

Susceptibility of *Candida albicans* Isolated from Blood to *Wickerhamomyces anomalous* Mycocins

Ana Paula Paris¹ · Cristiane Persel¹ · Cleber Fernando Serafin¹ · Rita de Cássia Garcia Simão² · Rinaldo Ferreira Gandra¹

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Abstract The occurrence of infections caused by *Candida albicans* in developed and developing countries and their resistance to some available antifungal drugs have been viewed as causing a great problem to human health worldwide. In order to find new researched molecules, there are some mycoses secreted by yeasts, especially mycocins produced by *Wickerhamomyces anomalous* with a broad antimicrobial spectrum of activity. Thus, this trial aimed at evaluating mycocins' activity obtained from environmental *W. anomalous* cell wall compared to thirty *C. albicans* strains isolated from blood. Mycocins were extracted from cell walls of three *W. anomalous* strains (WA40, WA45, and WA92). The 400 $\mu\text{g mL}^{-1}$ concentration of WA40M1, WA45M2, and WA92M3 mycocin extracts showed the following respective activity results: 96.6, 96.6, and 90.0 % *C. albicans* strains. WA45M2 and WA92M3 mycocin extracts showed some activity in 3.3 %

of *C. albicans* strains at 50 $\mu\text{g mL}^{-1}$ concentration. Mycocins extracted from cell walls of three *W. anomalous* strains named as WA40, WA45, and WA92 showed antifungal activity compared to *C. albicans* and low degree of hemolysis.

Introduction

Candida albicans fungus forms part of human microbiota, and it is also present in mucous membranes of gastrointestinal and genitourinary tracts of healthy individuals. It can cause potentially fatal infections in immune-compromised patients due to factors such as the Acquired Immunodeficiency Syndrome (AIDS), organ transplant, and chemotherapy treatment of cancer. Besides, *C. albicans* is one of the main causes of nosocomial infections as well as the most prevalent and pathogenic species of *Candida* genus [19, 21, 35].

Invasive fungal infections are essential in patients with impaired immunity. However, the available antifungal agents are limited due to the resistance of these fungi to themselves [15]. Thus, some researchers have been looking for new substances against multiresistant microorganisms and with less toxicity to human beings. Ergo, mycocins secreted by yeast are also studied as new molecules and potential candidates due to their broad spectrum of action [32].

Some yeasts can produce mycocins, since they consist of low molecular weight proteins or glycoproteins that inhibit their own growth [5]. This inhibition phenomenon was discovered by Bevan and Makower [1] in strains of *Saccharomyces cerevisiae*; since then, several genera such as *Wickerhamomyces*, *Candida*, *Pichia*, *Hansenula*, *Cryptococcus*, *Kluyveromyces*, *Schwanniomyces*, *Torulopsis*, and

✉ Ana Paula Paris
anapaula.paris@hotmail.com
Cristiane Persel
ctn.persel@hotmail.com
Cleber Fernando Serafin
cleberserafin@hotmail.com
Rita de Cássia Garcia Simão
ritabioq@yahoo.com.br
Rinaldo Ferreira Gandra
rinaldo.gandra@yahoo.com.br

¹ Hospital Universitário do Oeste do Paraná, Universidade Estadual do Oeste do Paraná–UNIOESTE, Av. Tancredo Neves, 3224, Cascavel, PR 85806-470, Brazil

² Laboratório de Bioquímica Molecular, Centro de Ciências Médicas e Farmacêuticas, Universidade Estadual do Oeste do Paraná, UNIOESTE, Cascavel, PR, Brazil

Williopsis were reported to be showing their inhibitory activities to other microorganisms [6, 9, 11, 13, 16, 27].

Wickerhamomyces anomalus can be isolated from different habitats as soil, plants, flowers, fruit peels, food-stuffs, contaminated oil, dairy products, wastewater, human tissues, insects, and marine environments [8, 17, 34]. Mycocins produced by this yeast have broad antimicrobial spectrum concerning their activity with high stability compared with mycocins from other yeasts; Furthermore, they have shown great promise by virtue of their potential applications in the fields of health and biotechnology [14, 30]. In the present study is reported mycocins' activity from environmental *W. anomalus* in relation to *C. albicans* strains, isolated from blood.

