Research in Molecular Medicine

Investigating the Antifungal Effects of Spirocyclopropane Oxindoles Derivatives Against *Aspergillus* Species

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Citation Ardebilifard A, Hoseinnejad A, Jafarzade J, Hajizadeh Jouybari N, Rajabnia Baboli A, Khalilpour A, et al. Investigating the Antifungal Effects of Spirocyclopropane Oxindoles Derivatives Against *Aspergillus* Species. Research in Molecular Medicine. 2023; 11(2):103-112. https://doi.org/10.32598/rmm.11.2.1287.1

https://doi.org/10.32598/rmm.11.2.1287.1

Article Type:
Research Paper

Article info:
Received: 10 Jan 2024
Revised: 25 Jan 2024
Accepted: 28 Mar 2024

Keywords:
Spirocyclopropane oxindoles derivatives, Itraconazole, *Aspergillus*

ABSTRACT

Background: Given the increasing resistance of *Aspergillus* spp. to azoles, finding effective new compounds, such as the spirocyclopropane oxindoles (4a-4b-4c) derivatives, seems necessary. The present study aimed to evaluate the antifungal activity of spirocyclopropane oxindoles (4a-4b-4c) derivatives against *Aspergillus* spp.

Materials and Methods: In vitro, the cytotoxicity of the synthesized compounds was evaluated against MCF-7 cancer cell lines using the MTT assay. In the next step, the antifungal susceptibility of 50 *Aspergillus* isolates of clinical origin to spirocyclopropane oxindoles (4a-4b-4c) derivatives and itraconazole was evaluated according to CLSI (Clinical and Laboratory Standards Institute) M38A2 guidelines. Statistical analysis was performed using SPSS software, version 20, and the significance level was considered $P<0.05$.

Results: The results revealed that 4c exhibited the lowest toxicity to MCF-7 cells among the three synthesized compounds. However, this level of toxicity was higher than control.

The present study shows a significant difference between the minimum inhibitory concentration (MIC) of 4a-4b-4c oxindole derivatives of spirocyclopropane and itraconazole against *Aspergillus* spp. Comparing the MIC values of 4a, 4b, and 4c oxindole spirocyclopropane derivatives with each other, 4b derivatives have lower MIC values for *Aspergillus flavus* and *Aspergillus fumigatus* isolates. In addition, 4c derivatives had the highest MIC for *Aspergillus terreus*.

Conclusion: Although the antifungal effects of spirocyclopropane oxindoles (4a-4b-4c) on *Aspergillus* spp. were significantly lower than itraconazole, we hope to increase the antifungal effects of these compounds with structural changes.

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Introduction

Aspergillosis is an infection caused by inhaling saprophytic *Aspergillus* species spores. Disease resulting from an aspergillosis infection usually affects the respiratory system, but the signs and severity of the disease vary greatly. The prevalence of invasive pulmonary aspergillosis is 1% to 15% [1]. Pathogenic agents include *Aspergillus fumigatus*, *Aspergillus flavus*, *Aspergillus nidulans*, *Aspergillus niger*, *Aspergillus terreus*, and *Aspergillus clavatus* [2, 3]. Background diseases such as immune deficiency, diabetes, corticosteroid usage, broad-spectrum antibiotics, malignancy, transplantation, chronic lung disease, alcoholism, etc. in the host, cause disease progression [4]. Treatment of these patients depends on the nature of the underlying disease, early diagnosis, and appropriate drug choice [5]. Over the last two decades, the susceptibility of *Aspergillus* spp. to anti-fungal drugs, particularly azoles, has decreased [6, 7]. Several studies indicate an increase in drug resistance in *Aspergillus* spp., and the consequences of this resistance between new triazoles and other antifungal drugs will be alarming [8, 9]. Spirocyclopropane oxindole is a crucial structural subunit as a stabilized pharmacophore found in many biologically active natural products and small molecules with wide therapeutic applications [10]. Several biological activities have been reported for spirocyclopropane oxindoles, including antitumor properties, pain reliever, treatment of central nervous system disorders, antiviral effects, etc. [11]. Recently, several studies have investigated the antifungal effects of oxindole derivatives of spirocyclopropane, providing relatively good results that by making changes in the skeleton of oxindole spirocyclopropane, compounds with potential and valuable antifungal effects can be obtained [12]. The antifungal susceptibility test for *Aspergillus* spp. has been standardized by the Clinical and Laboratory Standards Institute (CLSI) and the European Committee for Antimicrobial Susceptibility Testing (EUCAST) [13]. Given the increasing prevalence of Aspergillosis, the mortality's rate and increased drug resistance the importance of choosing an effective and appropriate treatment is evident. It should be noted that appropriate diagnostic and treatment strategies are required for this wide range of infections. Using oxindole spirocyclopropane compounds can be a suitable solution to deal with the cases mentioned. This study was conducted to investigate and compare the antifungal activity of oxindole spirocyclopropane derivatives (4a, 4b, 4c) and itraconazole against *Aspergillus* spp. isolated from clinical samples of Babol City, Mazandaran Province, Iran.

