

Inhibitory Effect of (-)-myrtenol alone and in combination with antifungal agents on *Candida* spp.

Efeito inibitório do (-)-myrtenol sozinho e em combinação com agentes antifúngicos sobre *Candida* spp.

Efecto inhibidor del (-)-myrtenol solo y en combinación con agentes antifúngicos sobre *Candida* spp.

Received: 10/28/2021 | Reviewed: 11/07/2021 | Accept: 11/10/2021 | Published: 11/17/2021

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Abstract

The aim of this study was to examine the effects of (-)-myrtenol alone and combined with antifungal agents against *Candida* spp. The Minimum Inhibitory Concentration (MIC) and Minimum Fungicidal Concentration of (-)-myrtenol and fluconazole against *C. albicans* and *C. parapsilosis* strains was obtained using CLSI guidelines. Combination of (-)-myrtenol with antifungal drugs was determined by checkboard test. The (-) myrtenol showed MIC ranging from 256 to 512 µg/mL against both species assay. And the MFC was 512 µg/mL, demonstrated nature fungicidal (MFC/MIC < 4). In addition, combination of antifungal agents (amphotericin B and fluconazole) and (-) myrtenol showed synergistic and additive effects on strains assays. Based on these results, the present study demonstrates that (-) myrtenol showed strong fungicide activity against *Candida* spp. In addition, Combination of antifungal agents and (-) myrtenol reduces the effective concentrations of both the agents with synergistic to additive effects. Therefore, (-) myrtenol has potential to be developed into an antifungal agent.

Keywords: Monoterpenes; Antifungal activity; Natural products; Alternative therapy.

Resumo

O objetivo deste estudo foi analisar os efeitos do (-)-mirtenol sozinho e combinado com agentes antifúngicos frente *Candida* spp. A Concentração Inibitória Mínima (CIM) e a Concentração Fungicida Mínima de (-) - mirtenol e

fluconazol frente as cepas de *C. albicans* e *C. parapsilosis* foram obtidas usando as diretrizes de CLSI. A combinação de (-) - mirtenol com drogas antifúngicas foi determinada pelo teste de checkboard. (-) mirtenol apresentou CIM variando de 256 a 512 µg / mL frente ambas as espécies. E o MFC foi de 512 µg / mL, demonstrado natureza fungicida (MFC / MIC <4). Além disso, a combinação de agentes antifúngicos (anfotericina B e fluconazol) e (-) mirtenol frente as cepas ensaiadas apresentou efeitos sinérgicos e aditivos. Com base nesses resultados, o presente estudo demonstra que (-) mirtenol apresentou forte atividade fungicida contra *Candida* spp. Além disso, a combinação de agentes antifúngicos e (-) mirtenol demonstraram efeitos sinérgicos a aditivos, com redução da CIM de ambos agentes. Portanto, o (-) mirtenol tem potencial para ser desenvolvido como um agente antifúngico.

Palavras-chave: Monoterpeno; Atividade antifúngica; Produto natural; Terapia alternativa.

Resumen

El objetivo de este estudio fue analizar los efectos del (-)-myrtenol solo y combinado con agentes antifúngicos contra *Candida* spp. La Concentración Inhibitoria Mínima (MIC) y la Concentración Fungicida Mínima de (-) - myrtenol y fluconazol contra cepas de *C. albicans* y *C. parapsilosis* se obtuvieron utilizando las pautas de CLSI. La combinación de (-) - myrtenol con fármacos antimicóticos se determinó mediante la prueba del tablero de ajedrez. El (-) myrtenol mostró una CIM que varió de 256 a 512 µg / mL contra ambas especies. Y la MFC fue de 512 µg / mL, demostró naturaleza fungicida (MFC / MIC <4). Además, la combinación de agentes antifúngicos (anfotericina B y fluconazol) y (-) myrtenol contra las cepas probadas mostró efectos sinérgicos y aditivos. Con base en estos resultados, el presente estudio demuestra que el (-) myrtenol tenía una fuerte actividad fungicida contra *Candida* spp. Además, la combinación de agentes antifúngicos y (-) myrtenol mostró efectos sinérgicos con los aditivos, con una reducción de la CMI de ambos agentes. Por lo tanto, el (-) myrtenol tiene el potencial de desarrollarse como un agente antifúngico.

