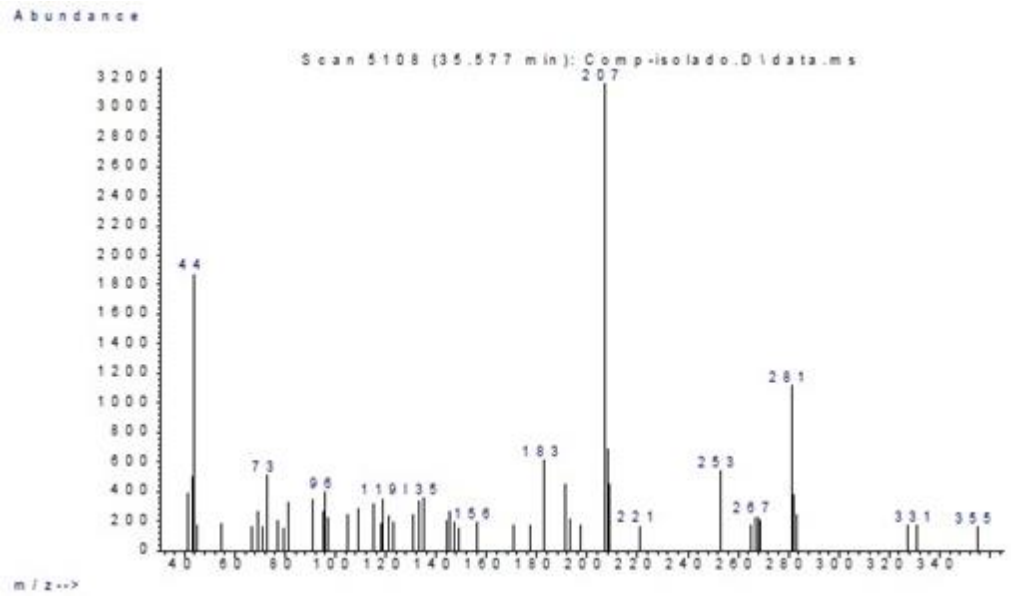

Figure 3. Effect of compound m/z 355 with cells of *Candida* spp.

Table 2. Growth inhibition of four of *Candida* species by components (2000 µg/mL) in *P. pubescens* extract. MICs are reported in µg/mL and measured visually. The unknown compound m/z 355 (A); geranylgeraniol (B); ester isomers 6α-hydroxy-7β-acetoxy-17β-vouacapan-oate and 7β-acetoxy-17β-hydroxy-vouacapan-oate (C); 6α-hydroxy-7β-acetoxy-17β-vouacapan-oate (D); Nyistatin (E).

Microorganisms	A	B	C	D	E
C. albicans	2.000	*	*	*	250
C. krusei	2.000	*	*	*	250
C. glabrata	2.000	*	*	*	250
C. tropicalis	2.000	*	*	*	250

*Values above 2000 µg/ml.

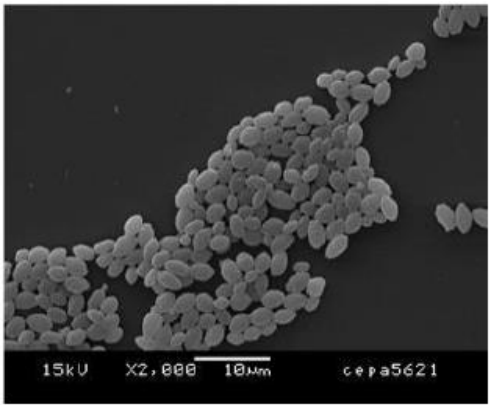

Figure 4. SEM image of *C. albicans* control cells. Cells are oval and have smooth, regular cell walls (2000x).

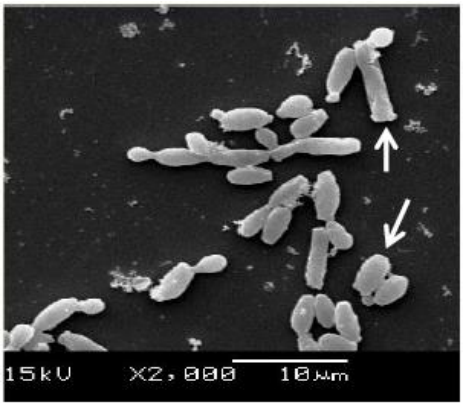

Figure 5. SEM image of *C. albicans* cells. A cell wall irregularity is indicated by the arrow (2000x).

(MIC 740 µg/ml), but tested fractions isolated from the seed of *P. pubescens* and extract with hexan and ethyl acetate. This study confirms the potential activity of the new compound isolated from *P. pubescens* in *Candida*

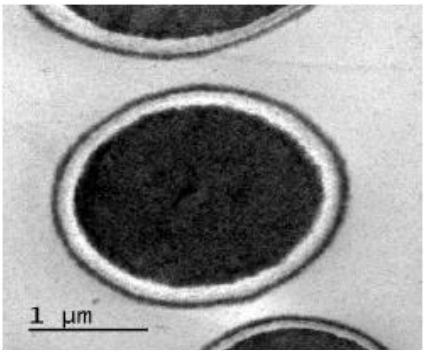

Figure 6. TEM image of *C. albicans* control cells. Cells are oval and regular (2000x).