Materials and Methods

Isolates

In this experimental and laboratory study, 50 *Aspergillus* isolates: *A. fumigatus* (15), *A. flavus* (15), *A. niger* (15), and *A. terreus* (5)) were available in the reserve bank of the Department of Parasitology and Mycology, Babol University of Medical Sciences. Fungal isolates were confirmed using the PCR–restriction fragment length polymorphism molecular technique [14]. To perform the antifungal susceptibility test, fungal isolates were cultured on Sabouraud dextrose agar culture medium containing chloramphenicol and incubated for 2 to 5 days at 30 ˚C.

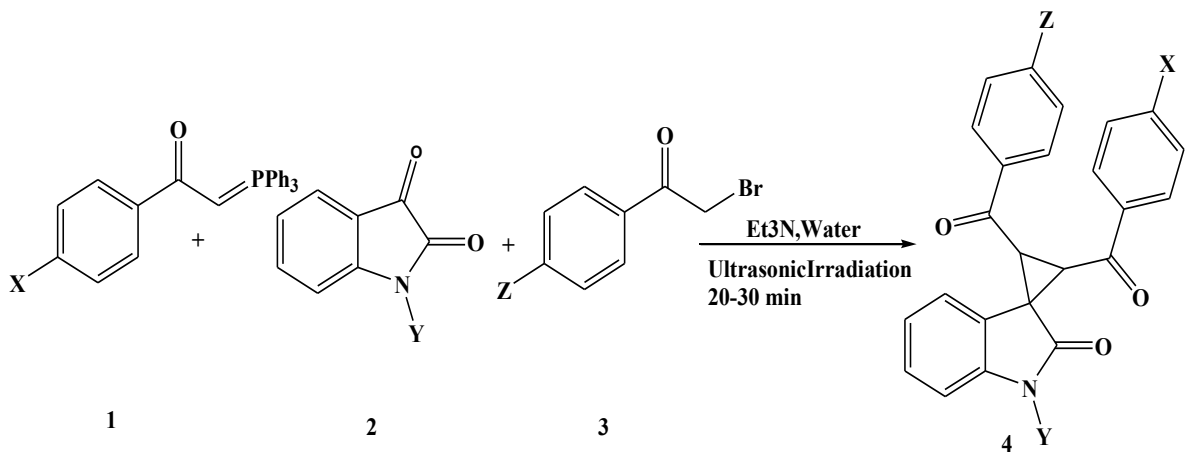
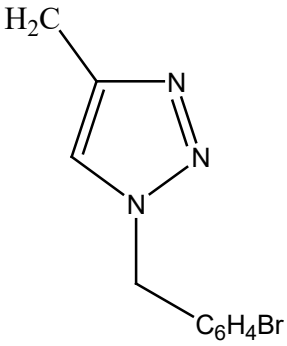
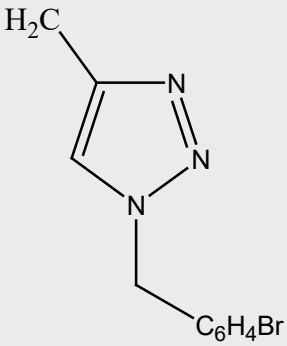
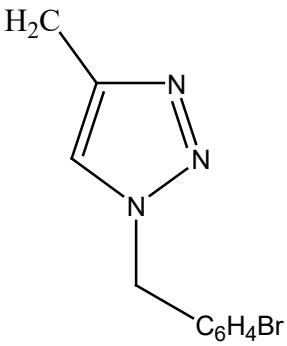


Figure 1. The structure of derivatives 4a-4b-4c

Table 1. Structure of (4a-4b-4c) spirocyclopropane oxindoles

Structure	y	x	z	Types of Compounds
4a		CL	H	2-Benzoyl-1-[(1-(4-bromobenzyl)-1H-1,2,3-triazol-4yl) methyl]-3- (4-chloro-benzoyl) spiro[cyclopropane-1,3-indol]-2(1H)-one
4b		CL	F	2-Benzoyl-1-[(1-(4-bromobenzyl)-1H-1,2,3-triazol-4yl) methyl]-3- (4-fluoro-benzoyl) spiro[cyclopropane-1,3-indol]-2(1H)-one
4c		H	F	2-Benzoyl-1-(1-(4-bromobenzoyl) 1,2,3-triazol-4yl)methyl]-3-(4-fluoro-benzoyl) spiro[cyclopropane-1,3-indol]-2(1H)-one

Synthesis of spirocyclopropane oxindole derivatives

Three derivatives of oxindole spirocyclopropane (4a-4b-4c) based on the study of Pourshab et al. (2019) were synthesized and prepared (Figure 1 and Table 1) [15].