Materials and Methods

Isolation of Soil Yeast

Two hundred soil samples were collected on the shores of Itaipu Lake-Paraná, Brazil, stored in bags hermetically sealed and put under cold storage in polystyrene boxes. First, the soil was homogenized, and 50 g was added into 250 mL of 0.9 % saline solution, which contained 0.1 % chloramphenicol and 0.05 % cycloheximide. This solution was kept under stirring at 150 r/min, at 25 °C for 2 h. Suspension was serially diluted to obtain 10^{-3} , 10^{-4} , and 10^{-5} UFC mL⁻¹ concentrations. Thereafter, they were plated in triplicates onto Sabouraud dextrose agar and incubated at 25 °C for 72 h. After this period, another isolation was carried out in Sabouraud agar, in which micro-morphological characteristics of the colonies were similar to yeasts. Thus, 74 strains of wild yeast were isolated again.

Screening of Mycocin-Producing Yeast

In order to record mycocin production, 74 wild yeasts isolated from soil were tested against four *C. albicans* strains, which were also isolated from soil. The wild yeasts were kept at 25 °C for 48 h in modified agar (2 % agar, 1 % peptone, 2 % glucose, 1.92 % citric acid and 3.48 % dibasic potassium phosphate, pH 4.7) before testing. For each *C. albicans* strain, one saline suspension was prepared containing 10^5 UFC mL⁻¹. Then, this suspension was mixed with agar in Petri dishes, modified by methylene blue (2 % agar, 1 % peptone, 2 % glucose, 1.92 % citric acid, dibasic potassium phosphate, 3.48 % methylene blue 0.003 %, and pH 4.7). When the temperature reached almost 55 °C, after homogenization and solidification, the wild yeasts were inoculated at equidistant points and incubated at 25 °C for 72 h.

The wild yeasts were considered as mycocin producers when they produced some colorless halo and/or an inhibition zone with blue colonies around itself. This showed that *C. albicans* strains were sensitive, and all the tests were carried out in triplicate. Among all the studied wild yeasts, three of them produced mycocins compared with the four tested *C. albicans* strains. Consequently, they were chosen to be completely identified and submitted to the other tests.

Identification of Mycocin-Producing Yeasts

Molecular identification was obtained by genomic DNAs of three wild yeasts, which were amplified at ITS region and comprised intergenic spacers ITS1 (18S rRNA) and ITS2 (5.8S rRNA), so that oligonucleotides were used as primers ITS1 (5'-TCC GTA GGT GAA CCT GCG G-3') and ITS4 (5'-TCC TCC GCT TAT TGA TAT GC-3').

Products of amplifications were sequenced, and their respective sequences were analyzed by the BLAST program in order to compare them to the sequences that were put in GenBank. The three wild yeasts, mycocin producers, showed 98, 99, and 99 % of similarity with *W. anomalus*, respective sequences of which were also put in GenBank. Thus, the numbers of access for the sequences of WA40, WA45, and WA92 *W. anomalus* nucleotides are KT580792, KT580794, and KT580796, respectively. Available at <http://www.ncbi.nlm.nih.gov/BLAST>.

Obtaining Mycocin Extracts

Mycocin extracts were obtained from the cell walls of strains WA40, WA45, and WA95 of *W. anomalus* isolated from the environment. *W. anomalus* strains were previously kept at 25 °C in modified agar (2 % agar, 1 % peptone, 2 % glucose, 1.92 % citric acid, and 3.48 % dibasic potassium phosphate, pH 4.7). The 10^6 UFC mL⁻¹ suspensions were obtained from cultures of isolated *W. anomalus* strains (WA40, WA45, and WA92) and transferred to the respective flasks containing 200 mL of modified broth (1 % bacteriological peptone, 2 % glucose, 1.92 % acid citric, and 3.48 % bibasic potassium phosphate, pH 4.7), incubated at 25 °C for 5 days under static cultivation.

Afterward, the studied cultures were centrifuged at 6000 r/min for 10 min, and the precipitated WA40, WA45, and WA92 *W. anomalus* cells were washed three times with sterile distilled water and oven dried at 40 °C. The dried cells were mixed to Coca liquid extractor (0.033 M sodium bicarbonate, 0.085 M sodium chloride, pH 9.1) 5 % (w/v) and kept at 4 °C for a duration of one week. Then, they were centrifuged at 6000 r/min for 10 min. Thus, WA40M1, WA45M2, and WA92M3 extracts of mycocins were obtained from the cell walls of WA40, WA45, and WA92

strains of *W. anomalous*, respectively. Mycocin extracts were sterilized by filtration through a 0.22- μm membrane and stored at 4 °C until further in vitro tests.

