JIDC | THE JOURNAL OF INFECTION IN DEVELOPING COUNTRIES

Original Article

In vitro antifungal activity of *Morinda citrifolia* (noni) extract against *Candida albicans*

Camila Botelho Miguel[1,2 #], Renato Ventresqui Oliveira[3 #], Wellington Francisco Rodrigues[2,4 #], Gustavo Tavares[5], Sandro Calderano Joinhas[5], Maria Auxiliadora Gomes da Cruz[5], Célia Fabrício de Souza Rezende[5], Andréa Maria Pasquini Alvarenga[5], Weder da Silva Mateus[3], Marcia Laina Silveira[3], Elizabeth Uber Bucek[5], Weslley Guimarães Bovi[2], Carlo José Freire de Oliveira[2,4], Thiago Alvares da Costa[2], Javier Emilio Lazo-Chica[6], Tony de Paiva Paulino[3,5]

[1] Unidade de Biociências, Centro Universitário de Mineiros, Mineiros, GO, Brazil
[2] Curso de Pós-graduação em Medicina Tropical e Infectologia, Universidade Federal do Triângulo Mineiro, Uberaba, MG, Brazil
[3] Laboratório de Pesquisas do Cefores, Universidade Federal do Triângulo Mineiro, Praça Manoel Terra, Uberaba, MG, Brazil
[4] Pós-graduação em Ciências da Saúde, Universidade Federal do Triângulo Mineiro, Uberaba, MG, Brazil
[5] Laboratório de Ciências Farmacêuticas, Universidade de Uberaba, Uberaba, MG, Brazil
[6] Laboratório de Biologia Celular, Instituto de Ciências Biológicas e Naturais, Universidade Federal do Triângulo Mineiro, Uberaba, MG, Brazil

# Authors contributed equally to this work.

Abstract

Introduction: *Candida albicans* is the main agent of the most common fungal infection, Candidiasis. It is an opportunistic and dangerous pathogen, especially in immunosuppressed patients. The biological properties of *Morinda citrifolia* (noni) make it a potent antifungal. In this study, antifungal effect of *M. citrifolia* was evaluated to verify its effect on human cells.

Methodology: Extract of *M. citrifolia* was used against strains of *C. albicans* (cEC 1291). Glucose consumption in *C. albicans* biofilm was determined at different concentrations of *M. citrifolia*, and germ tube formation was evaluated in the presence and absence of *M. citrifolia*. Fungicidal activity was determined by the kinetics of fungal cell death. THP-1 and HeLa cells were used for cell viability and apoptosis, and cell proliferation assays, respectively.

Results: Cells treated with *M. citrifolia* maintained higher concentration of glucose than the control group ($p < 0.05$). Germ tube formation was inhibited in cells treated with *M. citrifolia* ($p < 0.05$). *M. citrifolia* exerted a cytotoxic effect on *C. albicans* cells with 99.99% lethality after 6.82 h (1:1 and 1:2), and reduced the viability of THP-1 cells by 25% and 67% after 12 and 36 h, respectively. Annexin V expression in THP-1 increased in groups that received higher concentrations of *M. citrifolia* ($p < 0.05$), reducing the proliferation of THP-1 and HeLa cells (2.8-fold). A greater cytotoxic effect was observed in fungal cells.

Conclusions: These results indicate that *M. citrifolia* exerts biological activity against *C. albicans* and reduces the viability and proliferation of human cells.

Key words: *Morinda citrifolia*, noni; *Candida albicans*; THP-1; HeLa; cytotoxicity.

J Infect Dev Ctries 2022; 16(7):1206-1217. doi:10.3855/jidc.15835

(Received 20 September 2021 – Accepted 17 February 2022)

Introduction

A commensal relationship exists as long as the balance between the host's immune system and the virulence factors of *Candida albicans* is maintained; however, several factors influence the development of pathogenic and opportunistic infections, including systemic, local, hereditary, environmental, and virulence characteristics of *Candida*. Factors such as dysbiosis of the residential microbiota, immune dysfunction, and damage to the muco-intestinal barrier are also related to the development of *C. albicans* infection [1]. The most common type of infectious esophagitis, called esophageal candidosis, is caused by *C. albicans* [2,3]. *C. albicans* usually causes superficial skin infections, while “deep” mycoses, with the involvement of dermis and subcutis layers, are rare. The unifying and clinically relevant features of candidiasis are erythema, erosions, and easily removable cheesy

white plaques. While white plaques are more often seen on mucosa than on skin, erythema and erosions are widely unspecific presentations [1]. Vulvovaginal candidiasis is common in women; it affects approximately 75% of women at least once in their lifetime [4]. Although the presence of *Candida* spp. does not necessarily indicate a vaginal infection, its presence combined with inflammation and vulvovaginal symptoms such as itching, burning, and discharge indicate a clinical diagnosis of vulvovaginal candidiasis in the absence of other infectious agents [5,6]. Damage to the intestinal mucosa, mainly caused by surgery and chemotherapy, in addition to impaired neutrophil function due to tumor therapy or prolonged use of glucocorticoids favors intestinal infection by *C. albicans*. The infection has a wide range of clinical manifestations in the oral cavity, which can be divided into primary and secondary candidiasis, while it is associated with invasive candidiasis in the blood. Invasive candidiasis can cause a number of pathological disorders affecting many organs and tissues, including bones (osteomyelitis and spondylodiscitis), brain (brain abscess and meningoencephalitis), eyes (choroiditis, retinitis, and endophthalmitis), heart (endocarditis), kidney (candiduria, pyelonephritis, pyonephrosis, and renal abscess), lung (focal abscess), liver, and spleen (chronic disseminated candidiasis and focal abscess) [1].

Candidiasis is the most common yeast infection, and although other *Candida* species can also cause infection, *C. albicans* remains the most prevalent etiological agent [7,8]. About half of the world population is exposed to *C. albicans*, and its presence is not necessarily indicative of infection. However, it is a dangerous and opportunistic pathogen in immunocompromised individuals [9].

C. albicans is a dimorphic microorganism; its yeast form is common in the microbiota of human mucosal surfaces. However, its transition to the filamentous form can cause infections in the mucosa of the respiratory, digestive, and genitourinary systems, in addition to systemic infections, known as invasive candidiasis [10-16]. The change from the benign form (yeast) to the pathogenic form (filamentous) is triggered by a broad spectrum of factors, ranging from impaired immune response due to AIDS and corticosteroid therapies to disturbances in the mucosal microbiota ecosystem, for example, as a result of antibiotic therapy and diseases such as diabetes mellitus, Addison's disease, hypothyroidism, and leukemia [8,13].

