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Antifungal Evaluation Of The Combination Of Ferulic Acid And Nicotinamide Against Candida Spp.

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Abstract:
Background*: Candidiasis is the most important cause of fungal infections in humans. Limitations of antifungals used in treatment are reported in the literature. Prolonged treatment often leads to drug resistance, necessitating alternatives to conventional antimicrobial therapy. In this context, the aim of this study was to evaluate the antifungal activity of ferulic acid (FA), nicotinamide (NAM), and the FA:NAM combination against strains of Candida albicans, Candida tropicalis, and Candida krusei, and to determine their mode of action and synergistic effect when combined with the synthetic antifungal nystatin.*
Materials and Methods*: The minimum inhibitory concentration (MIC) was determined using a microdilution technique, and the minimum fungicidal concentration (MFC) was determined via subculture sowing. The mode of action of AF, NAM e AF:NAM was established by verifying fungal growth in the presence of sorbitol or ergosterol. The fractional inhibitory concentration index (FIC) was determined using the checkerboard method.*
Results*: NAM and the FA:NAM combination exhibited antifungal effects against C. albicans. The antifungal test results remained unchanged in the presence of ergosterol; however, the MIC value of the FA:NAM combination against C. albicans increased eightfold (125 to 1000 μg/mL) in the presence of exogenous sorbitol. The combination of the test substances and nystatin showed no antagonism, allowing for the association of these compounds in the treatment of fungal infections.*
Conclusion: *It was found that NAM and the AF:NAM mixture have a fungicidal effect on Candida species and show no antagonism when combined with nystatin.*
Key Word*: Ferulic Acid; Nicotinamide; Antifungal; Candida spp.; Caspofungin.*

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I. Introduction

Candidiasis is the infection caused by members of the fungal genus Candida, which is part of the commensal microbiota and is an opportunistic pathogen for humans. It is often associated with the mortality of susceptible individuals, including elderly patients, oncology patients, transplant recipients, and immunosuppressed individuals[1,2,3].

This genus includes *Candida tropicalis*, *Candida parapsilosis*, *Candida glabrata*, *Candida guilliermondii*, *Candida dubliniensis*, *Candida auris*, and *Candida krusei*. Collectively, these species are the causative agents of non-albicans candidiasis. Despite this, many studies have shown that systemic infections by *Candida albicans (C. albicans)* have a higher gross mortality rate, reaching approximately 40%[1,3,4,5].

Ferulic acid (FA) is a phenolic compound found in plants, fruits, and vegetables. It is known to be a potent antioxidant that plays a crucial role in maintaining human health and preventing various diseases. Free radicals generated through oxidation in the body lead to prolonged cellular damage, which can cause cancer and/or exacerbate conditions such as diabetes, hypertension, circulatory problems, inflammation, etc. Therefore, the consumption of antioxidants is essential to prevent these chronic diseases caused by free radicals. Simultaneously, the prevention of fungal diseases is equally important for better control of human health[6,7,8,9,10,11,12].

It is known that there are some difficulties regarding the bioavailability of phenolic acids because they are hydrophobic compounds, and this directly affects their therapeutic potential when administered orally. In view of this, new technological strategies have emerged to enhance this issue and improve their pharmacological

properties, such as co-crystals. Co-crystals are homogeneous crystalline structures formed by the union of two or more substances in a single crystalline phase[10,13,14,15,16].

Co-crystals of ferulic acid (AF) are well-documented in the literature with pharmacological, therapeutic, and food potential. Therefore, the synergy of FA with other molecules, as well as with nicotinamide, seems to be quite promising[17,18,19,20]. FA has shown in vitro antifungal activity by inducing apoptosis in *C. albicans* and *Candida glabrata* fungi, with more significant results when evaluated in combination with caspofungin, exhibiting a synergistic effect[21].