Biochemical Analyses of Mycocin Extracts

The concentrations of proteins and carbohydrates present in mycocin extracts obtained from cell walls of different strains of *W. anomalous* cell walls were determined by both Bradford's [4] and anthrone methods [29], respectively.

Antimicrobial Activity of Mycocin Extracts

In this trial, 30 strains of *C. albicans* were used, isolated from patients' blood admitted to the Intensive Care Unit [15 adults were from ICU: 8 of them were from NICU (neonatal) and 7 from PICU—pediatric] from Western Paraná University Hospital. Thus, 30 strains were identified by auxanogram search regarding the formation of germ tubes and chlamyospore production, according to the technical manual "The Yeasts" [17].

The susceptibilities of 30 *C. albicans* strains, corresponding to mycocin extracts of WA40M1, WA45M2, and WA92M3 *W. anomalous*, were tested by microdilution technique in broth, described by Clinical and Laboratory Standards Institute [10]—the M27-A3 method with some changes. Cell suspensions of each *C. albicans* strain were prepared in Mueller–Hinton broth and adjusted to obtain final concentrations of 10^3 UFC mL^{-1} .

The concentrations of mycocin extracts were based on the proteins present in the same extracts. Thus, they were tested at final concentrations of 10, 50, 100, 200, and 400 $\mu\text{g mL}^{-1}$. Sterile microplates were used from 96 wells. Each well received the inoculum, a culture medium consisting of Mueller–Hinton broth and mycocin extracts' concentrations. A growth control was performed with sterile medium and inoculum of each *C. albicans* strain as well as sterility control containing only sterile medium. The Petri dishes subjected to microdilution were incubated at 35 °C; after 48 h, we could observe the presence or the absence of visible growth as growth control reference. Also, 10 μL aliquots from the wells in which there was no turbidity were plated on nutrient agar to confirm the absence of growth. The test was carried out on samples in triplicate.

Viability Determination

C. albicans viability, compared to WA40M1, WA45M2, and WA92M3 mycocin extracts from WA40, WA45, and WA92 of *W. anomalous*, was determined by the fluorescence method (diacetate ethidium bromide fluorescein) described by Calich et al. [7] with some changes. 200 μL aliquots of each extract of WA40M1, WA45M2, and

WA92M3 *W. anomalous* mycocins were adjusted to 400 $\mu\text{g mL}^{-1}$ concentration and mixed with the same volume of suspension containing 10^6 UFC mL^{-1} of *C. albicans*. Thus, there were eight essays for each mycocin extract—one for each incubation time (30 min, 1, 2, 4, 6, 8, 10, and 12 h) at 37 °C. In addition, for each incubation time, a control composed of yeast suspension and sterile water was prepared for comparison.

After the right time, 300 μL aliquots were withdrawn, mixed with the same volume of a fluorescein diacetate (0.005 %) and ethidium bromide (0.005 %) solution and incubated for 15 min at 25 °C. Afterward, a drop of such mixture was placed on a slide, covered with a coverslip and examined in an immunofluorescence microscope. Subsequently, 100 cells were counted, and the percentage of dead cells (red blood cells) was evaluated. This test was also carried out in triplicate.

Hemolytic Action

The cytotoxic effects of WA40M1, WA45M2, and WA92M3 mycocin extracts from WA40, WA45, and WA92 of *W. anomalous* cell walls were determined according to Latoud et al.'s [18] method, but with some modifications. Human erythrocytes, collected from a healthy individual, were washed three times with phosphate-buffered saline (PBS), pH 7.4, by centrifugation at 1500 r/min for 10 min. Therefore, a 2 % suspension of erythrocytes was incubated at 37 °C for one hour with extracts from mycocins at concentrations of 10, 50, 100, 200, and 400 $\mu\text{g mL}^{-1}$. A parallel test was carried out with amphotericin B at the same concentrations. Then, the cells were pelleted at 1500 r/min for 10 min. The supernatant was collected, and the absorbance was determined using a spectrophotometer at 450-nm wavelength.

