

Antifungal activity of *Uncaria tomentosa* (Willd.) D.C. against resistant non-*albicans* *Candida* isolates



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ABSTRACT

This study investigated in vitro antifungal activity of the hydroethanolic extract (EXT), quinovic acid glycosides (QAPF), oxindole alkaloids (OAPF), water-soluble (WSF) and insoluble fractions of polyphenols (WIF) obtained from *Uncaria tomentosa* bark against non-*albicans* *Candida* resistant isolates. Cytotoxicity and genotoxicity of the major fractions were also assayed. Growth inhibition was assayed by the broth microdilution method according to the CLSI M27-A3 guideline. The most active fractions were evaluated regarding cell ultrastructure, sorbitol metabolism, and infrared (FT-IR) analysis of pseudomycelia of *C. krusei*, *C. glabrata* and *C. parapsilosis*. Fluconazole, terbinafine and anidulafungin were used as reference drugs. EXT and all fractions were able to inhibit non-*albicans* *C.* growth at concentrations ranging from 500 to 3.9 mg/L. Nonetheless, the WIF showed the best in vitro anti-*Candida* activity (3.9 mg/L–15.62 mg/L). This fraction was composed mainly by high molecular mass polyphenols (70.8%) and, to a lesser extent, oxindole alkaloids (7.9%) and quinovic acid derivatives (7.8%). No significant cytotoxicity and genotoxicity was seen. As observed by scanning electron microscopy (SEM), yeasts treated with WIF presented morphological alterations and loss of integrity of the cell wall. The *U. tomentosa* water-insoluble fraction (WIF), composed mostly by high molecular mass polyphenols, showed significant antifungal activity in several non-*albicans* species, among them isolates resistant to terbinafine, fluconazole and anidulafungin. Noticeable microscopic and physicochemical changes in the cell wall indicated that it was the main target of this activity.

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1. Introduction

Lately many fungal species have become resistant to the main currently used antifungal agents. Several approaches have been proposed to overcome antifungal resistance, including drug combinations in medical practice as well as research on new bioactive molecules from synthetic and natural sources (Barros et al., 2013).

Candida spp. are the most prevalent fungal species isolated from the oral, vulvovaginal and urinary tract cavities. They are detected recurrently in immunocompromised patients, but also in about 31–55% of healthy individuals (Samaranayake et al., 2002). Furthermore, the number of *Candida albicans* and *C. non-albicans* resistant to the main antifungal agents has increased worldwide. Clinical and in vitro resistance to antifungal agents can be inherent to the microorganism itself but also appears after previous contact with the drug. Several mechanisms are involved. Antifungal resistance implies, for instance, changes in the biosynthesis route of sterols and expression of the ERG 11 gene involved in the synthesis of enzyme 14 DMI (Morschhäuser, 2002); for antifungal allylamines, such as terbinafine, change in the genic expression of squalene epoxidase seems to be linked strongly to *Candida* spp. resistance (Klobucniková et al., 2003).

Abbreviations: EXT, hydroethanolic extract; QAPF, quinovic acid glycosides purified fraction; OAPF, oxindole alkaloids purified fraction; WSF, water-soluble polyphenols fraction; WIF, water insoluble polyphenols fraction; QAG, quinovic acid glycosides; MIC, minimal inhibitory concentration; TRB, terbinafine; FLZ, fluconazole; AND, anidulafungin.

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Popularly known as cat's claw, *Uncaria tomentosa* (Willdenow ex Roemer & Shultes) D.C is a woody vine, which is ubiquitous in the South and Central American rainforest (Pollito and Tomazello, 2006). Its bark comprises essentially alkaloid, triterpene and polyphenol derivatives (Aquino et al., 1989; Aquino et al., 1991; Stuppner et al., 1992; Laus and Keplinger, 1994; Laus et al., 1997). The alkaloid fraction includes oxindole and indole derivatives. Several biological activities have been ascribed to them, such as antiproliferative (Gonzales and Valerio, 2006), immunostimulant (Lemaire et al., 1999), anti-inflammatory (Aguilar et al., 2002), antiviral (Reis et al., 2008), antioxidant (Pilarski et al., 2006), and antimicrobial activity (Heitzman et al., 2005). The main triterpenes of cat's claw are quinovic acid, ursolic and oleanolic glycosides for which antiviral activity has been reported (Klobucniková et al., 2003). The polyphenol fraction includes phenolic acids, flavonoids monomers, but mainly high molecular weight compounds such as proanthocyanidins, and epicatechin and catechin-derived tannins, which can represent up to 20% of the dry weight of bark (Gonçalves et al., 2005). Nonetheless, no antifungal activity was reported before to any fraction quoted above, except for proanthocyanidins and epicatechin tannins from other plant sources (Ishida et al., 2006).

Although there are reports of microbial action, where *U. tomentosa* extract was effective in the inhibition of *Escherichia coli* and *Staphylococcus aureus*, with MIC values from 0.25 to 1 mg/mL, the antifungal activity of *U. tomentosa* has not been prospected in depth yet. There is only one report in which there is clinical improvement in patients with oral candidiasis after treatment with gel containing *U. tomentosa* extract.

In this context, the aim of this study was to investigate the anti-*Candida* activity of the hydroethanolic extract of *U. tomentosa*, fractions of quinovic acid glycosides, oxindole alkaloids, and both water soluble and insoluble fractions of polyphenols against resistant isolates in an attempt to contribute to their use as alternatives in antifungal treatment in humans. Cytotoxicity and genotoxicity of the major fractions were also studied.