This ability to change is used in techniques for the identification of *Candida* species, for example, the germ tube test, which allows for the testing of drugs with antifungal activity, given that preventing the transition to the filamentous form results in a positive prognosis for patients [14,15]. Although antifungal drugs exist, recent increase in the number of candidiasis cases caused by strains resistant to current treatments indicates a serious global problem [16]. Therefore, there is an urgent need to identify alternatives for the effective treatment of these pathogens.

With this in mind, our group has sought new therapeutic sources for this disease. In this context, the *Morinda citrifolia* (noni) plant represents a promising candidate. A member of the Rubiaceae family, this plant is native to Southeast Asia, but can also be found in tropical regions, such as Hawaii, Malaysia, Tahiti, Brazil, India, and Oceania [17]. Consumed as food and medicine by Polynesians for over 2,000 years [18], noni has recently gained popularity due to its supposed beneficial effects on health.

In recent years, a large number of studies have been conducted using different preparations of *M. citrifolia*, including the juice, leaf extract, bark, and root of the plant, showing good in vivo and in vitro results for the treatment of obesity [19,20], diabetes mellitus [21], hypertension [22], rebalancing the intestinal microbiota [23], control of tumors [24], immunological modulation [25,26] for the reduction of infection inflammation [27], and chronic intestinal inflammatory diseases [28]. However, the real potential of noni lies in its antimicrobial action. Studies have shown that it is effective in fighting bacteria, such as *Bacillus subtilis*, *Escherichia coli*, *Proteus vulgaris*, and *Staphylococcus aureus* [29] including methicillin-resistant *S. aureus* (MRSA) [30], in addition to parasites, such as *Leishmania amazonensis* [31].

Although the ability of noni to prevent the formation of filamentous structures of *C. albicans* and *Aspergillus nidulans* has been previously investigated in vitro [10], the antifungal properties of noni against *C. albicans* require further study. In this study, we further evaluated its antifungal capacity and verified the effects of noni extract on human cells.

Methodology

Preparation of M. citrifolia fruit extract

M. citrifolia plants were collected, and a specimen was deposited in the herbarium of the Universidade Federal de Uberlândia (HUFU-67210). The scientific name of the plant was confirmed to be *M. citrifolia* of the Rubiaceae family.

The fruits of *M. citrifolia* were randomly and manually collected from 100 plants growing in

Araguari city (–18.6441829, –48.194764413). They were washed in ozonized water, kept at a temperature of 25 ºC, and protected from wind and shade to completely mature over a period of 3 to 5 days. They were mechanically pulped in a fruit striper (Peel-All Fruit Peeler, Model CP44, Muro Corp., Tokyo, Japan). After removing the seeds, the resulting pulp was centrifuged in a refrigerated centrifuge at a temperature of 30 °C at 1300 × g until the supernatant was obtained, which was considered 100% (v/v) and stored at –20 °C.

Weight/volume ratio and concentration of protein quantification of M. citrifolia fruit

The weight and volume (w/v) ratio of *M. citrifolia* fruit was determined by measuring the dry matter (mg) after lyophilization. The protein concentration was determined using the Bradford method according to the manufacturer's instructions (Bio-Rad, Hercules, USA) and analyzed for absorbance at 595 nm. The protein concentration was obtained by comparing the absorbance with a standard curve, with the aid of the StatView program (Abacus Concepts Inc., Berkeley, CA, USA). In this study, the concentration of the plant sample derived from *M. citrifolia* was measured (14.4 mg/mL of w/v and 174 μg/mL of protein). Different dilutions were used for subsequent experiments.

Candida albicans

Strains of *C. albicans* (CEC1293) containing 10^6 viable cells/mL were provided by the Institute Biologie et Pathogenicité Fongigues Pasteur. Subsequently, the cells were seeded on Sabouraud Dextrose agar (SDA) (Difco, Detroit, MI, USA) and incubated for 24 h at 37 °C. After incubation, the microorganism was cultured in brain and heart infusion (BHI) (Difco) for 24 h at 37 °C. The fungal culture was then centrifuged at 1300 × g for 10 min and the supernatant was discarded; this procedure was repeated. The pellet was resuspended in 5 mL of sterile water and an aliquot was taken for quantification. The number of viable cells in suspension was determined using a spectrophotometer (λ = 530 nm, OD = 0.680) (B582; Micronal, São Paulo, Brazil).

Biofilm Glucose Consumption

The stationary phase of *C. albicans* was prepared at a concentration of 106 CFU/mL. This cell density was previously shown to be the optimum cell concentration for biofilm formation by *C. albicans*. BHI supplemented with glucose (1% w/v) was used as a biofilm formation medium, and 10^6 CFU/mL was added to a 24-well microplate and maintained at 37 °C for 24 h.

After biofilm formation, it was washed three times in phosphate-buffered saline (PBS) and incubated for 1 h with different concentrations of *M. citrifolia* extracts (1:1, 1:2, and 1:10 v/v). Thereafter, the biofilm was washed three times with PBS, BHI was added, and the glucose consumption was analyzed in media samples collected at 1, 2, 3, and 4 h. For each sample, the consumption of glycosides was determined using a commercial enzymatic kit.

Germ Tube Formation

To analyze the ability of *C. albicans* to form germ tubes after treatment with *M. citrifolia*, strains of *C. albicans* were incubated with fetal bovine serum (FBS) and *M. citrifolia* (1:2) for 3 h at 37 °C. The control treatment included strains of *C. albicans* incubated with only fetal bovine serum (FBS) for 3 h at 37 °C. Then, 20 μL of each suspension was placed in a Neubauer chamber and germ tube formation was calculated as the percentage of total cells forming germ tubes.