Nicotinamide (NAM) is an amide form of vitamin B3, known as a safe agent that can be used in the treatment of various diseases. It has been reported that NAM exhibits antimicrobial activities, inhibiting the growth of *Plasmodium falciparum*, *Mycobacterium tuberculosis*, human immunodeficiency virus (HIV), and also shows significant antifungal activity against *C. albicans*, including fluconazole-resistant isolates[22,23,24,25,26,27].

In this context, this study aimed to evaluate the antifungal activity of the substances FA and NAM individually, as well as the mixture of FA with NAM (FA:NAM) against different *Candida spp*. It also investigated the mechanisms of action and the potential synergistic effect with the antifungal nystatin.

II. Material And Methods

Microorganisms and molecules

Reference *Candida spp.* strains were obtained from the American Type Culture Collection (ATCC, Rockville, MD, USA): *C. albicans* ATCC 60193, *C. tropicalis* ATCC 750, and *C. krusei* ATCC 6258. Nystatin, Caspofungin, Tween 80%, and ergosterol were obtained from Sigma-Aldrich® Chemical Co. d-sorbitol anhydrous from INLAB® (São Paulo, Brazil). Ferulic acid (FA) was purchased from Pharmanostra (China), and nicotinamide (NAM) from Purifarma (Brazil). All raw materials presented purity greater than 99.5%.

Determination of Minimum Inhibitory Concentration (MIC) and Minimum Fungicidal Concentration (MFC)

The MIC was determined by the microdilution technique, as previously described by the adapted method from the Clinical and Laboratory Standards Institute[28]. Briefly, the biological activity of the test molecules was analyzed on *Candida spp*. strains through the serial microdilution technique in 96-well plates. Initially, the fungi were incubated in the presence of the test molecules diluted with DMSO and sterile distilled water (vehicle), analyzing the concentration range from 1000µg/mL to 7.8µg/mL for each molecule or mixture. Negative controls consisted of incubating *Candida spp*. strains only with the vehicle and/or culture medium. Nystatin (48 - 0.37µg/mL) was used as a positive control. After 24h at 35 ± 2°C, visible microbial cell growth was evaluated both qualitatively and using the TTC dye (2,3,5-triphenyl tetrazolium chloride, Sigma-Aldrich®, St. Louis, MO, USA). MIC was defined as the lowest concentration of the test substance that inhibits visible microbial growth.

Antifungal Mechanism of Action

Action of FA, NAM, and FA:NAM mixture on fungal cell wall and membrane (Sorbitol test): For this assay, the MIC value was defined as the lowest concentration of the substance that inhibits visible microbial growth in the presence of sorbitol (D-sorbitol anhydrous) (INLAB, São Paulo, Brazil). The microdilution technique was used to compare the MIC values of the compounds against *C. albicans* ATCC 60193, *C. tropicalis* ATCC 750, and *C. krusei* ATCC 6258 strains in the absence and presence of 0.8M sorbitol. The plates were incubated at 35°C, and readings were taken 24 hours after incubation. The positive control for this assay was caspofungin at an initial concentration of 4.0µg/mL (caspofungin diacetate - Sigma-Aldrich, St. Louis, MO, USA), which is known to disrupt the yeast cell wall. Based on sorbitol's ability to act as an osmotic protector in the fungal cell wall, higher MIC values in the presence of sorbitol (standard medium) suggest that the cell wall is a likely cellular target of the compound under analysis.

Action of FA, NAM, and FA:NAM mixture on the fungal cell membrane effect (Ergosterol test): For this assay, the MIC was defined as the lowest concentration of the substance that inhibits visible microbial growth in the presence of exogenous ergosterol. The assay was performed using the microdilution technique in the presence of exogenous ergosterol (Sigma-Aldrich, São Paulo, Brazil) at a concentration of 1008µM. The strains used in this test were *C. albicans* ATCC 60193, *C. tropicalis* ATCC 750, and *C. krusei* ATCC 6258. The plates were incubated at 35°C, and readings were taken after 24 hours. Nystatin was used as a positive control at an initial concentration of 48 µg/mL due to its known activity on yeast cell membranes, binding to membrane sterols and thereby disrupting membrane permeability.