2. Materials and methods

2.1. Preparation and characterization of crude extract and fractions

An authentic sample of *U. tomentosa* stem bark was kindly donated by Induquímica S.A. (Lima, Peru). The material was comminuted in a cutter mill (SK1 Retsch, Germany), provided with a 2 mm steel sieve. The crude extract (EXT) was prepared by 2 h-dynamic maceration using hydroethanolic solution 50% (v/v) and magnetic stirring (300 rpm) (RO 15 Power, IKA, Germany) at 23 ± 1 °C (Gonzales and Valerio, 2006). The extract was filtered (Whatmann N° 2) and concentrated under vacuum (Rotovaporator Büchi, B-480, USA) to half of its original volume. Next, the extract was cooled overnight at 10 °C allowing the separation of the insoluble solid phase. The water insoluble fraction (WIF) was separated from the water-soluble fraction (WSF) by filtration under vacuum and dried at 37 °C for 24 h. The oxindole alkaloids purified fraction (OAPF) and quinovic acid glycosides purified fraction (QAPF) were obtained as previously described (Pavei et al., 2010; CLSI, 2008). All samples were properly freeze dried immediately.

2.2. Quantification of polyphenols, oxindole alkaloids and quinovic acid glycosides

2.2.1. Content assay of low molecular mass polyphenols by HPLC-PDA

A previously validated HPLC-PDA method (Pavei et al., 2010) using chlorogenic acid, caffeic acid and rutin (Sigma–Aldrich, USA) as external standards was applied. All analyses were performed at

23 ± 1 °C. Polyphenol content was calculated by sum of individual contents, namely: chlorogenic acid, caffeic acid, rutin, peaks P1–P4 previously characterized as flavonoids by UV spectra (Pavei et al., 2010). Results were expressed as g% (w/w) of chlorogenic acid in dry material by the mean of three consecutive determinations.

2.2.2. Quantification of the condensed tannin content: the acid vanillin method

The analyses were performed as described elsewhere (Sun et al., 1998). Briefly, 1 mL-aliqouts of EXT, WSF and WIF were properly diluted in methanol (Nuclear®) and treated with 2.5 mL of reagent A (vanillin in methanol) and 2.5 mL of reagent B (HCl in methanol). The solution was heated at 60 °C for 10 min and the absorbance measured at 530 nm, at 25 °C (Hewlett Packard 8452A-Diode Array Spectrophotometer). Epicatechin (Sigma–Aldrich, USA) was used as reference substance, and water was used as blank. Results were expressed as g% (w/w) of epicatechin in dry material by the mean of three consecutive determinations.

2.2.3. Content assay of oxindole alkaloids by HPLC-PDA

The oxindole alkaloid content was determined using an HPLC-PDA method (Kaiser et al., 2013) employing mitraphylline (Sigma–Aldrich, USA) as external standard. Alkaloid content was calculated by the sum of individual alkaloid concentrations (ex: speciophylline, uncarine F, mitraphylline, isomitraphylline, pteropodine, isopteropodine). The results were expressed g% (w/w) of mitraphylline in dry material by mean of three determinations.

2.2.4. Content assay of quinovic acid glycosides (QAG) by HPLC-PDA

Quinovic acid glycoside content was analyzed using an HPLC-PDA method (Pavei et al., 2012) employing α -hederin (Sigma–Aldrich, USA) as external standard. The QAG content was calculated by the sum of individual concentrations of seven major peaks previously characterized as quinovic acid glycosides through UV and MS–MS data (Pavei et al., 2012), and expressed as g% (w/w) of α -hederin in dry material by mean of three determinations.

2.3. Microorganisms

The 13 yeasts tested included: *Candida krusei* ATCC 6258, CK01, CK02, CK04; *Candida glabrata* CG40039, CG10, CG03, RL02, RL03, *Candida parapsilosis* RL13, CP03, CP04, RL01. The isolates were obtained from the culture collections of the Laboratory of Applied Mycological Research, Universidade Federal do Rio Grande do Sul (UFRGS), Porto Alegre, Brazil.

2.4. Antifungal susceptibility tests

All samples from *U. tomentosa* were prepared at a concentration of 1.0 mg/mL using dimethylsulfoxide (DMSO) (Synth, Brazil) 2% (v/v) as a solvent. Terbinafine (TRB), fluconazole (FLZ) (Cristalia, Brazil), and anidulafungin (AND) (Pfizer, Brazil) were properly dissolved in water (control substances).

2.4.1. Screening

Screening for antifungal activity was carried out using broth microdilution, with each well containing approximately 10^3 yeast cells-inoculum and the respective test compound at 500 mg/L in RPMI 1640 (medium containing L-glutamine and without sodium bicarbonate), buffered to pH 7.0 with 0.165 M MOPS (Ludwig Biotec, Brazil). The plates were incubated at 35 °C for 48 h. The assay was performed in triplicate.

2.4.2. Minimal inhibitory concentration (MIC)

Reference drugs, EXT and fractions QAPF, OAPF, WSF, and WIF were assayed at concentrations ranging from 500 to 0.98 mg/L, and 10^3 yeast cells-inoculum of those *Candida* species that showed susceptibility in the screening test, by the broth microdilution method (CLSI, 2008a). MIC was defined as the lowest concentration of sample at which the microorganism tested did not demonstrate visible growth. Each assay was performed in triplicate.

