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Note

In vitro activity of juglone (5-hydroxy-1,4-naphthoquinone) against both fluconazole-resistant and susceptible *Candida* isolates

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ABSTRACT

Background: The rise in antifungal resistance and drug class limitations are causing higher morbidity and mortality rates all over the world. This issue highlights the urgent need for new and improved antifungal drugs with a novel target.
Aims: In order to evaluate whether juglone can be served as an alternative antifungal to cure drug-resistant *Candida* infections, we studied the *in vitro* susceptibility of juglone against fluconazole-susceptible and -resistance *Candida* isolates, alone and in combination.
Methods: Antifungal susceptibility testing was performed according to the CLSI (Clinical and Laboratory Standards Institute) guidelines.
Results: Juglone exhibited the highest minimal inhibitory concentration (MIC) values, followed by fluconazole and nystatin. Voriconazole showed significantly better antifungal activity than juglone, fluconazole, and nystatin, with MIC_{50} and MIC_{90} of 0.031 and 0.5 μg/mL. There were significant differences in MICs of fluconazole ($p < 0.001$) and juglone ($p < 0.0003$) between *Candida albicans* and the rest of the species. Combination of juglone with fluconazole revealed insignificant effects against fluconazole-susceptible and -resistant *Candida* isolates. Juglone increased the antifungal activity of fluconazole; however, no synergism effects were observed for any combination, and only an insignificant effect was found against all tested *Candida* species.
Conclusions: Although obtaining new antifungal drugs is a critical point, a completely novel approach should be implemented.

Actividad *in vitro* de juglona (5-hidroxi-1,4-naftoquinona) contra aislamientos de *Candida* resistentes y sensibles a fluconazol

Palabras clave:
Juglona
5-Hidroxi-1,4-naftoquinona
Sensibilidad
Resistencia al fluconazol
Candida

RESUMEN

Antecedentes: El aumento de la resistencia a los antifúngicos y las limitaciones propias de los fármacos son responsables de mayores tasas de morbimortalidad en todo el mundo. Este trabajo destaca la urgente necesidad de nuevos y mejorados fármacos antimicóticos contra una nueva diana.
Objetivos: Con el fin de evaluar si la juglona puede servir como un antifúngico alternativo para curar las infecciones por *Candida* resistentes a los fármacos antifúngicos, hemos estudiado la sensibilidad in vitro a la juglona de aislamientos de *Candida* sensibles y resistentes al fluconazol, solo y en combinación.
Métodos: La prueba de sensibilidad a los antifúngicos se realizó de acuerdo con las guías del Clinical and Laboratory Standards Institute (CLSI).

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Resultados: La juglona mostró los valores de concentración mínima inhibitoria (CMI) más altos, seguida por el fluconazol y la nistatina. El voriconazol mostró una actividad antifúngica significativamente mejor que la juglona, el fluconazol y la nistatina, con valores de CMI_{50} y CMI_{90} de 0,031 y 0,5 μg/mL. Hubo diferencias significativas en las CMI del fluconazol ($p < 0{,}001$) y la juglona ($p < 0{,}0003$) entre los aislamientos de *Candida albicans* y aquellos de otras especies. La combinación de juglona con fluconazol reveló efectos insignificantes contra cepas de *Candida* sensibles y resistentes al fluconazol. La juglona aumentó la actividad antifúngica del fluconazol; sin embargo, no se observaron efectos de sinergia para ninguna combinación y solo se encontró un efecto insignificante contra todas las especies de *Candida* ensayadas.
Conclusiones: Aunque el diseño o el descubrimiento de nuevos fármacos antimicóticos es una tarea crítica, es necesario planificar un abordaje completamente novedoso.

The antifungal resistance phenomenon, which is increasing, is the cause of serious and life-threatening infections in immunosuppressed patients.[1,11] It is important to note that no new antifungals that might fight against drug-resistant fungal pathogens have been developed in the recent past.[12] New and improved antifungal drugs with a novel target and a new molecular structure are necessary to prevent cross-resistance. Juglone, 5-hydroxy-1,4-naphthoquinone, is an allelochemical from trees of the *Juglans* genus (walnut), that may be active against *Candida* species.[9] Juglone has shown inhibitory activity on the tricarboxylic acid cycle of *Staphylococcus aureus*, as well as on the synthesis of deoxyribonucleic acid, ribonucleic acid, and proteins by this microorganism.[13] However, to the best of our knowledge, there is limited evidence on the efficacy of juglone as an antifungal agent. To assess the potential use of juglone as an alternative antimycotic drug to cure *Candida*-resistant infections, we checked the *in vitro* activity of juglone against a collection of fluconazole-susceptible and -resistant *Candida* isolates, and the results were compared with those obtained with fluconazole, as well as those from the combination of juglone and fluconazole.