Growth kinetics

Growth curves were employed to analyze the minimum time required for the initiation of microbial growth. In a flat-bottomed 96-well microdilution plate, each well received the culture medium (RPMI), followed by the substances FA, NAM, and FA:NAM at concentrations corresponding to MIC and 2x MIC. Then, 100µL of

C. albicans ATCC 60193 yeast inoculum (2.5 x 103 CFU/mL) was added to each well. The plates were incubated for 24 hours at 35ºC in a microplate reader (BIOTEK-EON), and absorbance values were read at 530nm every hour. Nystatin (Sigma-Aldrich, São Paulo, SP) was used as a positive control in assays at concentrations ranging from 1.5 to 12 µg/mL (1.61µM to 12.9µM). A growth control of the strain was also performed.

Synergy Assay – Checkerboard Method

The effect of the test substances alone and in combination with nystatin was determined using the checkerboard technique to derive the Fractional Inhibitory Concentration (FIC) index. Solutions of test products at certain concentrations (determined by their respective MICs) were used. Initially, 100µL of SDB was added to the wells. Then, 50µL of each test product at various concentrations (MIC x 8, MIC x 4, MIC x 2, MIC, MIC ÷ 2, MIC ÷ 4, and MIC ÷ 8) were added horizontally (the substances under analysis) and vertically (nystatin). Finally, the fungal inoculum from the tested strains was added (2,5 x 103 UFC/mL). Growth and sterility controls were also performed. Results were visually read, and fungal growth was shown using the TTC dye. The assay was conducted in triplicate, and the microplate was incubated for 24 hours at 35°C [29,30]. The FIC index was calculated as the sum of FIC A + FIC B, where A is the test substance, and B is nystatin. FIC A, in turn, is calculated using the MIC A combined/MIC A alone ratio, while FIC B = MIC B combined/MIC B alone ratio. This index was interpreted as follows: synergy (<0.5), additivity (0.5-1.0), indifference (>1), or antagonism (>4.0).

Statistical analysis

All assays were performed in triplicate in three independent experiments. Descriptive and inferential statistical analysis was conducted using appropriate tests with a significance level of 5%. Growth kinetics assays were carried out using GraphPad Prism® version 7.3 (GraphPad Software Inc., USA).

III. Result

Determination of MIC and MFC

Table 1 shows the results of the assay for determining the antifungal activity of the studied substances. NAM exhibited better results in both MIC (250µg/mL) and MFC (250µg/mL) against the *C. albicans* strain and maintained this outcome when combined with FA:NAM (250µg/mL). The MIC/MFC ratio indicated that the substances are fungicidal against the studied strains.

Table 1: Antifungal activity of substances (FA, NAM, and FA:NAM mixture) against *Candida spp*.

Strains									
Substances	*C. albicans*			*C. tropicalis*			*C. krusei*		
	CIM	CFM	CFM/ CIM	CIM	CFM	CFM/ CIM	CIM	CFM	CFM/ CIM
FA	1000	1000	1	2000	2000	1	2000	2000	1
NAM	250	250	1	1000	1000	1	1000	1000	1
FA:NAM	250	250	1	1000	1000	1	1000	1000	1
Nystatin	1,5	1,5	1	1,5	1,5	1	1,5	1,5	1

Antifungal Mechanism of Action

The results showed that the antifungal properties of the substances are not related to the exogenous ergosterol pathway. In contrast, the antifungal properties of NAM and the FA:NAM mixture are related to the biosynthetic pathways of the cell wall; given that, the results showed that the MIC value against *C. albicans* increased fourfold for NAM (125 to 500µg/mL) and eightfold for the FA:NAM mixture (125 to 1000µg/mL) (Table 2). While in *C. tropicalis*, the MIC increased fourfold (500 to 2000µg/mL) for both NAM and the FA:NAM mixture (Table 3). This indicates that NAM and the FA:NAM mixture seem to act on the fungal cell wall, suggesting this mechanism as one of the possible targets. The caspofungin result worked as expected; the MIC value in the absence of sorbitol was 0.062µg/mL and increased significantly when in contact with sorbitol, presenting a value of 1.0µg/mL. Therefore, its mechanism of action via the fungal cell wall is confirmed.