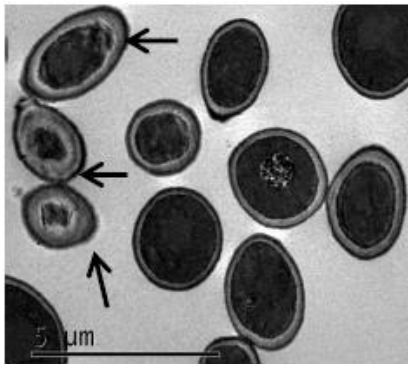

Figure 7. Thickening of the cell wall of *C. albicans* indicated by arrows (2000x).

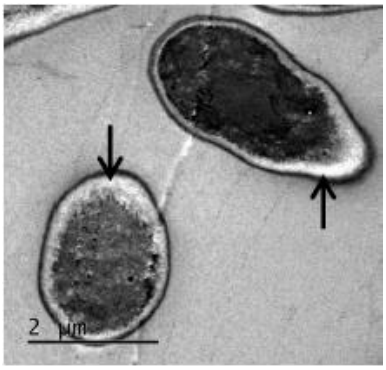

Figure 8. TEM image of *C. albicans* cells with irregular shape and cell wall thickening indicated by arrows (2000x).

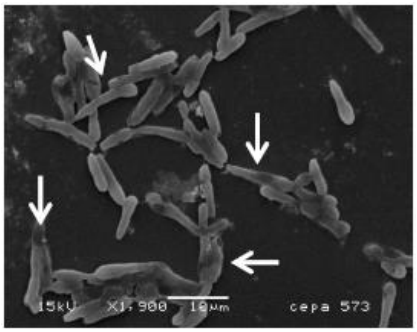

Figure 9. SEM image of *C. krusei* cells with irregularities and electrodense areas indicated by arrows (1900x).

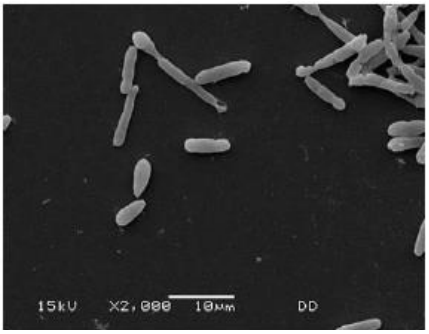

Figure 10. SEM image of *C. krusei* control cells, which exhibit a regular cell wall (2000x).

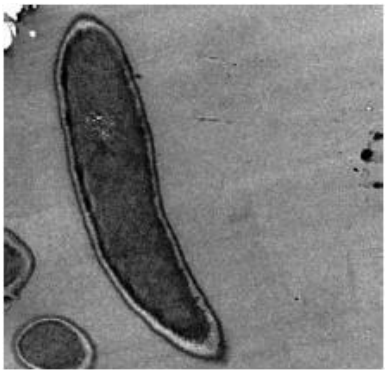

Figure 11. TEM image of *C. krusei* control cells (2000x).

spp. Santos et al. (2010) observed that the essential oil showed activity against the Gram-positive bacteria, and inactive on clinical isolates of *Candida* spp. But used the leaves from *Pterodon emarginatus* and the extraction of the compounds was carried out using n-hexane and dichloromethane provided steroids.

The literature reports studies with filamentous fungi and bacteria. Silva et al. (2005) confirms the seed oil fungicidal action *Pterodon* spp. which significantly reduced the mycelial growth of *Alternaria brassicae, Fusarium oxysporum, Rhizoctonia solani* and *Ceratocystis fimbriata,* using crude extract. Ferreira et al. (2014) evaluated the antimicrobial activity of essential oil *P. emarginatus* against *Staphylococcus aureus*,

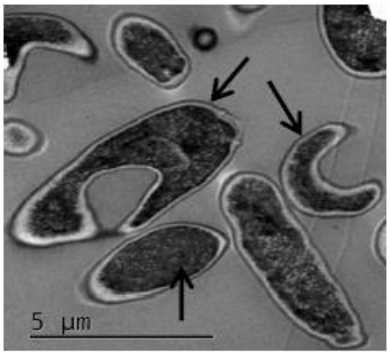

Figure 12. TEM image of *C. krusei* cells Irregularities indicated by arrows (2000x).

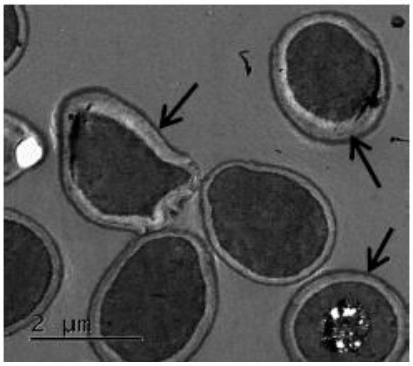

Figure 13. TEM image of *C. krusei* cells exhibiting cell wall thickening indicated by arrows (2000x).

Pseudomonas aeruginosa and *Escherichia coli* and has no antimicrobial activity against any of the tested strains. The alcoholic extract of sucupira seed showed antimicrobial activity against *Proteus mirabilis* (Gonçalves et al., 2005).

Conclusion

We have demonstrated that the unknown compound (m/z 355) isolated from *P. pubescens* has modest antifungal activity against four species of *Candida.*

This compound may represent a novel anti-fungal pharmacological target that bears little structural resemblance to presently available antifungals. This new scaffold may have use in battling strains of *Candida* resistant to antifungals presently in use. Further studies evaluating mechanism of action animal-cell toxicity remain to be completed.

This study also highlights the rich chemistry of *P. pubescens* that may be further studied using other solvents (hexane, butanol, and methanol), additional chromatographic fractions, and diverse tests (that is, anti-cancer, anti-bacterial, etc.).

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