The negative control consisted of erythrocytes suspension and PBS buffer, while the positive control consisted of lyse buffer (2 % acetic acid) to lyse completely the erythrocytes. Percentages of Hemolysis were calculated and plotted against protein concentrations used for determining cytotoxic effects of dose for human erythrocytes. The test was conducted in triplicate. The percentages of intact erythrocytes as well as red blood cell percentages of hemolysis were calculated according to the following formulas:

$$\begin{aligned} & \% \text{ Intact erythrocytes} \\ & = 1 - \left(\frac{A \text{ the extract from mycocins} - A \text{ PBS}}{A \text{ the buffer lysis} - A \text{ PBS}} \right) \times 100 \end{aligned}$$

$$\% \text{ Hemolysis} = 100 - (\% \text{ of intact erythrocytes})$$

Results

Biochemical Analyses of Mycocin Extracts from the Cell Wall

Protein contents of WA40M1, WA45M2, and WA92M3 of *W. anomalous* mycocin extracts were, respectively, 9.643, 6.308, and 4.107 mg mL⁻¹, while carbohydrate concentrations were, respectively, 1.678, 0.766, and 0.842 mg mL⁻¹ (Fig. 1).

Antimicrobial Activity of Mycocin Extracts

It was observed that, at 400 µg mL⁻¹ concentrations of WA40M1, WA45M2, and WA92M3 *W. anomalous*, mycocin extracts showed the following activities, respectively: 96.6, 96.6, and 90.0 % *C. albicans* strains, while for 200 µg mL⁻¹ concentrations, the highest percentage of susceptible strains was 70.0 % with WA45M2 mycocin extract. Also, WA45M2 and WA92M3 mycocin extracts showed 3.3 % susceptible *C. albicans* strains at 50 µg mL⁻¹ concentration. However, at 10 µg mL⁻¹ concentration, none of the mycocin extracts was able to inhibit *C. albicans* strains (Table 1). *C. albicans* (ATCC-90028) has been chosen as reference strain, and it was susceptible to WA40M1, WA45M2, and WA92M3 mycocins extracts at concentrations of 200, 400, and 100 µg mL⁻¹, respectively.

Viability Determination

Cell viability of *C. albicans* in the presence of mycocin extracts from WA40M1, WA45M2, and WA92M3 *W. anomalous* is shown in Fig. 2. After 30-min contact of mycocin extracts from WA40M1, WA45M2, and WA92M3 *W. anomalous* with yeast, the respective percentages of unviable cells were recorded as 26, 37, and

38 %. However, when there was a 12-h contact, 100 % *C. albicans* cells were found to be unviable.

Hemolytic Action

The cytotoxic effects of WA40M1, WA45M2, and WA92M3 mycocin extracts from WA40, WA45, and WA92 of *W. anomalous* in human erythrocytes have shown some very weak hemolytic activity up to 200 µg mL⁻¹ concentration. The highest percentage of hemolysis was 5.2 % with 400 µg mL⁻¹ regarding WA40M1 *W. anomalous* mycocin extract. However, the amphotericin B showed a 100 % hemolytic activity up from 100 µg mL⁻¹ concentration (Fig. 3).

Discussion

The breakthrough achieved with the discovery of new substances with antifungal activity has been of fundamental importance, since there are infections caused by *C. albicans* in developed and developing countries as well as restrictions of efficacy and serious side effects of available antifungal drugs [23].

Carbohydrates and proteins are required to form yeasts' cell walls. Some proteins are considered as enzymes that modify the wall structure or digest extracellular nutrients; they also have glycoproteins, which make part of the cell recognition mechanisms. The cell walls of ascomycetes, such as *W. anomalous*, consist mainly of (1–3)/(1–6) β-glucan-chitin, (1–3) α-glucan, and (glyco) proteins [20].

In this trial, it was possible to dose the amount of carbohydrates and proteins from mycocin extracts of WA40, WA45, and WA92 *W. anomalous* cell walls. This ensures the effectiveness of Coca fluid as liquid extractor. According to Mendes [22], when fungal cells are in contact with Coca liquid for a week at 4 °C, the extraction of cell wall components is optimized. In addition, it does not promote glycoproteins hydrolysis. Thus, it was possible to keep the antimicrobial potential of extracts from mycocins.