The strains consisted of fluconazole-resistant ($n = 14$) and -susceptible ($n = 26$) *Candida* isolates (according to non-species-specific *Candida* breakpoints of ≥4 μg/mL for fluconazole-resistant isolates), including *Candida albicans* ($n = 11$), *Candida glabrata* ($n = 6$), *Candida tropicalis* ($n = 5$), *Candida parapsilosis* ($n = 5$), *Candida krusei* ($n = 5$), *Candida kefyr* ($n = 4$), *Candida haemulonii* ($n = 3$) and *Candida auris* ($n = 1$) from urine ($n = 15$), biopsy ($n = 9$), peritoneal fluid ($n = 6$), blood ($n = 6$), and vagina ($n = 4$).[6,7,5,8] All isolates had been identified by conventional and molecular methods (i.e., sequencing of the Internal Transcribed Spacer region of ribosomal DNA using specific primers) in our previous studies.[6,7,5,8] Antifungal susceptibility testing was performed according to the guidelines provided by the Clinical and Laboratory Standards Institute (CLSI).[3] Antifungal agents were dispensed into microdilution trays at final concentration ranges of 0.016–16 μg/mL for amphotericin B (Sigma, St. Louis, MO, USA), nystatin (Sigma, St. Louis, MO, USA), itraconazole (Janssen Research Foundation, Beerse, Belgium), and voriconazole (Pfizer Central Research, Sandwich, UK); 0.063–64 μg/mL for fluconazole (Pfizer, Groton, CT, USA) and juglone (Sigma, St. Louis, MO, USA); and 0.008–8 μg/mL for micafungin (Astellas Pharma, Ibaraki, Japan) and anidulafungin (Pfizer, Central Research, Sandwich, United Kingdom). The *in vitro* interaction of fluconazole and juglone against 20 *Candida* isolates from eight different species was assessed by using a checkerboard method based on the microdilution broth reference technique of the CLSI, as described previously.[10] Concentrations ranged from 0.125 to 64 μg/mL for fluconazole and juglone. The fractional inhibitory concentration index (FICI) was calculated and the interaction was interpreted as synergistic for FICI ≤ 0.5, indifferent for FICI = 0.54, and antagonistic for FICI > 4.[10] The Students *t*-test was used to find out a statistical significance difference between juglone and fluconazole MICs in *C. albicans* and non-*C. albicans Candida* isolates, using the SPSS statistical package (version 7.0; SPSS Inc., Chicago, IL). The *p*-values lower than 0.05 were considered statistically significant.

The *in vitro* activities of juglone and other antifungals against 40 fluconazole-resistant and -susceptible clinical *Candida* isolates are summarized in Table 1. Juglone exhibited the highest MICs (MIC range, 0.5–>64 μg/mL; MIC_{90}, 16 μg/mL), followed by fluconazole (MIC range, 0.125–>64 μg/mL; MIC_{90}, 8 μg/mL), nystatin and amphotericin B. *Candida albicans* isolates were more sensitive to juglone (MIC range, 0.52 μg/mL; MIC_{90}, 2 μg/mL) than non-*C. albicans Candida* isolates (MIC range, 0.5–>64 μg/mL; MIC_{90}, 16 μg/mL). There were significant differences in the MICs of fluconazole ($p < 0.001$) and juglone ($p < 0.0003$) between *C. albicans* and the rest of the species. Voriconazole showed significantly better antifungal activity than juglone, fluconazole, and nystatin, with $MIC_{50/90}$ values of 0.031 and 0.5 μg/mL. All *Candida* isolates had low MICs of anidulafungin (MIC range, 0.004–0.5 μg/mL; MIC_{90}, 0.5 μg/mL) and micafungin (MIC range, 0.008–0.5 μg/mL; MEC_{90}, 0.5 μg/mL). The checkerboard analysis of the juglone and fluconazole is summarized in Table 2. The FICI results revealed indifferent effect against fluconazole-susceptible and -resistant *Candida* isolates when juglone was combined with fluconazole. Overall, no antagonistic effects were observed against *Candida* isolates with this combination.

The limited therapeutic options to cure infections caused by strains resistant to one or more antifungal agents has increased the mortality.[12] It is essential to design new classes of antifungals, as well as novel combination therapies to treat invasive fungal infections caused by drug-resistant pathogens. Based on the results of this study, juglone did not show good antifungal activity against *Candida* isolates. The MIC_{50} values for juglone and fluconazole against *Candida* isolates were 2 μg/mL and 1 μg/mL, respectively, whereas MIC_{90} values were 16 and 8 μg/mL. However, D'Angeli et al. reported potent activity of *Juglans regia* pellicle extract against most of the tested *Candida* strains.[4] The findings of a study conducted by Clark et al. also showed a moderate antifungal activity of juglone against *Trichophyton mentagrophytes* and *Microsporum gypseum*.[2] The antifungal activity of juglone was also observed against *Aspergillus* strains, *Penicillium* strains, *Hansenula* strains, and *Saccharomyces carlsbergensis*.[14,15] The reasons for these discrepancies in the results may rely on multiple factors such as the fungal strain, the method used, or even the way in which MIC endpoints have been determined. In this study, we also used the checkerboard microdilution method to analyze the drug-drug interactions of juglone with fluconazole against 20 fluconazole-resistant and -susceptible *Candida* isolates. Juglone increased the antifungal activity of fluconazole; however, no synergism effect was observed for any combination and an indifferent effect was found against all tested *Candida*. Finally, the increasing antifungal resistance, along with the lack of newly developed drugs, is an alarming signal for global public health. Although the critical point is the difficulty in obtaining new antifungal drugs, it is needed to plan a completely novel approach.