Table 2: Effect of exogenous sorbitol (0.8 M) on the MIC of the molecules and caspofungin in the absence and presence of sorbitol (0.8 M) against the C. albicans strain ATCC 60193

C. albicans											
AF			**NAM**			**FA:NAM**			**caspofungin**		
Concentration (µg/mL)	Absence of sorbitol	Presence of sorbitol	Concentration (µg/mL)	Absence of sorbitol	Presence of sorbitol	Concentration (µg/mL)	Absence of sorbitol	Presence of sorbitol	Concentration (µg/mL)	Absence of sorbitol	Presence of sorbitol
4000	-	-	4000	-	-	4000	-	-	4	-	-
2000	-	-	2000	-	-	2000	-	-	2	-	-

1000	-	-	1000	-	-	1000	-	+	1	-	+
500	+	+	500	-	+	500	-	+	0.5	-	+
250	+	+	250	-	+	250	-	+	0,25	-	+
125	+	+	125	+	+	125	+	+	0,125	-	+
62,5	+	+	62,5	+	+	62,5	+	+	0,062	+	+
31,25	+	+	31,25	+	+	31,25	+	+	0,031	+	+

Observations: +, fungal growth; -, absence of fungal growth.

Table 3: Effect of exogenous sorbitol (0.8 M) on the MIC of the molecules and caspofungin in the absence and presence of sorbitol (0.8 M) against the *C. Tropicalis* strain ATCC 750

C. tropicalis											
AF			**NAM**			**AF:NAM**			**caspofungina**		
Concentration (µg/mL)	Absence of sorbitol	Presence of sorbitol	Concentration (µg/mL)	Absence of sorbitol	Presence of sorbitol	Concentration (µg/mL)	Absence of sorbitol	Presence of sorbitol	Concentration (µg/mL)	Absence of sorbitol	Presence of sorbitol
4000	-	-	4000	-	-	4000	-	-	4	-	-
2000	-	-	2000	-	+	2000	-	+	2	-	-
1000	+	+	1000	-	+	1000	-	+	1	-	+
500	+	+	500	+	+	500	+	+	0.5	-	+
250	+	+	250	+	+	250	+	+	0,25	-	+
125	+	+	125	+	+	125	+	+	0,125	-	+
62,5	+	+	62,5	+	+	62,5	+	+	0,062	+	+
31,25	+	+	31,25	+	+	31,25	+	+	0,031	+	+

Observations: +, fungal growth; -, absence of fungal growth

Growth kinetics

It is known that an ideal microbial growth curve should be measured through a graph that expresses the number of viable cells over time, i.e., the recording of countable cells at certain time intervals[31].

Our growth kinetics assay indicated that FA started to inhibit the growth of *C. albicans* ATCC 60193 after the 9ºth hour of incubation, while NAM and the FA:NAM mixture showed inhibition starting from the 8ºth hour (Figures A, B, C), for any evaluated concentration. This result is consistent with others already reported in the literature, reinforcing the fungicidal effect of the substances[21,27].