Palabras clave: Monoterpeno; Actividad antifúngica; Producto natural; Terapia alternativa.

1. Introduction

Fungal infections have achieved considerable importance over the last few decades, especially candidiasis, the most frequent opportunistic fungal infection, caused by strains of the *Candida* spp. With the development of superficial or problematic infections, such as invasive candidiasis, with a high mortality rate (Ben-Ami, 2018).

Invasive candidiasis affecting mainly immunocompromised patients in intensive care units (ICUs). Among the different *Candida* species isolated, *C. albicans* is the most common isolated, followed by non-*albicans* species such as *C. glabrata*, *C. parapsilosis*, *C. krusei*, *C. tropicalis* and the most recent *C. auris*, pathogen-resistant multidrug emerging (Jeffery-Smith et al., 2018; Pappas et al., 2018).

The strategy for the treatment of candidiasis depends on the patient's immune status, location, and severity of the infection, however a lack of rapid diagnostic assay, mainly invasive candidiasis, normally empiric antifungal therapy was initiated. This approach can lead to the unnecessary use of antifungal agents and promote the emergence of resistance in individuals (Bhattacharya et al., 2020).

A major factor that contributes to the high levels of morbidity and mortality of fungal diseases is the limited number of antifungal drugs. Currently, there are only three classes of antifungal drugs approved for the treatment of invasive mycoses, echinocandins, polyene and azoles. The efficacy of these agents is compromised by the development of drug resistance in pathogen populations, respective adverse effects and treatment costs (Lee et al., 2020; Wall & Lopez-Ribot, 2020).

Therefore, it is necessary to search for alternatives to this scenario. Through the development of new drugs that are more effective and have fewer adverse effects or that can be used in combination with current antifungals. One of the main sources of search is natural products and their derivatives, such as bicyclic monoterpene alcohols that have been shown to have several biological effects in the literature. (Nikitina et al., 2021)

(-) Myrtenol is an example of this group with several reported biological effects in literature: angiogenesis (Huang et al., 2021), gastric protector (Viana et al., 2019), anti-bacterial (Cordeiro et al., 2020). Thus, the aim of this study was to examine the effects of myrtenol alone and in combination with antifungal drugs against *Candida* spp.

2. Material and Methods

2.1 Type of study

This is an experimental laboratory study of a quantitative nature (Tacconi, 2018)

2.2 Microorganisms:

Candida spp. used in the antifungal assay was obtained from the archival collection of the Federal University of Paraíba Laboratory of Mycology (LM). They included *C. albicans* (ATCC 76485, LM-117, LM-516, LM-587 and LM-699), and *C. parapsilosis* (ATCC 22019, LM-439, LM-546, LM-577 and LM- 689). The inoculum preparation was carried out by M27 A2 document (CLSI., 2008)

2.3 Chemicals

The product tested was the (-) myrtenol, amphotericin B, Fluconazole and RPMI-1640-L-glutamine (without sodium bicarbonate) obtained by Sigma-Aldrich®, São Paulo, SP, Brazil. Sabouraud dextrose agar (SDA) were purchased from Difco Laboratories (Detroit, MI, USA), culture media were used.

2.4 Minimum Inhibitory Concentration (MIC) and Minimum fungicide Concentration (CFM)

The MIC of myrtenol and fluconazole was carried out using the microdilution technique in a microplate containing 96 wells with a U-shaped bottom (ALAMAR®), described by M27 A2 document (CLSI, 2008), with some modifications. The myrtenol had their test concentrations ranging between 1024 µg/mL and 0.5 µg/mL, Fluconazole was used as a positive control in assays at concentrations ranging from 64 to 0.125 µg/mL. Tension viability controls, average sterility, DMSO and Tween 80%, solvents used to prepare myrtenol emulsion, were performed simultaneously with the assay. All the tests were performed in duplicate and the plates were incubated at 35 ± 2°C for 24 hours to be conducted later playback. MIC was defined as the lowest concentration of products capable of producing visible inhibition on fungal growth when compared to its control.