Antifungal Activity

The killing kinetics of the fungicidal activity was analyzed by fitting the mean data at each time point to an exponential equation: $N_t = N_0 \times e - K_t$, where N_t denotes the number of viable yeasts at time t, N_0 is the number of viable yeasts at the beginning of the experiment, K is the lethality rate, and t is the incubation time. The exponential equation was transformed into a line by applying natural logarithms ($\log N_t = \log N_0 + K_t$). The goodness of fit was determined from the correlation coefficient ($r^2 = 0.8$–0.98). The fungicidal activities were compared using K values, with positive values indicating growth and negative values indicating death. Thus, the seven time points on each killing curve were reduced to one value, K. The following parameters were derived from the killing equation: the mean times to achieve reductions in the proportions of viable cells up to 50% (t_{50} = 0.30103/K), 90% (t_{90} + 1/K), and 99% (t_{99} + 2/K), and the time to reach the fungicidal endpoint ($t_{99.9}$ + 3/K) for each *M. citrifolia* concentration and each strain [32-34].

Human Cell Culture

Monocyte cell line THP-1, derived from acute monocyte leukemia, and HeLa cells were obtained from Fundação Oswaldo Cruz (Rio de Janeiro, Brazil). The cells were cryopreserved in liquid nitrogen at −186 °C and maintained at the Immunology Laboratory of the Universidade Federal do Triângulo Mineiro (Uberaba, Brazil). The cells were thawed and cultivated in Roswell Park Memorial Institute (RPMI) medium

supplemented with 10% Fetal Bovine Serum (FBS), 50 mM 2-mercaptoethanol, 1 mM sodium pyruvate, 25 mM sodium bicarbonate, 10 mM hydroxyethyl piperazineethane sulfonic acid (HEPES), 100 IU/mL penicillin, 100 µg/mL streptomycin, and 20 mM L-glutamine. The cells were incubated at 37 °C in a humidified atmosphere containing 5% CO_2. The medium was periodically replaced until an optimum value was obtained.

Treatment

THP-1 and HeLa cells were distributed in 96-well plates at a concentration of 1×10^5 cells/well. *M. citrifolia* extract was added to the culture at protein concentrations of 87, 17.4, and 8.7 µg/mL (in total extract) in RPMI medium for 12, 24, and 36 h, respectively. These protein values matched the respective concentrations (1:2, 1:10, and 1:20 (v/v)) based on the previous results, in which the dilutions 1:1 and 1:2 showed similar results. We chose to replace the more concentrated dilution for a more diluted one for our proposed treatment.

Viability of THP-1

The viability of the monocyte cell line THP-1 was determined after treating the cells with the new concentrations of *M. citrifolia* 1:2 (87 µg/mL of protein), 1:10 (17.4 µg/mL of protein), and 1:20 (8.7 µg/mL of protein). Cells were collected from one well containing 1×10^5 cells in 10 µL of solution and mixed with 10 µL of trypan blue (0.4%). Cell viability was measured using a Countess™ Automated Cell Counter (Invitrogen, Carlsbad, CA, USA).

Apoptosis Assay

Next, the THP-1 cells were washed twice with cold PBS (137 mM NaCl, 9.7 mM Na_2HPO_4, 1.5 mM KH_2PO_4, 2.7mM KCl, pH 7.0) and resuspended with binding buffer (10 mM Hepes/NaOH, 140 mM NaCl, 2.5 mM $CaCl_2$, pH 7.4). Annexin V-fluorescein isothiocyanate (annexin V-FITC) (7 pM) and propidium iodide (PI) (374 pM) staining was performed according to the manufacturer's specifications (BD Biosciences, Palo Alto, CA, USA). A minimum of 3×10^4 cells were analyzed by fluorescence-activated cell sorting (FACS), and Annexin V^+ PI^+ cells were considered as apoptotic cells. THP-1 cells in RPMI medium were used as negative controls in this assay, and THP-1 cells treated for 30 min at 57 °C were used as positive controls for apoptosis and cell death. The data obtained using a FACS Calibur (Becton Dickinson Biosciences, San Jose, CA, USA) cytometer were analyzed using CellQuest 5.1 (Becton Dickinson, San Jose, CA, USA) and FlowJo 10 software (Treestar).

HeLa Proliferation Assay

HeLa cell proliferation was monitored by resazurin reduction in the culture medium. After the addition of the different treatments of *M. citrifolia*, resazurin was added (1%) in a solution containing 1×10^5 cells. Spectrophotometry was performed, and the variation between two wavelengths (570 and 600 nm) was determined. Evaluation was done 18 and 24 h after the addition of resazurin solution.

Statistical Analysis

The experiments described in this section were performed in triplicates, and their respective statistical analyses were performed using GraphPad Prism 5.0 (GraphPad Software, San Diego, CA, USA). Continuous variables were expressed as mean ± standard deviation (SD). One-way analysis of variance (ANOVA) with Tukey's post hoc test was used for the comparison of four independent groups. Differences were considered significant at $p < 0.05$.

Results

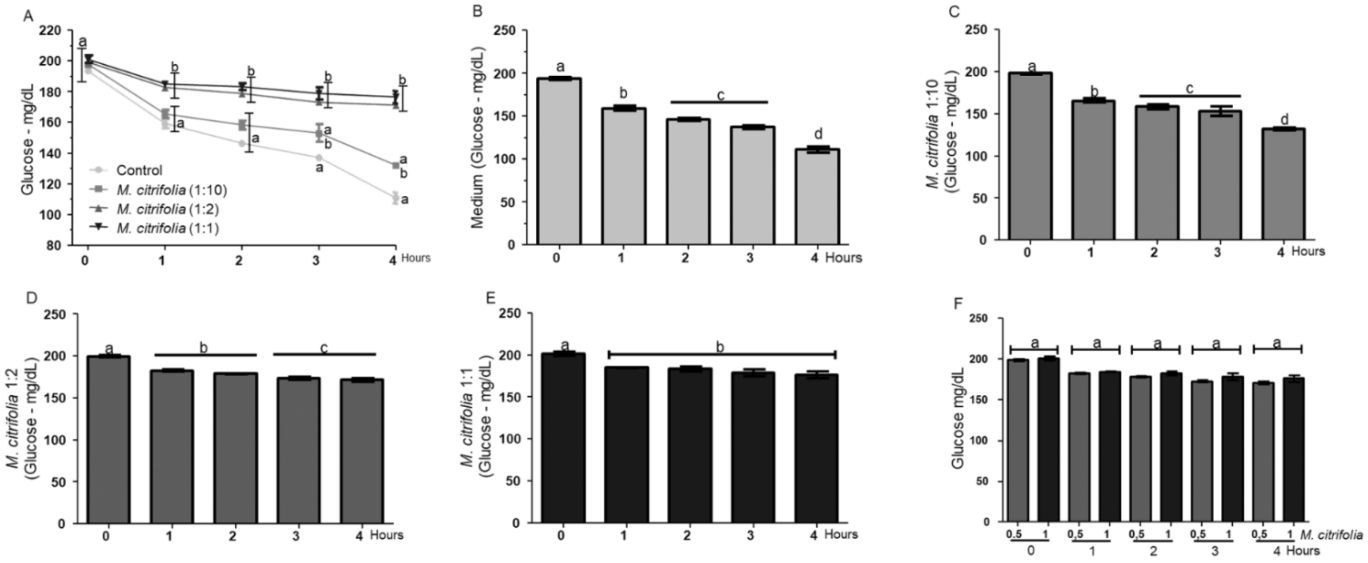
Effects of M. citrifolia treatment on glucose consumption by C. albicans in cultures