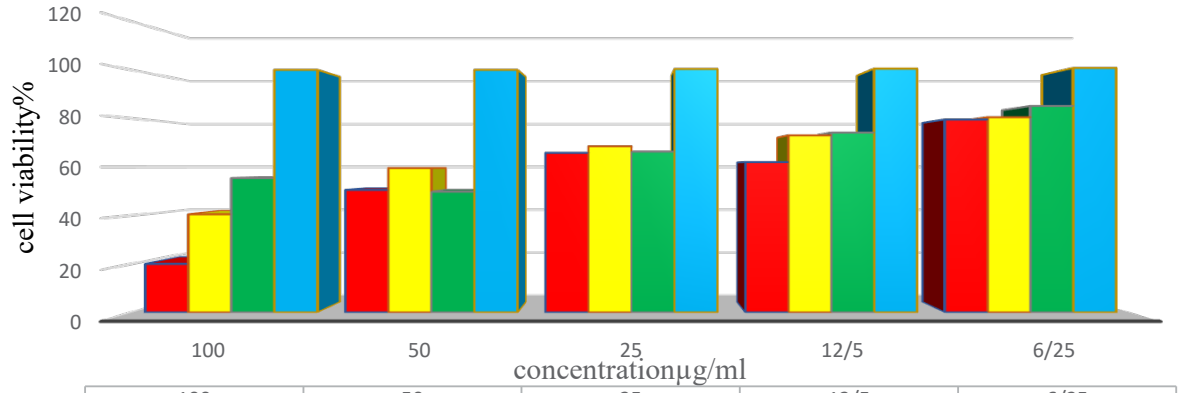
Cell viability assay

Cell viability of synthesized compounds (4a–c) was determined using the MTT assay described by Mosmann [16]. The cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM), supplemented with 10% fetal bovine serum and 1% antibiotics (penicillin/streptomycin) in a humidified incubator at 37 °C and 5% CO_2. For the experiment, cells were seeded at a density of 1×10^4 cells/well in a 96-well microplate and incubated overnight. The cells were then exposed to a diluted series of test samples with DMEM (at different concentrations ranging from 0 to 100 µg/mL and incubated at 37 °C for 24 and 48 h. As a negative control, standard culture media without treatment was also used. Standard doxorubicin was used as positive control. For cytotoxicity assay, 50 µL of MTT stock solution (5 mg/mL) was added to each culture medium, and the plates were incubated at 37 °C. After 4 h incubation, the result was that MTT formazan purple crystals were dissolved in acidic isopropanol dissolved in 1 mL dimethyl sulfoxide (DMSO) at ambient temperature. Using a microplate reader, the solubilized formazan crystals' optical density (OD) was measured at 570 nm (Rayto, Shenzhen, China). The cell

Table 2. The results of antifungal susceptibility testing *Aspergillus* spp. against spirocyclopropane-oxindole derivatives and itraconazole

Aspergillus spp.	MIC_{50}/MIC_{90}/GM (µg/mL)	4a	4b	4c	Itraconazole
A. fumigatus (n=15)	MIC_{50}	16	8	32	0.25
	MIC_{90}	32	16	64	0.25
	GM	20.158	8	35.098	0.180
	Maximum	64	32	64	0.5
	Minimum	8	4	16	0.062
	SD	13.812	7.539	18.995	0.110
A. flavus (n=15)	MIC_{50}	32	8	64	0.25
	MIC_{90}	64	16	128	0.25
	GM	33.513	12.699	53.199	0.180
	Maximum	32	32	128	0.5
	Minimum	16	8	16	0.062
	SD	17.803	6.566	46.319	0.208
A. niger (n=15)	MIC_{50}	16	128	32	0.125
	MIC_{90}	64	128	64	0.25
	GM	25.398	61.109	40.317	0.157
	Maximum	128	128	32	0.5
	Minimum	8	16	16	0.062
	SD	32.519	65.932	17.596	0.112
A. terreus (n=5)	MIC_{50}	128	256	512	2
	MIC_{90}	128	256	512	4
	GM	97.005	128	388.023	2.297
	Maximum	256	265	512	4
	Minimum	32	16	256	1
	SD	85.865	108.281	140.216	1.341
Total isolates (n=50)	MIC_{50}	32	16	32	0.25
	MIC_{90}	64	128	128	0.5
	GM	29.446	22.315	52.709	0.223
	Maximum	256	256	512	4
	Minimum	8	4	16	0.062
	SD	42.756	72.245	119.954	0.828

MIC: Minimum inhibitory concentration; GM: Geometric mean.