The result was expressed by the arithmetic mean of the MICs obtained in the three tests. According to Sartoratto et al., 2004, results is considered strong antifungal activity MIC values between 0.05-0.50 mg/mL, MIC values between 0.6 to 1.50 mg/mL are considered moderately active and values MIC higher than 1.50 mg/mL are related to a weak antifungal activity.

After determination of the MIC, 10 µl aliquots of the supernatant from the wells corresponding to the MIC, MIC × 2 and MIC × 4 were subculture in an SDA containing plate, which was then incubated at 37°C for 24–48h. The MFC was the lowest drug concentration that showed either no growth or fewer than three colonies. All assays were performed in triplicate, and the geometric mean values were calculated. The MFC/MIC ratio was calculated to determine if the substance had fungistatic (MFC/MIC ≥ 4) or fungicidal (MFC/MIC < 4) activity (Siddiqui et al., 2013)

2.5 Combination study (checkerboard assay)

The (-) myrtenol was used in combination with fluconazole and amphotericin, following a method described previously Ahmad et al., 2015. (-) Myrtenol was combined with the antifungal drugs in a 1:1 volume ratio to the first row of microtiter plate and were serially diluted. A 100 µl of culture inoculum was added into each well and the plates were then incubated at 37 °C for 24 h, followed by MICs recording as described above. The experiment was performed in triplicate to validate the results. Based on lower additivity zero-interaction theory, combination interaction was calculated by determining the fractional inhibitory concentration index (FIC) indices were calculated as $FIC^A + FIC^B$, where FIC^A and FIC^B represent the Minimum Concentrations inhibiting the fungal growth for drugs A and B, respectively:

$FICA = MIC^A \text{ combination} / MIC^A \text{ alone}$ and $FIC^B = MIC^B \text{ combination} / MIC^B \text{ alone}$. A mean FIC index was

calculated based on the following equation: $FIC\ index = FIC^A + FIC^B$.

The interpretation was made as follows: synergistic (< 0.5), additivity (0.5-1.), indifferent (> 1) or antagonistic (> 4) (Ahmad et al., 2015)

3. Results and Discussion

The strategy for the treatment of candidiasis depends on the patient's immune status, location, and severity of the infection, however a lack of rapid diagnostic assay, mainly invasive candidiasis, normally empiric antifungal therapy was initiated. This approach can lead to the unnecessary use of antifungal agents and promote the emergence of resistance in individuals (Bhattacharya et al., 2020). Therefore, there is a dire need to discover novel antifungal agents.

The MIC and MFC values are presented in Table 1. The molecule showed inhibitory activity against all tested strains, with a MIC ranging from 256 to 512 µg/mL against both species assay. And the MFC was 512 µg/mL, demonstrated nature fungicidal (MFC/MIC < 4) (Siddiqui et al., 2013).

Table 1: MIC and MFC of (-) myrtenol and fluconazole against strains of *C. albicans* and *C. parapsilosis*.

Microorganisms	(-) Myrtenol				Flu	Controls			
	MIC	MFC	$\frac{MFC}{MIC}$	Nature activity	MIC	Mo	Ste	Sol	
<i>Candida albicans</i>	ATCC 76485	512	512	1	Fungicide	32(R)	+	-	+
	LM-117	512	512	1	Fungicide	08(R)	+	-	+
	LM-516	256	512	2	Fungicide	64(R)	+	-	+
	LM-587	256	512	2	Fungicide	16(R)	+	-	+
	LM-616	256	512	2	Fungicide	08(R)	+	-	+
<i>Candida parapsilosis</i>	ATCC 22019	512	512	1	Fungicide	16(R)	+	-	+
	LM-439	512	512	1	Fungicide	32(R)	+	-	+
	LM-546	512	512	1	Fungicide	16(R)	+	-	+
	LM-689	512	512	1	Fungicide	32(R)	+	-	+
	LM-577	512	512	2	Fungicide	64(R)	+	-	+

Note: Flu: fluconazole; (R): Resistant (according to CLSI, 2008); Mo: microorganism; Ste: sterility; Sol: RPMI + solvents; +: growth; -: no growth. Source: author himself. Fonte: Autores.