The amount of glucose in media is an important indicator of the metabolic activity of microorganisms. Therefore, we evaluated the effect of treatment with different concentrations of *M. citrifolia* extract and for different times periods on glucose (Figure 1). Interestingly, treatment reduced the glucose present in the medium within the first hour at dilutions of 1:2 and 1:1, but maintained higher levels of glucose in the medium over the entire treatment period ($p < 0.05$) (Figure 1a). A comparison of each treatment profile in relation to the treatment period indicated a reduction of glucose in the medium over time in all groups ($p < 0.05$) (Figure 1b-e). Given that a more significant result was observed in the dilutions 1:2 (0.5) and 1:1 (1), the different periods of treatment were compared to identify any differences (Figure 1f). However, no differences were observed ($p > 0.05$).

Effects of M. citrifolia on germ tube formation in C. albicans culture

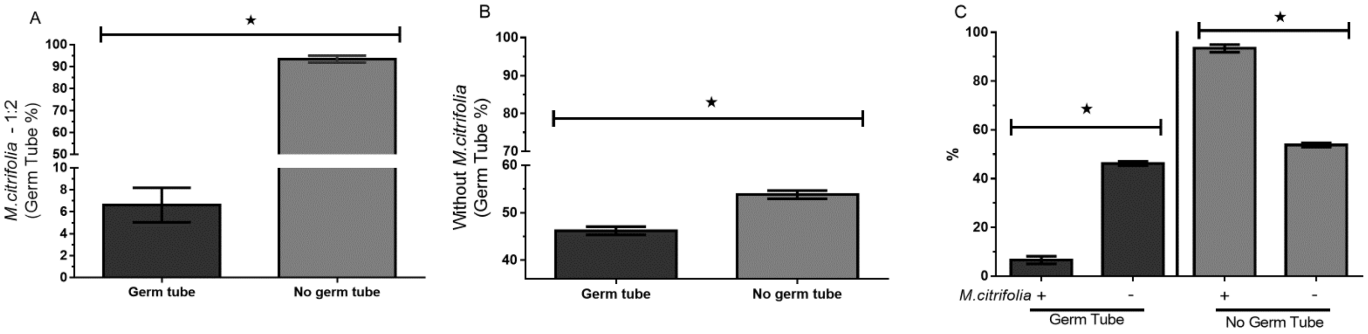
Following the observation that glucose uptake by yeast cells was decreased after treatment with *M. citrifolia* (1:2), we evaluated the ability of *C. albicans* to form germ tubes after treatment with the same dilution of *M. citrifolia* (Figure 2).

Figure 1. Determination of glucose in *Candida albicans* culture after treatment with *Morinda citrifolia*.



Glucose consumption by *C. albicans* was measured after biofilm formation and 1, 2, 3, and 4 h after treatment with *M. citrifolia* using the colorimetric technique (commercial kit). The concentrations were expressed in milligrams per deciliter. **A:** Quantification of glucose concentrations at different times and concentrations of *M. citrifolia* extract. **B-E:** Quantification of glucose concentrations in relation to different times, culture medium, and concentrations (1:10, 1:2, and 1:1). **F:** Comparison of glucose concentrations at different times at different *M. citrifolia* concentrations 1:2 (0.5) and 1:1 (1). The letters a, b, and c indicate statistically significant differences between groups ($p < 0.05$).

Figure 2. Germ tube formation in *Candida albicans* culture treated with *Morinda citrifolia*.



Germ tube formation was evaluated after treatment with *M. citrifolia* (1:2). Germ tubes were evaluated in a Neubauer chamber (expressed as a percentage). **A, B:** Quantification of germ tubes was performed in medium containing *C. albicans* culture in the presence (**A**) and absence (**B**) of *M. citrifolia*. **C:** Comparison between different media for the presence and/or absence of germ tubes. Asterisk indicates significant differences between groups ($p < 0.05$).

This treatment resulted in inhibition of germ tube formation by over 15-fold (Figure 2a). The same effect was not observed in the absence of *M. citrifolia*, which resulted in a difference of less than 1-fold (Figure 2b). These differences were best expressed when the groups treated with *M. citrifolia* were compared with those without treatment (Figure 2c).

Activity of M. citrifolia as an antifungal agent

The reduction of glucose consumption and the inhibition of germ tube formation after treatment with *M. citrifolia* were associated with the capacity of the plant to reduce viable fungal cells and the potential lethality of *M. citrifolia* in *C. albicans*. Thus, *M. citrifolia* was evaluated as a potential cytotoxic agent to fungal cells (Figure 3). Toxicity against fungus was found at a concentration of 1:2 (Figure 3a), with no differences in the mortality rate when compared with the pure extract (1:1) (Figure 3b). The duration of the activity and the lethality of treatment was determined by the minimum inhibitory concentration (MIC) shown for *M. citrifolia* (1:2), as detailed in Figure 3c. A total of 6.82 h of exposure to *M. citrifolia* were required for 99.99% lethality ($r^2 = 0.89$).