2.4.3. Sorbitol: osmotic protector of fungal cell wall

MIC values of WIF fraction against *C. krusei*, *C. glabrata* and *C. parapsilosis* were determined in the absence and in the presence of 0.8 M of sorbitol (used as osmotic support) (Sigma–Aldrich, USA) added to the assay medium. AND was used as control drug. MICs were read at 2 and 7 days.

2.4.4. Scanning electron microscopy

A sample of *C. krusei* strain (CK04) treated with a subinhibitory concentration of WIF (positive control) was fixed with glutaraldehyde 2.5% for 24 h at 10 °C. Post-fixation was performed with osmium tetroxide 1% in cacodylate buffer solution for 1 h. Subsequently, the samples were dehydrated in upward gradient acetone, critical-point dried in CO₂, coated with gold and examined (Carl Zeiss AG-EVO 50, Germany) (Gil-Lamaignere and Müller, 2004). A sample of the same isolate but without any further treatment was fixed and prepared as described above (negative control).

2.4.5. Infrared spectroscopy (FT-IR) analysis of the pseudomycelia

C. krusei strain (CK04) treated with sub-inhibitory concentration (4 mg/L) of WIF was purposely cultivated as follows. A 100 µL-aliquot of fungal suspension containing 1×10^6 to 5×10^6 CFU/mL was uniformly applied on petri dishes containing potato dextrose agar. Separately, 100 µL of WIF fraction at sub-inhibitory concentration and ultrapure water (Milli Q, Millipore, Bedford, MA, USA) were placed in a 7 mm diameter central hole, excavated beforehand for that purpose. Next, each plate was incubated at 32 °C for 48 h. The pseudo-hyphae samples were carefully collected by scraping, transferred into sterile tubes, frozen at –20 °C, freeze dried as usual and stored until analysis. The assay was performed in triplicate (Galindo et al., 2013).

FT-IR spectra were recorded in a frequency range of 4000–600 cm^{–1}, using a resolution of 4 cm^{–1} and 40 accumulations (IR spectrophotometer, Shimadzu DR-8001, USA). KBr discs containing about 1.5 mg of each sample were prepared by compression and analyzed immediately.

2.5. Toxicity evaluation

For the toxicity test, EXT, WSF and WIF fractions were evaluated. All procedures were previously approved by the Research Ethics Committee of the Federal University of Santa Maria (protocol no.

23081), and informed consent was obtained from all individuals whose information was collected prospectively.

Human peripheral blood samples were collected by venipuncture using a syringe (BD Diagnostics, Plymouth, UK) and tubes with heparin. Blood specimens (3 mL) were centrifuged within 30 min of collection for 10 min at 3000 rpm. Next, leukocytes were isolated to obtain a 8×10^3 cell mm³. One aliquot of each sample was used for DNA damage analysis.

2.5.1. Single cell gel electrophoresis (comet assay)

The alkaline comet assay was performed as described earlier (Dos Santos Montagner et al., 2010) in accordance with the general guidelines for use by the comet assay (Tice et al., 2000; Hartmann et al., 2003; Nadin et al., 2001). One hundred cells (50 cells from each of the two replicate slides) were selected and analyzed. The human leukocytes were visually scored according to tail length and received scores from 0 (no migration) to 4 (maximal migration). Therefore, the damage index for cells ranged from 0 (all cells with no migration) to 400 (all cells with maximal migration). This assay was performed in triplicate. Each slide was analyzed by two different individuals at least.

2.5.2. Viability test

The cell viability assay was carried out by the method of Mischell and Shiingi (Bishell et al., 1980), with minor modifications. Cells were exposed to the same concentration of both compounds as in the comet test for 1 h. After incubation, 100 µL of the cell suspension was mixed with 100 µL of Trypan Blue solution 0.4% for 3 min. Cell viability was determined microscopically (400× magnification) and two categories of cells were scored: (1) live cells, which appeared uncolored or light blue, (2) dead cells, which appeared blue. At least 300 cells were counted for each survival determination.

2.6. Statistical analysis

The statistical analysis was performed by Wilcoxon test and by ANOVA followed by Tukey's test (Minitab 15.0 software, USA). Data are expressed as mean ± SEM. Differences were considered statistically significant when $p < 0.05$.

3. Results

3.1. Chemical characterization of EXT and fractions of *U. tomentosa*

Results of the content determination assays of EXT and related fractions are shown in Table 1.

Compared to the crude extract and other fractions, the proanthocyanidin (high molecular mass polyphenols) content in WIF was the highest of all the compounds and exceeded that of the low molecular mass polyphenols. Apparently, the oxindole alkaloid and

Table 1

Alkaloid, triterpene and polyphenol contents in the crude extract and related fractions from cat's claw bark.

Samples	Oxindole alkaloids g% (w/w)	Quinovic acid glycosides g% (w/w)	LMM polyphenols ^a g% (w/w)	Proanthocyanidins ^b g% (w/w)
EXT	2.49 ± 0.04	2.12 ± 0.28	4.72 ± 0.18	34.11 ± 5.16
WIF	7.91 ± 0.08	7.79 ± 1.78	3.77 ± 0.14	70.80 ± 6.73
WSF	2.66 ± 0.05	0.57 ± 0.36	4.96 ± 0.14	21.07 ± 3.60
OAPF	75.26 ± 3.26	ND	ND	ND
QAPF	ND	21.78 ± 0.46	ND	ND

Result expressed as $\bar{x} \pm SD$ from three determinations; ND: not detected; LMM: low molecular mass.