This result showed that the (-) myrtenol has showed strong fungicide activity against *Candida* spp. with MIC: 512 µg/mL, according criteria marked by Sartoratto et al., 2004. Supporting these results, Nikitina et al., 2021 showed that (-)-myrtenol has high antimycotic activity against all tested fungal species (yeast and filamentous fungi), MIC range (23.5 µg/mL – 47 µg/mL).

The literature data suggest that antifungal activity of (-)-myrtenol, possibly, probably damages the fungal membrane, affecting the change in the functional state of integrin-like proteins, which can lead to disruption of the morphogenesis of the fungal cell (Gomes et al., 2017)

In addition to their inherent antifungal properties, natural products and their derivatives may alter the effects of standard antifungal drugs. The potential benefits of combination therapy include: different mechanisms acting together allow complementary targeting within the fungal cells; expansion of the action spectrum, toxicity reduction due to lower dosage use and lower number of resistant organisms (Chang et al., 2017; Fuentesfria et al., 2018).

In Tables 2 and 3, the results were observed to combinations of the (-) myrtenol with antifungal drugs (amphotericin B and fluconazole) against *C. albicans* ATCC 76485 and *C. parapsilosis* ATCC 22019. Additive effect (FIC: 1) was observed for the combinations of (-) myrtenol with fluconazole against *C. albicans* ATCC 76485. Synergistic effects (FIC: 0.187 and 0.625) were observed for the combinations of (-) myrtenol with amphotericin B against *C. albicans* ATCC 76485 and *C. parapsilosis* ATCC 22019. The same synergistic effect was observed from the combinations of (-) myrtenol with fluconazole against *C. parapsilosis* ATCC 22019.

Table 2: MIC of Antifungal drugs and effect of combination with (-) myrtenol against *C. albicans* ATCC 76485.

Antifungal + myrtenol	MIC ($\mu\text{g/mL}$)	FIC INDEX (Type of interaction)
(-) Myrtenol	512	
Amphotericin B	1	
Fluconazole	32	
(-) Myrtenol/ Amphotericin B	32/0,125	0.187 (Synergism)
(-) Myrtenol/ Fluconazole	256/16	1 (Additivity)

Note: FIC: Fractional Inhibitory Concentration. MIC: Minimal Concentration Inhibitory. Source: Author himself

Table 3: MIC of Antifungal drugs and effect of combination with (-) myrtenol against *C. parapsilosis* ATCC 22019.

Antifungal + (-) myrtenol	MIC ($\mu\text{g/mL}$)	FIC INDEX (Type of interaction)
(-) Myrtenol	512	
Amphotericin B	1	
Fluconazole	16	
(-) Myrtenol/ Amphotericin B	64 / 0,5	0.625 (Synergism)
(-) Myrtenol/ Fluconazole	128 / 0,5	0.75 (Synergism)

Note: FIC: Fractional Inhibitory Concentration. MIC: Minimal Concentration Inhibitory. Source: Author himself.

In this study, synergistic and additivity effects were demonstrated when was combine the (-) myrtenol with the antifungal agents (amphotericin B and fluconazole) against *Candida* spp., depending on the strain assay, suggesting the (-) myrtenol positively modulated the *in vitro* action of this drugs, suggesting future pharmacological use as an adjuvant for these drugs.

Several reports have been made concerning different antifungal combinations assayed *in vitro* and applied in the clinic (Dąbrowska et al., 2021; Fuentefria et al., 2018; Perea et al., 2002; Shaban et al., 2020). However, combinations of the (-) myrtenol with antifungal agents against *Candida* spp. are reported here for the first time.

4. Conclusion

Based on these results, the present study demonstrates that (-) myrtenol showed strong fungicide activity against *Candida* spp. with MIC 512 $\mu\text{g/ml}$. In addition, Combination of antifungal agents and (-) myrtenol reduces the effective concentrations of both the agents with synergistic to additive effects. Therefore, (-) myrtenol has potential to be developed into an antifungal agent. However, future studies on this product are necessary, such as search to mechanism of action, toxicity tests, aiming their possible application in the clinic or as a source of new antimicrobial compounds.

Acknowledgments

These studies were partially funded by National Counsel of Technological and Scientific Development (CNPq).

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