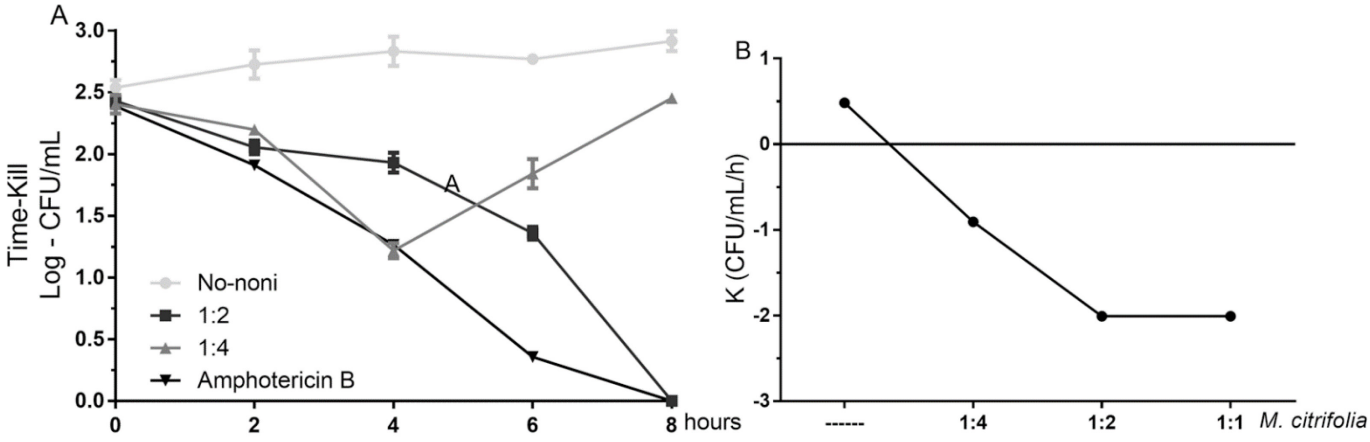
Effect of M. citrifolia on the viability of human monocytic cells

After evaluating the cytotoxicity and time of lethality of *M. citrifolia* treatment in *C. albicans* cells, its cytotoxicity in human cells was evaluated by determining the viability of THP-1 cells (Figure 4). An initial analysis indicated a significant difference ($p < 0.05$) in viability after 36 h of culture in all groups (Figure 4a). Interestingly, after 12 h of treatment, a reduction of the cell viability of approximately 25% was observed in cells treated with the 1:2 dilution. This reduction was maintained, reaching 67% after 36 h, but was ultimately unable to affect all cells (Figure 4b). Overall, cell viability was found to be negatively correlated with time (Spearman r = – 0.66) and extract concentration (Spearman r = – 0.62) (Figure 4c, d).

M. citrifolia treatment induces apoptosis markers in THP-1 cells

After confirming that treatment with *M. citrifolia* decreased the viability of THP-1 cells, its effect on the induction of cellular apoptosis was evaluated (Figure 5). A significant increase was observed ($p < 0.05$) in markers of cellular death (annexin V+ and propidium iodide+) in the group treated with the highest concentration of extract (1:2) (Figure 5o).

Figure 3. Assessment of lethal capacity of *Morinda citrifolia* on *Candida albicans* cells.

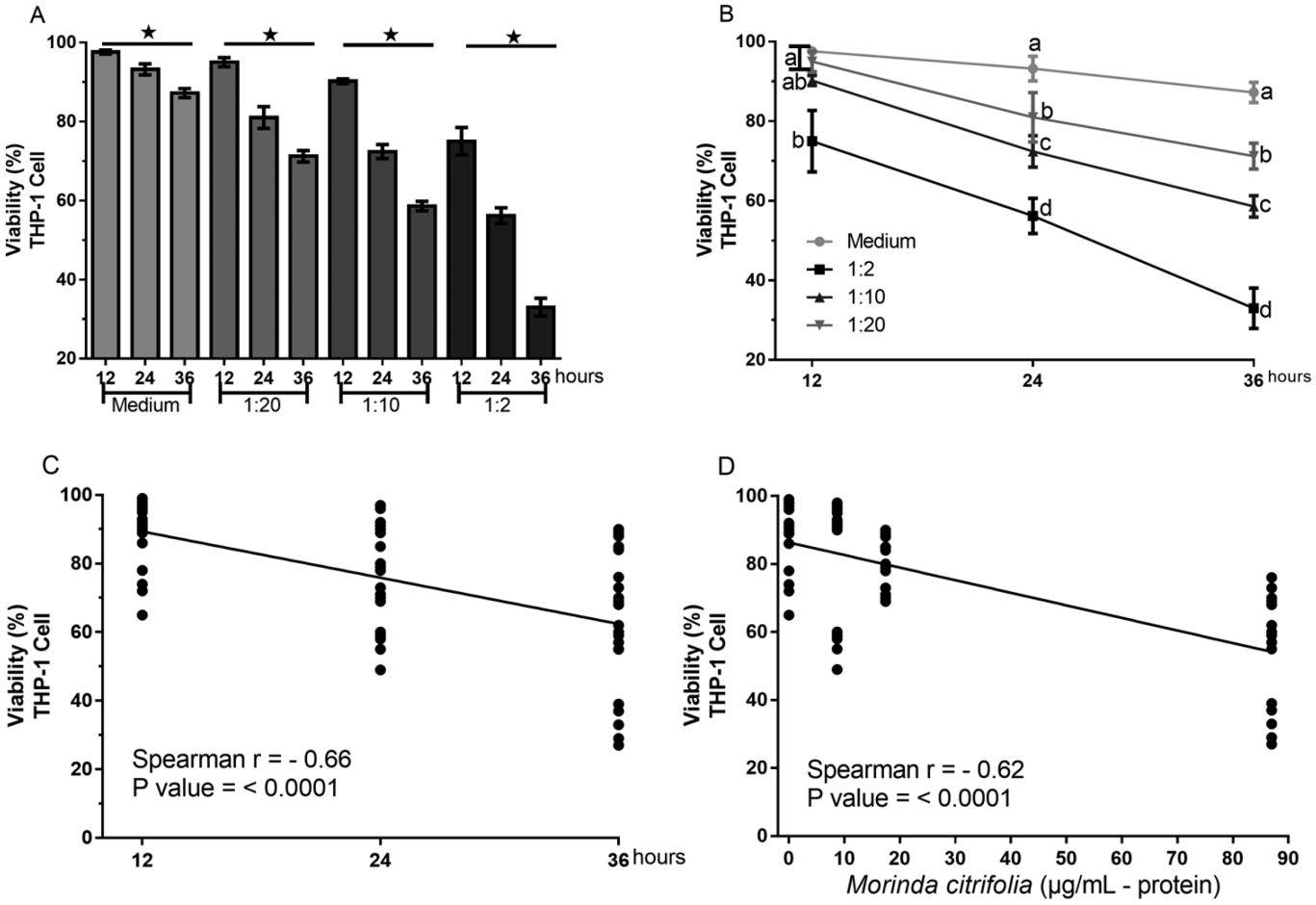


C

M. citrifolia - 87 µg/mL - protein (1:2 - MICI)									
Activity time (hours)	3.03	3.79	4.55	5.31	6.07	6.45	6.67	6.75	6.82
Lethality (%)	50	60	70	80	90	95	98	99	99.99
r^2									0.89