^a Contents of low molecular mass polyphenols determined by HPLC-PDA.

^b Contents of proanthocyanidins determined by vanillin assay. EXT: hydroethanolic crude extract; WIF: water-insoluble fraction; WSF: water-soluble fraction; OAPF: oxindole alkaloid purified fraction; QAPF: Quinovic acid glycosides purified fraction.

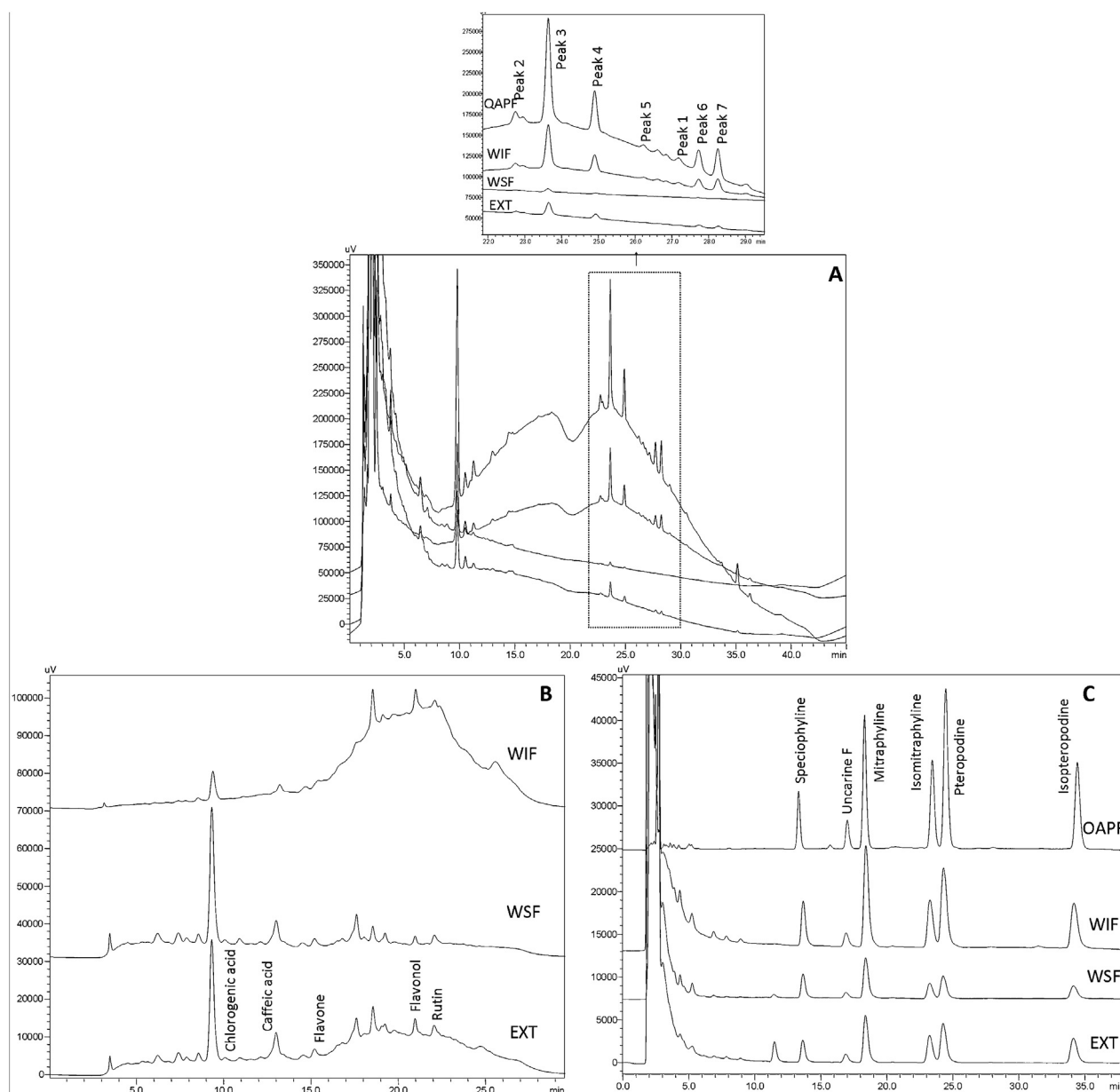


Fig. 1. HPLC-PDA profile of oxicindole alkaloid, quinovic glycoside and polyphenol contents of EXT and related fractions. (A) Comparison among the QAPF, WIF, WSF and EXT in relation to quinovic acid glycosides, (B) to the low molecular mass polyphenol content, and (C) to the oxicindole alkaloid content.

quinovic acid glycoside contents in WIF are also higher than in EXT and WSF. However, this can be explained by the concentration processing to obtain WIF, and the low water solubility of quinovic acid glycosides.

Analyzing the chromatographic profile of different fractions and EXT of *U. tomentosa*, some relevant differences are noticeable (Fig. 1).