The thirty clinical *C. albicans* strains, isolated from blood and used in this study, were inhibited by one or all mycocin extracts from the environmental isolates of *W. anomalous* (WA40, WA45, and WA92). Since mycocin extracts of WA40M1, WA45M2, and WA92M3 *W. anomalous* at 400 µg mL⁻¹ concentration have shown the best antifungal activity, thus, inhibiting more than 90 % *C. albicans* strains. Sawant et al. [28] found out that mycocin produced by a *Pichia anomala* strain showed a fungistatic action compared with *C. albicans*.

However, a mycocin obtained from the supernatant of *W. anomalous* culture and isolated from the environment was not active against *C. albicans*, *Candida parapsilosis*,

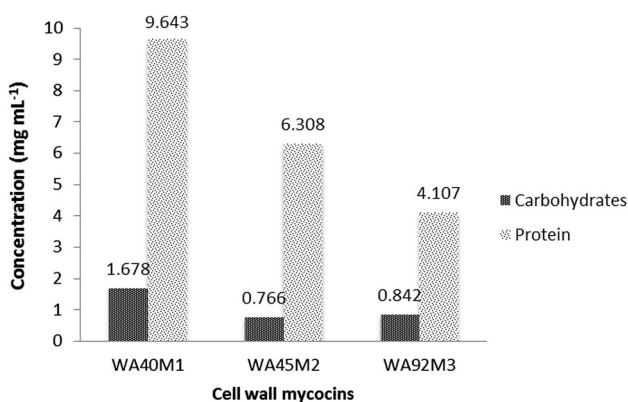


Fig. 1 Biochemical analysis of proteins and carbohydrates concentrations present in mycocin extracts from cell walls of WA40, WA45, and WA92 *W. anomalous* strains

Table 1 Susceptibility percentages of thirty *C. albicans* strains isolated from blood according to different concentrations of Wof A40M1, WA45M2, and WA92M3 mycocin extracts obtained from cell wall of *W. anomalous*, strains WA40, WA45, and WA92

Extracts of <i>W. anomalous</i> mycocins	Percentages of susceptible <i>C. albicans</i> strains (%)				
	Mycocin Extract Concentrations ($\mu\text{g mL}^{-1}$)				
	400	200	100	50	10
WA40M1	96.6	46.6	6.6	0	0
WA45M2	96.6	70.0	16.6	3.3	0
WA92M3	90.0	16.6	6.6	3.3	0

and *Candida krusei*, but against a clinical strain of *Candida mesorugosa* [33]. Heterogeneity, in response to mycocins extracts, was also observed in this study, as well as differences that were observed in the responses of thirty *C. albicans* strains.

W. anomalous' ability on inhibiting other microorganisms' growth maybe occurs by the synthesis of volatile compounds (ethyl acetate, isoamyl acetate, and ethyl propionate), as well as by production and excretion of glycoproteins (mycocins) [24, 34]. Moreover, lately, research has laid great emphasis on searching mycocins with antimicrobial activity. Meanwhile, the antimicrobial

activity of products secreted by yeast is not so common, or the interactions between mycocin-producing yeasts and strains of other microorganisms are not well elucidated [3]. Yet, some peculiarities on cell wall composition of those yeasts have indicated that mycocins secretion could be the most likely mechanism of inhibition [6].

In this study, essays were used to extract glycoproteins of cell walls from *W. anomalous* strains, in order to verify the fact that these mycocins present in cell wall have antimicrobial action. The antimicrobial activity test was also carried out with supernatant mycocins of WA40, WA45, and WA92 culture; however, the obtained results were inferior to the cell wall mycocin ones (these data are not shown). In future study with the purification of mycocins extracts, it will be possible to obtain an increase in anti-*Candida albicans* activity.