Figure A

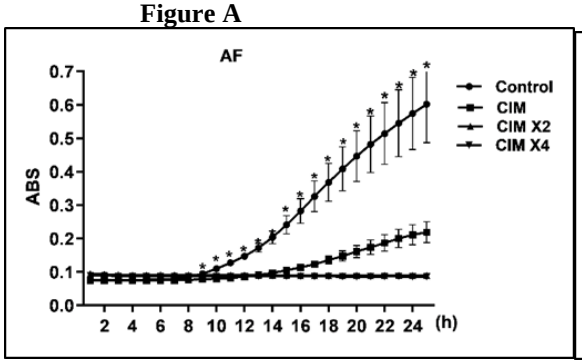


Figure B

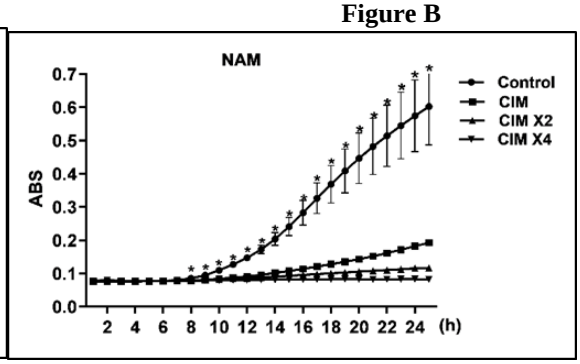
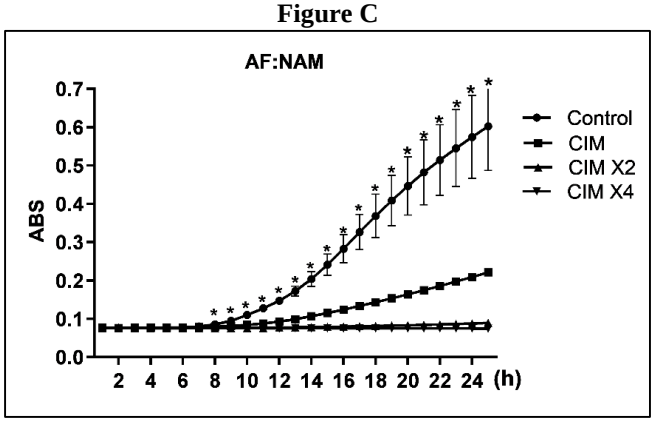


Figure C



Synergy Assay – Checkerboard Method

The combination of antifungals has been widely investigated due to its advantages in combating resistant infections. It increases effectiveness against target pathogens, minimizes the emergence of fungal resistance, and expands the spectrum of action. Consequently, it reduces the duration of antifungal therapy and shortens mortality rates[32,33].

Based on the combination assay, it was observed that there was no reduction in MIC values for *C. albicans* ATCC 90028 for the test substances. Considering that the Fractional Inhibitory Concentration Index (FICI) was equal to 0.5, it is evident that the combination of FA, NAM, FA:NAM, and nystatin exhibited an indifferent effect. These results are described in Table 4.

The above-mentioned results indicate relationships of indifference and consequently, the absence of antagonism among the tested compounds, suggesting the possibility of combining these compounds in the treatment of fungal infections without interfering with each other's mechanisms of action. However, further *in vitro* and *in vivo* studies are needed to understand the safety and efficacy of this combination, supporting our hypothesis.

Table 4. Fractional Inhibitory Concentration Index (FICI) after combination between *AF, NAM e AF:NAM* and Nystatin on *C. albicans* strain ATCC 90028

	Fractional Inhibitory Concentration (FIC)	$FICI=FIC_A + FIC_B$
FA	$FIC_A = 0,5$	1 (Indifferent)
NAM	$FIC_A = 0,5$	1 (Indifferent)
FA:NAM	$FIC_A = 0,5$	1 (Indifferent)
Nystatin	$FIC_B = 0,5$	
Synergism: FICI ≤0,5; Indifferent 0,5 < ICIF ≤ 4; antagonism >4		

IV. Discussion

With the aim of studying the antifungal action of plant-derived products, we evaluated FA, NAM, and the combination of these two substances. FA, as a representative of phenolic compounds, exhibits various biopharmacological activities reported in the literature[6,8,9,12,34]. Similarly, NAM also has therapeutic effects on various diseases, including anti-inflammatory action[35,36].

To enhance the effect of FA, by improving its solubility and consequently availability, we suggest that the combination with NAM could increase aqueous solubility. In our study, we aimed to explore the opportunity to combine these compounds, and them with nystatin, with the intention of increasing the efficacy of the anticandida medication. The effect against *Candida spp*. and the mechanisms of action in anticandida activity were investigated. No study has evaluated the effect of combining FA with NAM and the FA:NAM mixture with nystatin on *C. albicans*, *C. tropicalis*, and *C. krusei* until now.