HPLC-chromatograms of QAPF revealed that a smaller portion of these compounds (peaks 1–7) remained in WIF but not in WSF (Fig. 1A). The comparison of OAPF, WSF and WIF (Fig. 1C) showed that the major cat's claw alkaloids were distributed proportionally according to their solubility in water. As a result, they still were an important portion of WIF composition. In addition, the low mass polyphenol contents (Fig. 1B), were drastically reduced in WIF, as observed for the more water-soluble chlorogenic acid, caffeic acid, and rutin. Conversely, peaks related to the high molecular mass proanthocyanidins (Fig. 1B, 15–25 min-retention time) were

prevalent in WIF, thus corroborating the content difference described earlier in Table 1.

3.2. Antifungal susceptibility tests

The susceptibility test for antifungal activity of the four selected *Candida* spp. showed MIC values ranging from 3.90 to 500 mg/L (Table 2), except for *C. tropicalis* that showed no activity (data not shown).

Compared to the crude extract and other fractions the insoluble polyphenol fraction (WIF) showed the highest antifungal activity (lowest MIC values). Nonetheless, the antifungal activity evidenced for EXT was lower when compared to the related fractions. This finding almost certainly results from the aforementioned concentration and fractioning process.

A categorical conclusion after comparison of these results to cat's claw itself and other vegetable species seems to be undefined

Table 2
Antifungal activity of hydroethanolic crude extract and related fractions against non-albicans *Candida* isolates.

Specie	Samples						Antifungal		
	Isolates	EXT (mg/L)	QAPF (mg/L)	OAPF (mg/L)	WSF (mg/L)	WIF (mg/L)	FLZ (mg/L)	TRB (mg/L)	AND (mg/L)
<i>Candida parapsilosis</i>	RL13	>500	>500	>500	>500	>500	8	32	2
	CP03	>500	62.5	62.5	>500	3.9	2	2	2
	CP04	>500	125	125	>500	3.9	2	1	2
	RL01	>500	62.5	62.5	>500	7.81	8	32	0.5
<i>Candida glabrata</i>	CG40039	7.81	125	62.5	125	3.9	64	32	0.5
	CG10	15.62	125	125	500	15.62	1	32	0.125
	RL02	>500	125	125	>500	7.81	8	1	0.25
	RL03	>500	125	62.5	>500	7.81	1	32	0.25
<i>Candida krusei</i>	CK02	3.9	>500	125	15.62	3.9	2	1	2
	ATCC6258	7.81	>500	62.5	31.25	3.9	64	32	0.5
	CK01	>500	>500	62.5	250	7.81	64	32	0.25
	CK04	15.62	>500	31.25	>500	15.62	64	32	1
	Median*	500 ^a	125 ^{a,b}	62.50 ^c	500 ^b	7.81 ^d	8 ^{d,e}	32 ^f	0.5 ^g

* Same letters represent statistically equivalent MIC values according to the Wilcoxon test ($p > 0.05$). EXT: hydroethanolic crude extract; WIF: water-insoluble fraction; WSF: water-soluble fraction; OAPF: oxindole alkaloid purified fraction; QAPF: quinovic acid glycosides purified fraction; TRB: terbinafine; FLZ: fluconazole; AND: anidulafungin.

Table 3
Minimum inhibitory concentration values (MIC) determined at 2 and 7 day-treatment of *Candida* spp. isolates with anidulafungin (AND) and the water-insoluble fraction (WIF), in the presence and absence of sorbitol solution.

Isolates	MIC AND 2 days/7days (mg/mL)		MIC WIF 2 days/7days (mg/mL)	
	Without sorbitol	With sorbitol (0.8 M)	Without sorbitol	With sorbitol (0.8 M)
	RL13	2/2	2/16	>500/>500
CP03	2/2	4/32	3.9/3.9	3.9/31.25
CP04	2/2	4/16	3.9/3.9	15.62/125
RL01	0.5/0.5	1/8	7.81/7.81	15.62/125
CG40039	0.5/0.5	1/4	3.9/3.9	15.62/31.25
CG10	0.125/0.125	0.25/2	15.62/15.62	62.5/125
RL02	0.25/0.25	0.5/2	7.81/15.62	15.62/125
RL03	0.25/0.25	0.5/2	7.81/7.81	15.62/62.5
CK02	2/2	4/16	3.9/7.81	7.81/31.25
ATCC6258	0.5/0.5	1/4	3.9/3.9	7.81/31.25
CK01	0.25/0.25	0.25/8	7.81/7.81	15.62/62.5
CK04	1/1	2/8	15.62/15.62	62.5/125

owing the scarce number of antifungal data related to cat's claw and derived fractions, as well the widespread use of chemical undefined crude extracts from other species.

A comparison with the antifungals FLZ, TRB and AND allowed further inferences regarding antifungal resistance (Table 2). The resistance criteria were those found in CLSI M27-A3 and M38-A2 guidelines, except for TRG. *Candida* spp. was considered resistant to TRB if the MIC value ≥ 16 mg/L (Gil-Lamaignere and Müller, 2004). Thus, six isolates multiresistant to FLZ and TRB could be detected according to CLSI criteria, namely, FLZ at a concentration >4 mg/L for *C. parapsilosis* and >32 mg/L for *C. glabrata*; anidulafungin >0.25 mg/L for *C. glabrata* and >4 mg/L for *C. parapsilosi* and >0.5 mg/L for *C. krusei*. Five of the above-mentioned multiresistant isolates (RL01, CG40039, ATCC6258, CK01 and CK04) were susceptible to WIF at concentrations ranging from 3.9 to 15.6 (mg/L). In addition, of the isolates shown in the table, two are multiresistant to FLZ, TRB and AND (CK04 and CG40039), since they are sensitive to the fraction of WIF at low concentrations.