It can also be observed that mycocin extracts from WA40M1, WA45M2, and WA92M3 *W. anomalous* were able to cripple *C. albicans* cells via the viability method using fluorescein diacetate and ethidium bromide. Calich et al. [7] applied this method to several species of fungi based on the ability on accumulating fluorescein (fluoride chromasy) of fungal cells. Thus, it is easily visualized in green color on an immunofluorescence microscope. On the other hand, ethidium bromide quickly penetrates into damaged cells by binding to DNA, resulting in a red-

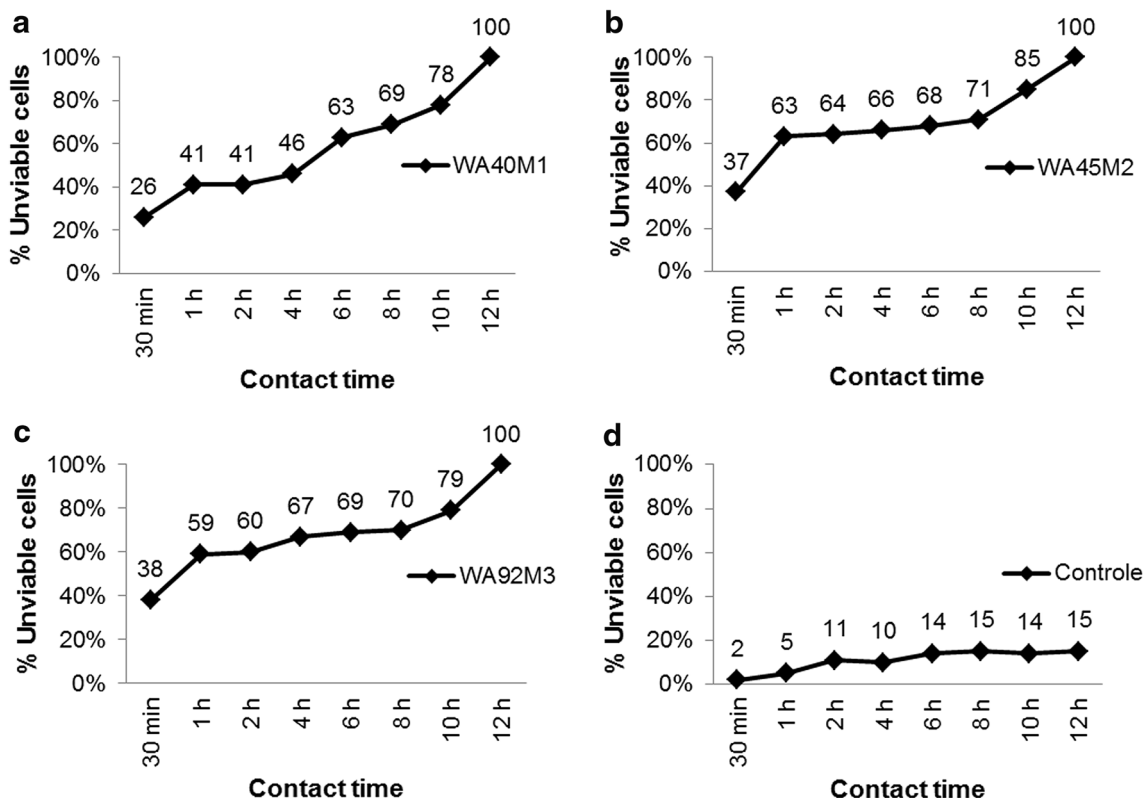


Fig. 2 *C. albicans* viability after contact with WA40M1, WA45M2, and WA92M3 mycocin extracts, obtained from WA40, WA45, and WA92 *W. anomalous* cell walls at 400 $\mu\text{g mL}^{-1}$ concentration