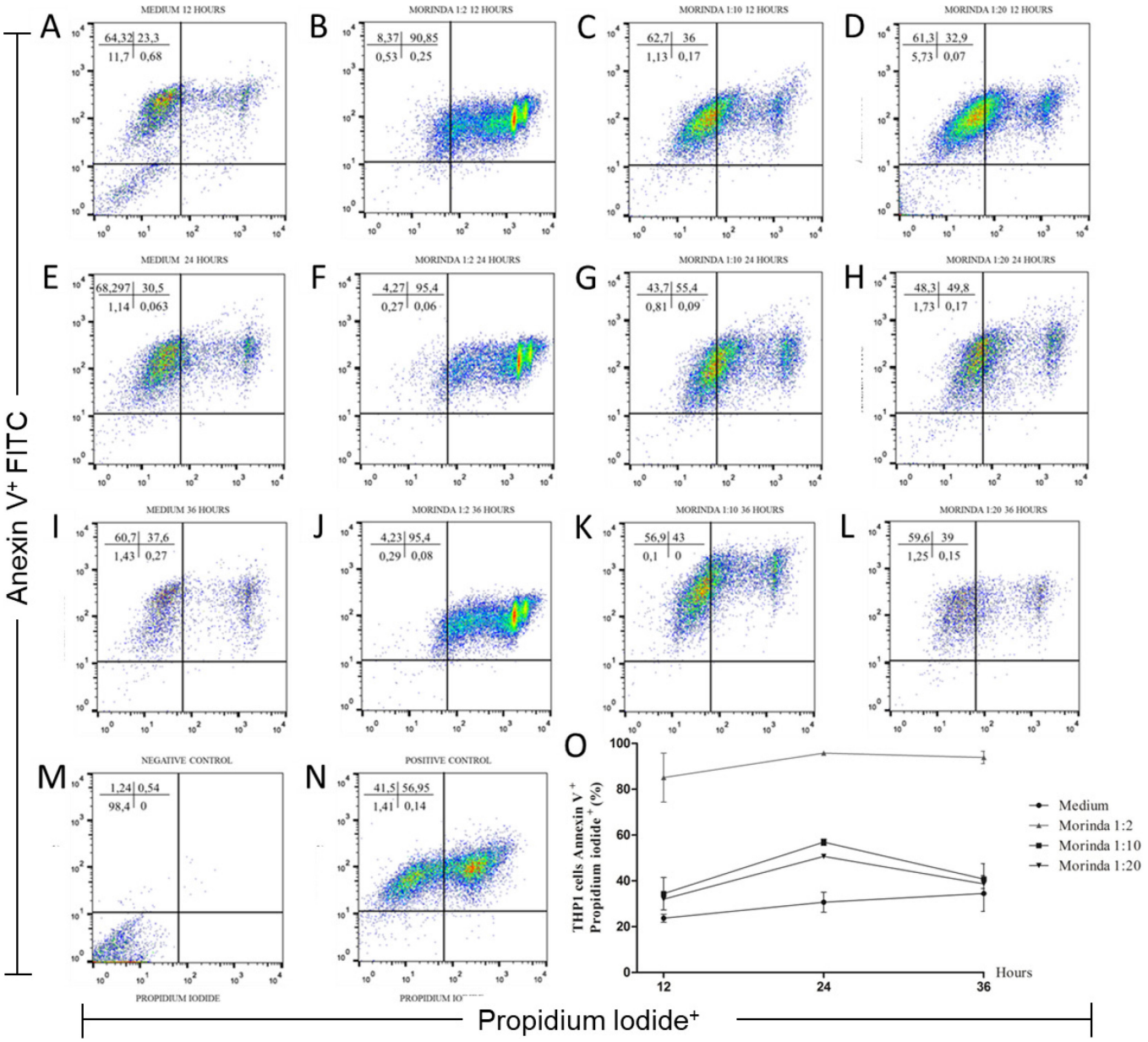
After accounting for fungal cells affected by treatment with *M. citrifolia*, the death curve (**A**) and lethality rate (**B**) were determined. To calculate the lethality estimates in percentages, the minimum inhibitory concentration (MIC) for *M. citrifolia* (1:2) was used (**C**), with r2 = 0.89.

Figure 4. Evaluation of cytotoxicity induced in THP-1 cells by treatment with *Morinda citrifolia*.



THP-1 cells were cultured in RPMI complete medium and treated with different concentrations of *M. citrifolia* (1:20, 1:10, and 1:2). Cells were monitored for cell viability at different times (12, 24, and 36 h). **A:** Comparison of cell viability in the different groups at different times. **B:** Comparison of cell viability between groups at different times. **C:** Correlation between the cell viability of THP-1 cells and the evaluation time. **D:** Correlation between cell viability and *M. citrifolia* concentration. Asterisk and the letters a, b, c, and d indicate statistically significant differences between groups ($p < 0.05$).

Figure 5. Detection of positivity to propidium iodide and annexin V in cells treated with *Morinda citrifolia*.



THP-1 cells were cultured and treated with different concentrations of *M. citrifolia* (1:2, 1:10, and 1:20) before staining with propidium iodide and annexin V. Positivity for annexin V and propidium iodide were determined after 12, 24, and 36 h. **A:** Comparison of staining between groups. **B-O:** Representations of staining distributions among the different groups. Data are expressed in percentages (%).

We considered the status of different groups in terms of apoptosis markers and normalization with positive and negative controls for this evaluation (Figure 5a-n).