In addition, *C. glabrata* isolates resistant to FLZ were susceptible at low concentrations of WIF (3.9–7.8 mg/L). Similarly, *C. krusei* isolates resistant to FLZ were sensitive to WIF at 3.9–15.6 mg/L, despite being naturally resistant to FLZ (Morschhäuser, 2002). Conversely the EXT and WSF had no activity against *C. parapsilosis*.

Noteworthy, all *Candida* resistant isolates, which were tested in this work, were susceptible to QAPF and OAPF to different degrees, except for *C. krusei*, which proved to be resistant to QAPF.

Nevertheless, these results were inferior to those recorded from WIF.

3.2.1. Sorbitol protection assay

Cell wall weakness can be enhanced by the addition of sorbitol. In the presence of 0.8 M sorbitol, the MIC values of WIF against *C. krusei* were increased about 8–32 times (Table 3) as well as that of anidulafungin (control).

3.2.2. Cytotoxic and genotoxic effect of EXT, WSF and WIF

The percentage of viable cells for both control groups and for each group of 3 trials, each one with different concentrations of the extract and fractions, is shown in Fig. 1A. According to Collins et al. (2008), high viability is required as a precondition for performing the comet assay. Thus, the positive control presented the right conditions for this purpose. The two lower concentrations tested (4 and 40 mg/L) represent those with anti-*Candida* activity, while the third one was set deliberately higher aiming to challenge cell sensitivity (400 mg/L).

No perceptible cell injury could be detected after treatment with the crude extract (EXT) and related fractions from cat's claw bark, as well as any DNA fragments forming the 'comet'.

3.2.3. Ultrastructural analysis

The effect of WIF on the morphology and ultrastructure of *C. krusei*, was evaluated by scanning electron microscopy. After

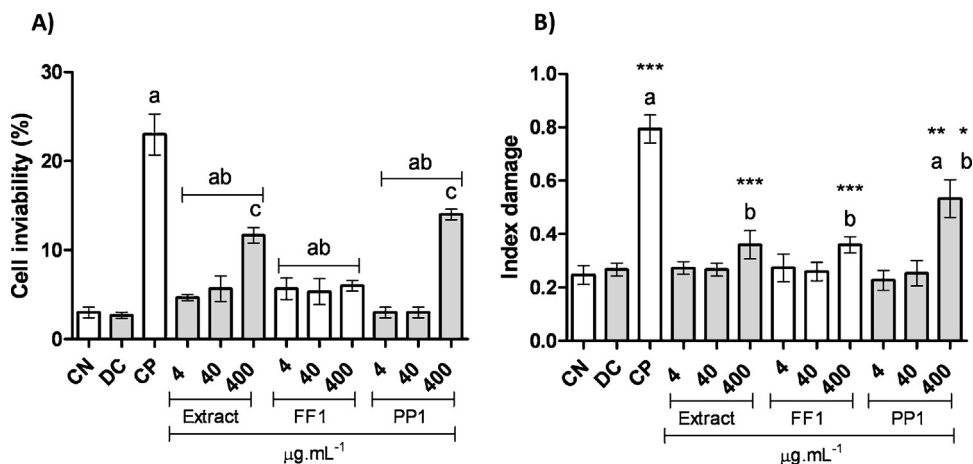


Fig. 2. (A) The cell viability assay of the crude extract (EXT) and related fractions at three different concentrations. (***) $p < 0.001$; ^astatistically different from NC; ^bstatistically different from CP; ^cstatistically different from other concentrations of the same extract or fraction. (B) Comet assay. * $p > 0.05$; ** $p > 0.01$; *** $p > 0.001$; ^astatistically different from negative control; ^bstatistically different from positive control. Positive control (CP); negative control (CN); DMSO control (DC) and extract concentrations. One-way ANOVA followed by Bonferroni multiple comparison test. The data are expressed as percentage; $n = 3$.