Table 1
In vitro activity of juglone and seven antifungal drugs against clinical *Candida* isolates.

Strains and antifungal drugs	MICs (μg/mL)															
	Range	MIC_{50}/MIC_{90}	G mean	≤0.008	0.016	0.031	0.063	0.125	0.25	0.5	1	2	4	8	16	32	≥64
All *Candida* isolates (40)																	
AmB	0.031–1	0.25/0.5	0.213			2	2	**13**	12	8	3						
FLZ	0.125–>64	1/8	1.275					2	5	7	**8**	4	5	4		2	3
ITZ	0.016–1	0.125/0.5	0.101		4	**9**	5	7	**9**	4	2						
VRZ	0.008–0.5	0.031/0.25	0.036	1	**16**	13	2	2	3	3							
NYT	0.125–4	1/2	0.982					1		10	**18**	10	1				
AFG	0.004–0.5	0.125/0.5	0.068	9	2	1	7	**10**	5	6							
MFG	0.008–0.5	0.5/0.5	0.043	**15**	1	4	4	7	4	5							
Juglone	0.5–>64	2/16	2.639							3	**13**	4	5	4	4	2	5
Candida albicans (11)																	
AmB	0.31–1	0.125/0.5	0.117			2	2	**5**		1	1						
FLZ	0.125–1	0.5/1	0.413					1	**4**	3	3						
ITZ	0.016–0.25	0.031/0.125	0.035		3	**6**		1	1								
VRZ	0.016–0.063	0.016/0.031	0.020		**8**	2	1										
NYT	0.5–2	1/2	0.938							3	**6**	2					
AFG	0.004–0.031	0.008/0.016	0.009	**8**	2	1											
MFG	0.008	0.008	0.008	**11**													
Juglone	0.5–2	1/2	1.065							1	**8**	2					
Other *Candida* species (29)																	
AmB	0.125–1	0.25/0.5	0.268					8	**12**	7	2						
FLZ	0.125–>64	2/8	2.054					1	1	4	**5**	4	5	4		2	3
ITZ	0.016–1	0.125/0.5	0.151		1	3	5	6	**8**	4	2						
VRZ	0.008–0.5	0.031/0.5	0.046	1	8	**11**	1	2	3	3							
NYT	0.125–4	1/2	1					1		7	**12**	8	1				
AFG	0.008–0.5	0.125/0.5	0.144	1			7	**10**	5	6							
MFG	0.008–0.5	0.125/0.5	0.083	4	1	4	4	7	4	5							
Juglone	0.5–>64	4/16	4							2	**5**	2	5	4	4	2	**5**

AMB: amphotericin B; FLZ: fluconazole; ITZ: itraconazole; VRZ: voriconazole; NYT: nystatin; AFG: anidulafungin; MFG: micafungin. Figures in bold are modal values.

Table 2
In vitro combination of fluconazole with juglone against resistant and susceptible *Candida* species.

		MIC (μg/mL)			
Isolate	*Candida* species	Fluconazole	Juglone	Fluconazole/Juglone	FICI/INT
FDC1	*C. albicans*	0.5	1	0.125/1	1.25/IND
FDC3	*C. albicans*	0.125	2	0.125/1	1.5/IND
FDC22	*C. albicans*	1	2	0.5/1	1/IND
FDC27	*C. albicans*	0.25	1	0.125/0.5	1/IND
FDC8	*C. glabrata*	2	4	1/4	1.5/IND
FDC9	*C. glabrata*	4	16	1/8	0.75/IND
FDC15	*C. glabrata*	2	8	2/4	1.5/IND
FDC43	*C. parapsilosis*	8	32	4/16	1/IND
FDC48	*C. parapsilosis*	0.5	2	0.25/0.5	0.75/IND
FDC52	*C. parapsilosis*	1	4	0.5/2	1/IND
FDC17	*C. krusei*	≥64	≥64	≥64/≥64	2/IND
FDC51	*C. krusei*	32	≥64	16/≥64	1.5/IND
FDC38	*C. kefyr*	2	8	1/2	0.75/IND
FDC6	*C. kefyr*	8	32	4/8	0.75/IND
FDC41	*C. tropicalis*	1	1	0.5/1	1.5/IND
FDC14	*C. tropicalis*	0.5	1	1/1	3/IND
FDC21	*C. tropicalis*	0.5	0.5	0.5/0.5	2/IND
FDC71	*C. haemulonii*	8	8	2/8	1.25/IND
CMRC 386	*C. haemulonii*	4	16	2/4	0.75/IND
FDC229	*C. auris*	≥64	≥64	16/≥64	1.25/IND

MIC: minimum inhibitory concentration; FICI: fractional inhibitory concentration index; INT: interpretation; IND: indifference.

Conflict of interest

All authors report no potential conflicts of interest. The authors alone are responsible for the content and writing of the paper.

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