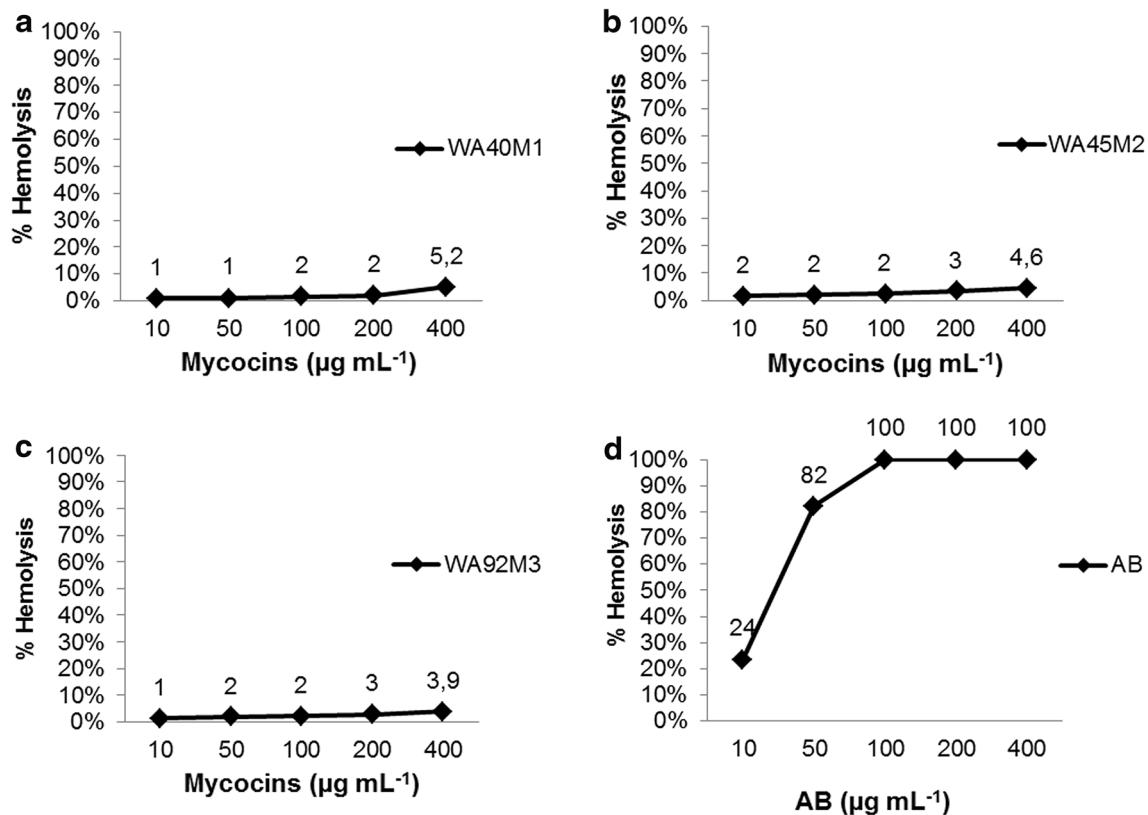


Fig. 3 Cytotoxic activity of WA40M1, WA45M2, and WA92M3 mycocin extracts obtained from WA40, WA45, and WA92 *W. anomalous* cell walls compared with amphotericin B. **a** Hemolysis

percentage of WA40M1 mycocin extract. **b** Hemolysis percentage of WA45M2 mycocin extract. **c** Hemolysis percentage of WA92M3 mycocin extract. **d** Hemolysis percentage of amphotericin B (AB)

colored fluorescent complex [36]. This method shows advantages such as being fast to execute, high sensitivity, simplicity, and good reproducibility due to its sharp difference between viable and non-viable cells [12].

Bhattacharyya et al. [2] observed that compounds present in *Aspergillus flavus* culture filtrate demonstrated ability to inhibit *C. albicans* and were considered nontoxic to human cells. In this study, the results of toxicity trials in human erythrocytes are highly important, since mycocin extracts showed no toxicity, with maximum hemolysis percentage being 5.2%. Besides, amphotericin B has shown much higher percentage compared with WA40M1, WA45M2, and WA92M3 *W. anomalous* mycocin extracts at all concentrations that have been tested (Fig. 3). However, some substances with antimicrobial activity, which arise from microorganisms, can cause toxicity. This fact was shown by Sorensen et al. [31] who observed some cytotoxic activity of peptides with antifungal activity produced by *Pseudomonas syringae* bacterium, which was greater than that produced by amphotericin B when tested in sheep erythrocytes.

Amphotericin B is an antifungal drug that has important side effects such as nephrotoxicity, with some *Candida* species having already demonstrated their resistance to it as

well [25]. Therefore, there is a great need on finding out and developing new antifungal drugs according to the increased incidence of infections caused by *C. albicans* in debilitated patients with high resistance to available antifungal treatments [26].

In this study, WA40M1, WA45M2, and WA92M3 mycocin extracts produced by WA40, WA45, and WA92 *W. anomalous* strains showed some inhibitory activity in *C. albicans* strains as well as no cytotoxic activity. These properties have evidenced that *W. anomalous* mycocins are potential candidates to develop new antifungal drugs that can also be used in synergism with other antifungal drugs.

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Compliance with Ethical Standards

Conflict of Interest No competing financial interests exist.

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