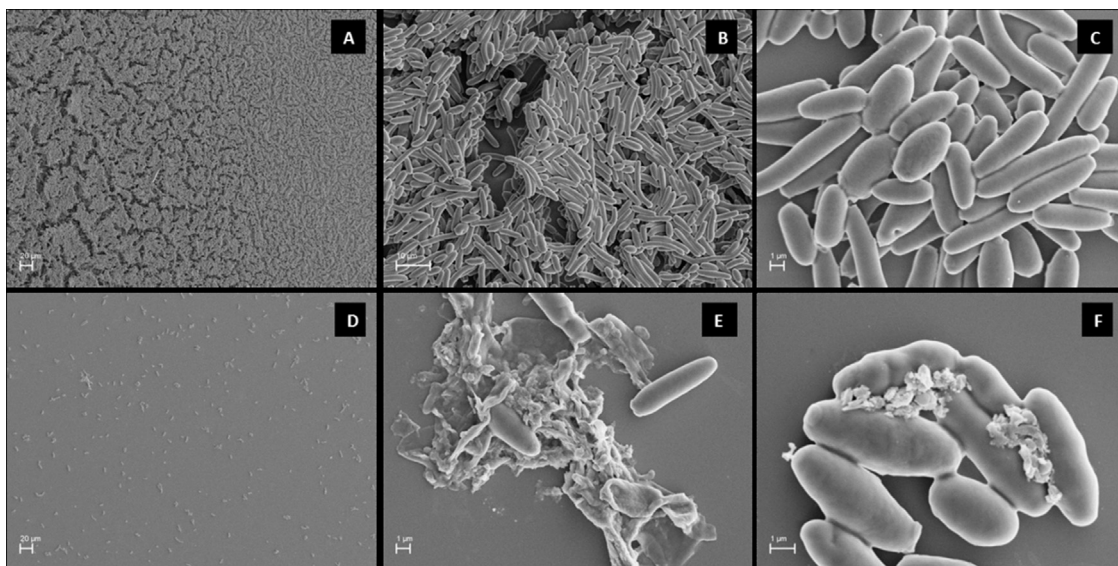


Fig. 3. SEM-micrographs of *Candida krusei* (CK04) treated with fraction WIF from *Uncaria tomentosa*. (a) Control 450 \times ; (b) Control 2500 \times ; (c) Control 10,000 \times ; (d) WIF 7.81 mg/L 450 \times ; (e) WIF 7.81 mg/L 10,000 \times ; (f) WIF 7.81 mg/L 18,000 \times .

treatment with WIF the decreasing number of cells (Fig. 3A and D), and material deposited on the cell walls of the fungi was visible compared to the control (Fig. 3F). In addition, yeasts treated with WIF underwent morphological alterations and loss of integrity of the cell wall (Fig. 3E and F). Conversely, untreated cells (Fig. 3A–C) showed a normal shape and a well-defined smooth surface.

3.2.4. The FT-IR analysis of pseudomycelium

The FT-IR analysis of the pseudomycelium of fungus treated with 7.81 mg/L of WIF (here exemplified with *C. krusei* isolate CK 04) exhibited a different spectrum pattern compared to untreated fungus used as control (Fig. 4). No regions with major changes were detected either in wavelength regions ascribed to carboxyl, hydroxyl, phenol and amide functional groups (3700–2996 cm^{-1}) or to lipids (2996–2800 cm^{-1}). On the other hand, a broader signal in 1407 cm^{-1} and disappearance of the peak at 1542 cm^{-1} were associated with changes in the protein cell wall. In addition, four new peaks located at 1185–900 cm^{-1} were observed in pseudomycelium. The four new peaks located at 1185–900 cm^{-1} could not be assigned to any of the compound classes detected in WIF

and therefore seem to be a characteristic of the effect on the cell wall after treatment with that fraction.

4. Discussion

In the past 20 years, various types of fungal infection occurring in patients with immunosuppressive disorders have received increasing attention. The causal agents were mostly non-*albicans* *Candida* species, in particular *C. glabrata*, *C. parapsilosis* and *C. krusei* (Nucci and Colombo, 2002; Sharma et al., 2009; Zhang et al., 2014). Although fluconazole was widely indicated for the treatment of infections caused by *Candida* species, today it is considered ineffective against *C. krusei* and the therapeutic response regarding *C. glabrata* is rather variable (Rodriguez-Tudela et al., 2007). In that context, the results gained using defined fractions from cat's claw can provide a new perspective to overcome antifungal resistance.

In the present study we investigated the antifungal activity of a crude extract and fractions from cat's claw bark in some multi-resistant non-*albicans* *Candida* species. The crude extract (EXT) and

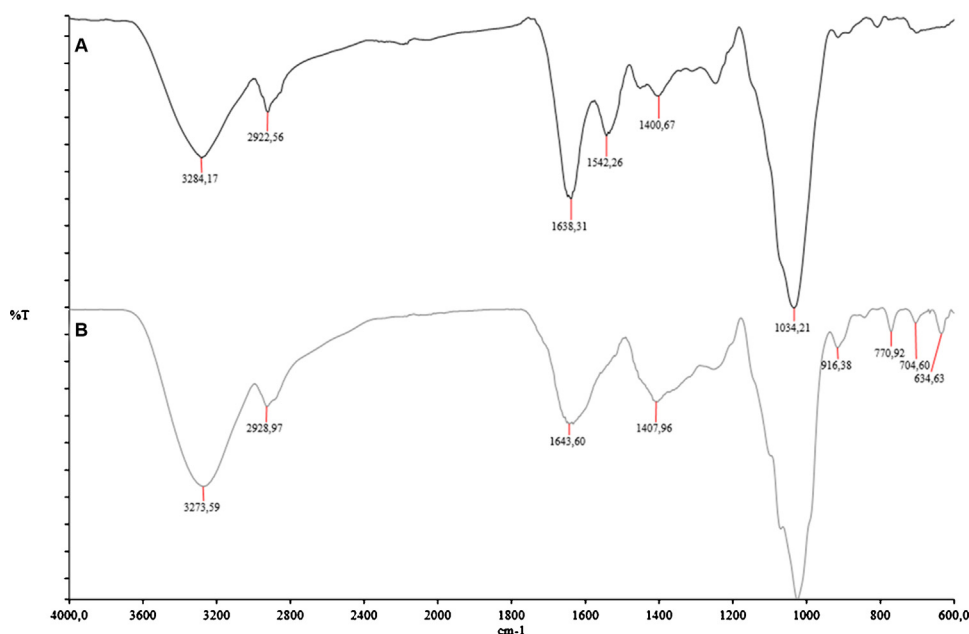


Fig. 4. FT-IR-spectra of *Candida krusei* (CK 04) pseudo-mycelium exposed to (A) no treatment control and (B) WIF 7.81 mg/L.