Effect of M. citrifolia on HeLa cell proliferation

In addition to evaluating the cytotoxicity of *M. citrifolia* extract in human THP-1 cells, we evaluated the proliferative effect of this extract on a lineage of human cervical (HeLa) cells (Figure 6). We observed that the HeLa cells were evenly distributed among the different groups (Figure 6a). Interestingly, all groups demonstrated proliferation rates that were lower at higher extract dosages (approximately 2.8-fold) in the first 18 h of treatment. This ratio decreased after 24 h (2.3-fold) ($p < 0.05$) (Figure 6b). Furthermore, we evaluated the different groups in paired form at 18 h and 24 h and found significant differences only in the groups that either received treatment at a dilution of 1:20 or did not receive treatment (Figure 6b), indicating proliferation of HeLa cells.

Absence of correlation between fungal cell deaths and cytotoxic action in THP-1 and HeLa cells

The increased mortality observed in fungal cells after treatment with *M. citrifolia* suggests that this plant extract has potential antifungal applications in humans. Furthermore, by assessing the cytotoxicity of *M. citrifolia* in human cells (blood and uterine cervix), the extract was found to reduce cellular viability and proliferation, but also maintained an index of viable cells. To determine whether a correlation exists between fungal lethality (antifungal capacity) and decreased viability and/or cellular proliferation, we analyzed the results obtained for cells treated with *M. citrifolia* extract at a dilution of 1:2 (Figure 7). Surprisingly, no significant correlations were observed for the kill curve data, THP-1 cell viability, or HeLa cell proliferation (Figure 7a, b).

Discussion

As previously discussed, parts of the *M. citrifolia* plant, including its fruit, roots, and leaves, have been widely studied, especially with regard to their potential negative effect on microorganisms. In a preliminary study, the fruit extract of *M. citrifolia* was found to possess antifungal activity. Even at a low concentration (1:10), the extract was able to significantly reduce the consumption of glucose by fungal cells after 4 h of treatment.

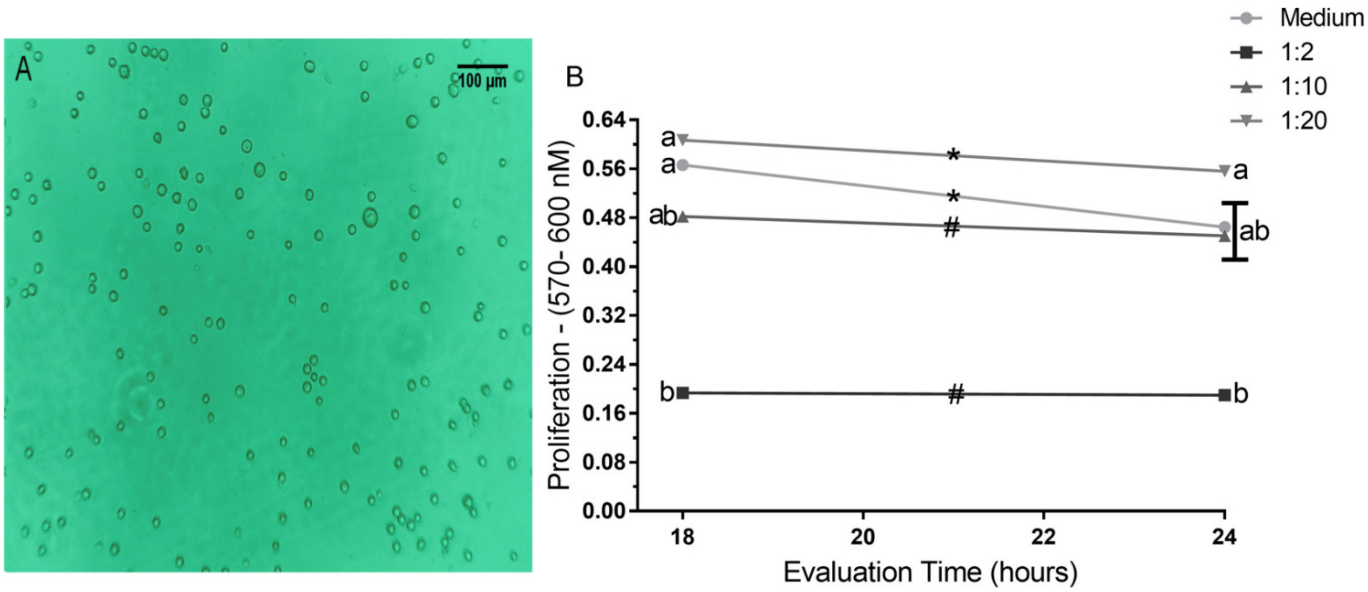
Glucose is one of the main nutrients found in culture medium. Thus, a decrease in its concentration in the medium indicates that microorganisms are viable and feeding. In the present study, a reduction in glucose uptake indicated a decrease in viable fungal cells in the culture. In addition to this reduction, the formation of germ tubes by *C. albicans* was reduced 15-fold after treatment with noni (1:2) compared to the control, which was also observed by Banerjee *et al*. [10].

Based on these results, we evaluated the lethal capacity of the extract in cultures of *C. albicans*. Once the MIC was reached in planktonic cells after treatment with the extract at a dilution of 1:2, we verified its beneficial aspects and pharmacodynamic properties for use in clinical applications. The evaluation of the MIC value (1:2) in time-dependent apoptosis studies showed that *M. citrifolia* completely inhibited the growth of *C. albicans* for 8 h before a second dose (if in clinical use) was required. The ½ MIC values (1:4) were also tested, and the resulting time-death curve indicated that the metabolism of the *C. albicans* cells was affected at the beginning of their growth. However, after 6 h of treatment, the cells returned to the normal linear growth. This could be due to the fact that ½ MIC is a sub-lethal dose and, despite an initial effect, cells returned to their regular growth. Thus, if the extract was to be used in clinical applications, a second dose after 6 h may be needed to completely inhibit *C. albicans* growth.

The antifungal effects of *M. citrifolia* demonstrated in the present study have revealed some discrepancies in the literature. Studies conducted by the Jainkittivong *et al.* confirmed the time-dependent antifungal ability of *M. citrifolia* extract [35], while Jayaraman *et al.* found that noni extract had no antifungal effect on *C. albicans* [36], contrary to Banerjee *et al*., Jainkittivong *et al.,* and the results of the present study. However, the methodological differences between these studies should be considered while comparing their results. For example, Jayaraman *et al.* used a very low concentration of *M. citrifolia* and conducted their analysis after 4 days of culture, while our work showed that the dose and the time of contact are very important, where a sub-lethal dose was unable to prevent the growth of fungus for a long time.