FA is a promising fungicide for food preservation, demonstrating efficacy against *Fusarium graminearum* by inhibiting ergosterol synthesis in the cell membrane in a dose-dependent manner. The fungal cell membrane is a crucial target for FA to exert its antifungal effect, supported by the loss of membrane integrity, leakage of cytoplasmic content, and the absence of essential components for membrane synthesis[37]. It also inhibited and induced resistance against *P. expansum* in apples by biosynthesizing secondary metabolites in the phenylpropanoid metabolism, increasing gene expressions, and activities of enzymes involved in reactive oxygen species (ROS) metabolism[38].

Many plant secondary metabolites have antifungal and immunomodulatory effects. For instance, chlorogenic acid exhibits antifungal effects against phytopathogenic fungi through a mode of action dependent on ROS and alteration of fungal cell membrane permeability. Another phenolic acid, gallic acid, has antifungal effects against *C. albicans*, and FA shows anti-inflammatory action in bovine endometrium, primarily reducing pro-inflammatory cytokines. Therefore, plant secondary metabolites, particularly phenolic acids, have the potential for developing new antifungals[39,40].

The fungal cell wall plays a crucial role in protecting the cell from lysis and is important in pathogenesis. The main structural components of the fungal cell wall include β-glucans, chitin, and mannosylated proteins (manoproteins), which are efficiently regulated during cell growth[41]. Phenolic compounds isolated from *V. vinifera* have shown a strong ability to bind to proteins or glycoproteins. For example, caffeic acid in *C. albicans* inhibits the activity of the isocitrate enzyme, which is a key enzyme in the glyoxylate cycle and is an important virulence factor[42].

Femonstrated that NAM treatment led to a decrease in the level of mannose in the outer layer of the cell wall and an increase in chitin; moreover, it can expose the underlying β-glucan in the intermediate layer of the cell wall[27]. Previously, it was observed that agents affecting one component of the fungal cell wall often lead to modification of other cell wall components to compensate and try to maintain wall integrity. Therefore, our result

corroborates with reports from the literature, as we showed that the FA:NAM mixture has a fungicidal effect by acting on the cell wall of *C. albicans* and *C. tropicalis*[43].

Indeed, it seems that this effect was due to NAM since FA alone did not demonstrate a direct action on the cell wall. However, when we analyzed the FA:NAM mixture in *C. albicans*, the MIC was higher than when exposed only to NAM; taken together, these data suggest that FA contributed to greater stress on the fungal cell wall. Another polyphenolic compound, quercetin, acts as an antifungal against *C. albicans* through a combined action damaging cell wall integrity, increasing permeability, and causing cell wall damage[44].

The study of the synergistic action of substances has been one of the possibilities to combat infections resistant to antifungals. Synergisms have been found between caffeic acid and fluconazole, thymol with nystatin, fluconazole with nystatin, and the essential oil of *Mentha suaveolens* with antifungal drugs against *C. albicans*[45,46,47,48]. In our study, there was no synergistic effect of FA, NAM, or the FA:NAM mixture with nystatin. Despite this, the data show indifferent relationships and, consequently, an absence of antagonism, opening perspectives for the combination of these compounds for future use in the treatment of fungal infections.

Therefore, further *in vitro* and *in vivo* research is necessary for a more conclusive understanding of the suitability of FA and NAM as potential antifungal agents. New findings may assist in identifying interesting drug combinations with novel mechanisms of action.

V. Conclusion

In conclusion, this study demonstrated the fungicidal effect of FA and NAM on *Candida spp*. We consider that these effects are likely due to nicotinamide, but it is worth noting that the mechanism of action on the fungal cell wall and the time of inhibition of fungal growth were better when the FA:NAM mixture was used. Substantial evidence is available regarding the synergistic effect of ferulic acid and existing antifungal agents, which may become a promising anticandidal treatment. This study evaluated the combination of plant-derived substances and opened up different possibilities for the treatment of fungal infections against Candida species.

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