enriched fractions of quinovic acid glycosides (QAPF) and alkaloids (OAPF) were prepared for this purpose, and EXT was also separated into a water soluble fraction (WSF) and a water-insoluble fraction (WIF). Remarkably, the latter fraction has been omitted in many chemical and biological studies, so there are few reports in the literature referring to its characterization and composition. Nonetheless, WIF was the fraction with the highest antifungal activity (MICs from 3.90 to 15.62 mg/L). Both HPLC and chemical analyses demonstrated that this fraction was predominantly composed of high molecular weight polyphenols (70.8% w/w), oxindole alkaloids (7.9% w/w) and quinovic acid glycosides (7.8% w/w). Taking into account that fractions OAPF (alkaloid total content of 75.3%) and QAPF (quinovic acid content of 21.8%) were less effective than WIF, the antifungal activity of WIF should be ascribed to the water-insoluble polyphenols. In turn, the low antifungal activity of WSF (500 mg/L) most likely derived from its lower content of insoluble polyphenols (21.07% w/w). Since the water-soluble polyphenol content was higher in WSF (4.96%) than in WIF (3.77%), it is almost certain that these compounds are unrelated to the antifungal activity observed with WIF.

While the antifungal activity of high molecular mass polyphenols has been documented elsewhere, particularly for water-soluble extracts, the study on the activity of the water-insoluble fraction has been relegated to a secondary role, and currently information about the structure-activity relationship regarding specific polyphenols is scarce (Mendoza et al., 2013; Liu et al., 2009; Razzaghi-Abyaneh and Rai, 2013).

When looking for new drugs, besides biological activity, it is important to verify that the drug is not harmful to human cells (Ndhala et al., 2013). Some tests, such as the comet assay, are able to identify genotoxic agents and substances having potential risks to human health. Thus, regarding the crude extract and related fractions prepared from cat's claw bark specifically, no evidence of cytotoxic and genotoxic effects on leukocytes was found at concentrations up to 400 mg/L (Fig. 2). This finding corroborated previous studies, although these were performed on water-soluble fractions from *U. tomentosa* at concentrations as high as 100 mg/mL.

Compared to fluconazole and terbinafine, about 66% of the *Candida* isolates were more susceptible to WIF (Table 1). In addition, all

strains resistant to anidulafungin were susceptible to WIF fraction, while the crude extract and other fractions showed lower activity. Therefore, it seems reasonable to ascribe the antifungal activity of WIF to the water insoluble polyphenols (high molecular mass polyphenols) from cat's claw bark.

The FT-IR analysis of the fungal pseudomycelium of a *C. krusei* multiresistant isolate showed major changes in the bands associated with the proteins and polysaccharides of the fungal cell membrane (Fig. 4) (Galindo et al., 2013) caused by treatment with WIF. In that sense, some literature data (Souza-Moreira et al., 2013; Haslam, 1996) allow us to support a reliable interaction between proanthocyanidins and proteins and polysaccharides of the fungal cell wall, thus reducing the matrix strengthening process. Since the cell wall composition varied from specie to specie regarding chitin, glucans, galactomannans (Adams, 2004; Fukuda et al., 2009), it is expected that the activity of WIF will be displayed differently according to the species and isolates studied.

Antifungal resistance against drugs from the echinocandin class has been ascribed to the mutation-induced thickening of the cell wall (Liu et al., 2009) and blocking of the inhibition of beta-(1–3)-D-glucan synthase (Frost et al., 1995). In that sense, fungal cell death caused by the cell wall damage induced by inhibitors of its synthesis can be avoided using osmoprotecting substances such as sorbitol. Since the sorbitol supply was able to inhibit the antifungal activity of WIF, this finding reinforces the results gained from the FT-IR analysis. Moreover, a similar mechanism based on the interaction of high molecular weight polyphenols with the outer glycoprotein-rich layer of *C. albicans* was initially proposed respecting some rich tannin plants, such as *Stryphnodendron adstringens* (Ishida et al., 2006) and *Pinus cauliflora* (Souza-Moreira et al., 2013). In addition, Haslam (1996) and Quideau et al. (2011) suggested that tannins are able to complex with other molecules, including macromolecules such as proteins and polysaccharides present in the cell wall of yeasts.

Cells treated with drugs that interfere in cell wall synthesis often have different morphological characteristics. The SEM-micrographs (Fig. 3) from the *C. krusei* pseudomycelium previously treated with WIF revealed the presence of material deposited on the cell wall (Fig. 3F), with loss of cell integrity (Fig. 4) and cell lysis (Fig. 3E).

5. Conclusion

The evidence gained in this study allows establishing consistently the antifungal activity of the water insoluble, rich polyphenol, and little studied fraction from *U. tomentosa* bark against some selected non-*albicans* *Candida* species. The superior inhibition of the cell growth seems to be related to morphological and chemical changes in the cell wall, without indication of cell-toxicity by the comet test.

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