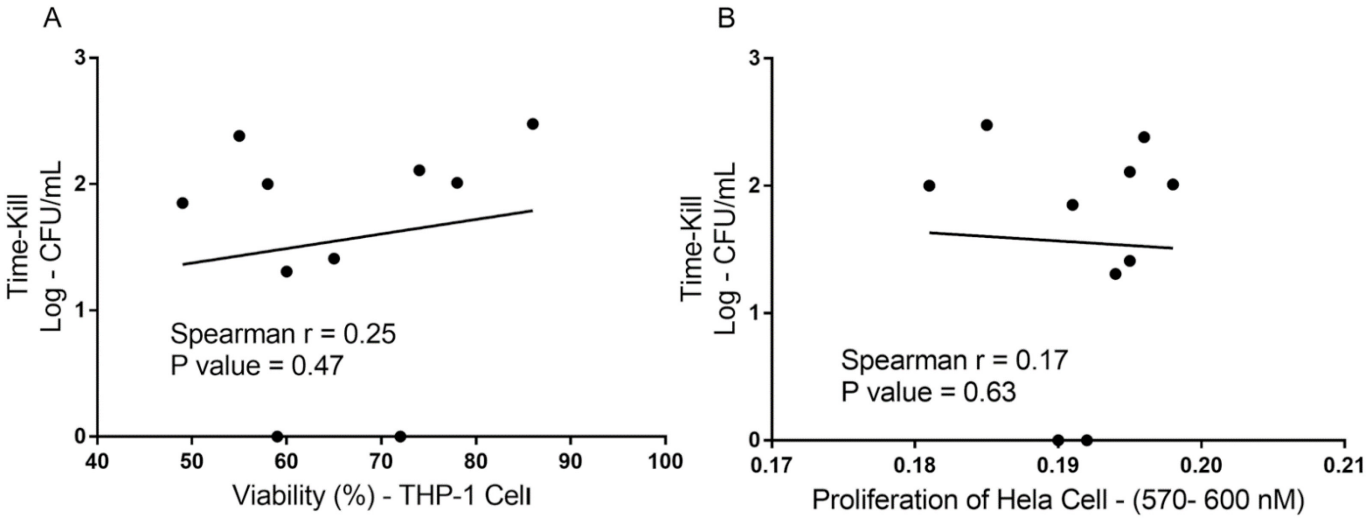
The antifungal activity of *M. citrifoilia* that we observed is promising, given that the search for new therapies that can also act on multidrug-resistant strains has become paramount. The antifungal activity of *Ruta graveolens* essential oil has been demonstrated against clinical strains of *Candida*, including *C. albicans* [37]. The activity of plant derivatives against *Candida* appears to be dependent on the lineage and phytochemical characteristics of the plant compound.

Figure 6. Evaluation of proliferation in HeLa cells after treatment with *Morinda citrifolia*.



HeLa cells were cultured and treated with different concentrations of *M. citrifolia* (1:2, 1:10, and 1:20). To evaluate cell proliferation, consumption of resazurin was measured at two different times (18 and 24 h). **A:** Cellular distribution of resazurin in culture medium. **B:** Comparison of cell proliferation in the different groups at different times. The letters a and b indicate statistically significant differences between groups ($p < 0.05$).

Figure 7. Correlation between human and fungal cells.



The correlation between the time of fungal death and (i) the cell viability of THP-1 cells and (ii) the proliferation of HeLa cells after exposure to *M. citrifolia* was measured. **A:** Correlation between the time of death of yeast cells (CFU/mL) and the viability of THP-1 cells (%). **B:** Correlation between the time of fungal death and the proliferation of HeLa cells (nanometers). Results were considered statistically significant at $p < 0.05$, and spearman correlation was considered valid at $r \geq 0.8$.

In an evaluation of 30 clinical isolates belonging to 5 different *Candida* spp., inhibition of *C. glabrata* and *C. krusei* was better with the use of *H. microphyllum* macerated oil than with *H. perforatum* [38].

Clinically, the proliferation of *C. albicans* strains that are resistant to currently known synthetic antifungals is concerning. The mechanisms of fungal resistance are complex, but the antimicrobial activity of natural compounds has been effective in blocking an important resistance mechanism, which is the efflux pump, already described for the multidrug-resistant bacterium, *Staphylococcus aureus* [39]. Thus, we believe that biological activity of the fruit extract of *M. citrifolia* possibly caused inhibition of the efflux pump. In a previous study, researchers evaluated three P-glycoprotein efflux inhibitors in 10 clinical strains of *Candida* and found a reduction in the development of fungal cells [40].

In the present study, the antifungal effect of *M. citrifolia* was verified. Furthermore, the cytotoxic action of this plant in humans was determined to evaluate its potential clinical use for the treatment of candidiasis. As a result, *M. citrifolia* was found to induce apoptotic markers, reduce the viability of THP-1 monocytes up to 67%, and inhibit HeLa cell proliferation in a dose- and time-dependent manner. These findings correlate with the cytotoxic and genotoxic effects of noni extract reported by De Moraes *et al.* [41]. However, our findings are in contrast to a large number of studies that have found noni to be safe for clinical use [42,43]. In this context, it is worth mentioning that plants, such as *M. citrifolia*, may undergo significant changes in nutrient bioavailability due to soil conditions, harvesting, and storage [44,45].

Noni continues to be reported as a miracle remedy for a range of disorders in humans. Based on our own observations, noni does indeed have the potential to improve a variety of human health conditions. However, further studies will be needed to fully elucidate its effects and mechanisms of action and, above all, to ensure the safety of its use in the general population.

Acknowledgements

The authors would like to thank the kindness of Dr. Christophe d'ENFERT in providing the *Candida* strain used in this study. We also appreciate the support of research and post-graduation pro rectory and CEFORES from the Federal University of the Triângulo Mineiro. WFR, receives a Junior Postdoctoral Fellowship (PDJ) from the National Council for Scientific and Technological - CNPq (process: 152889/2022-1).

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Corresponding author

Wellington Francisco Rodrigues, PhD
Cell Biology Laboratory, Federal University of Triângulo Mineiro, Av Tutunas, 490, 38051327, Uberaba city, MG, Brazil.
Tel: +5534996309185
Fax: +553437006626
Email: wellington.frodrigues@hotmail.com

Conflict of interests: No conflict of interests is declared.