	100	50	25	12/5	6/25
4a	20	50/5	65/8	62	79/54
4b	40/45	59/52	68/5	73	80/5
4c	55/4	49/8	66/32	74/15	85/12
Control	100	100	100/4	100/5	100/8

Figure 2. The MCF-7 cell viability after 24 h incubations with compounds 4a-c

viability (%), as the ratio between the amount of formazan determined for cells treated with synthesized compounds and for control non-treated cells, was calculated as follows (Equation 1):

1. % Cell viability=(OD treated/OD control)×100

Antifungal susceptibility testing

In vitro, antifungal susceptibility testing of *Aspergillus* isolates was done using the broth microdilution method as recommended by the protocol CLSI M38-A3 [17]. Itraconazole (Sigma-Aldrich USA) was used as a control drug to compare the antifungal activity of new derivatives. First, 2.3 mg of pure powder of drug and derivatives were dissolved in 1 mL of DMSO, and serial dilutions were prepared for final concentrations ranging from 0.256 to 128 µg/mL for spirocyclopropane oxindole derivatives, and 0.032 to 16 for Itraconazole. In the next step, 200 µL of spirocyclopropane oxindole derivatives and itraconazole were seeded into the first column of a flat-bottomed 96-well plate, and then 100 µL of RPMI medium (Sigma-Aldrich, USA) was added to the remaining wells (except the first column) and serial dilution was done. Columns 11 and 12 were considered the negative control (drug only, no organism) and the positive control (organism only, no drug). The suspension was adjusted spectrophotometrically to ODs between 80% to 83% transmission at a 530 nm wavelength. Lastly, 100 µL of the fungal suspensions prepared were added to all columns except the negative control column, and the plates were incubated at 35 ˚C for 48 hours. After incubation, the minimum inhibitory concentration (MIC) was visually determined as the lowest drug concentration that inhibited fungal growth by 100% or more. The reference strains of *Candida parapsilosis* (ATCC 22019) and *Candida krusei* (ATCC 6258) were used as quality control for each new set of isolates. All antifungal susceptibility tests were replicated to ensure reproducibility.

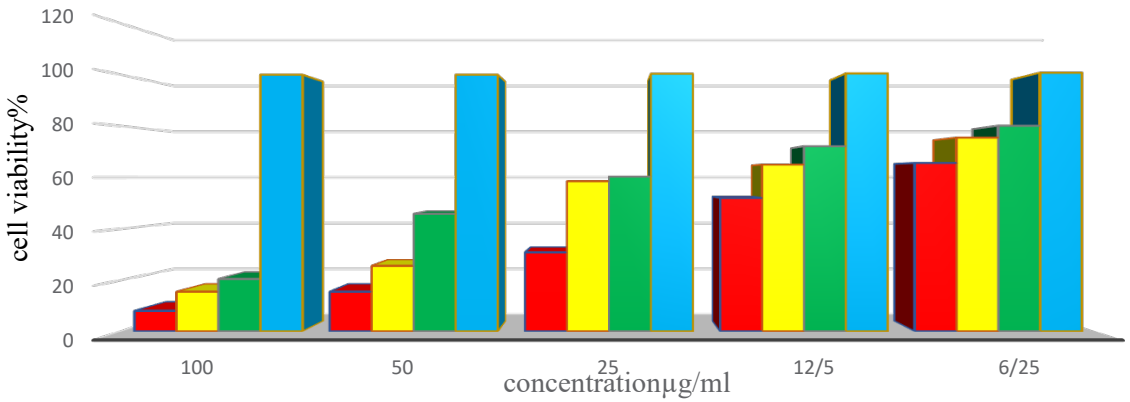
Statistical analysis

The data were analyzed using SPSS software, version 27 (IBM) software. The independent t-test was used to analyze quantitative results, and the chi-squared test was used to analyze qualitative variables, with $P \leq 0.05$ considered significant. Also, using Excel version 2018, $MIC_{50,}$ MIC_{90}, and GM (geometric mean) were calculated for all isolates.

Results

Derivatives of oxindole spirocyclopropane (4a-4b-4c) toxicity on MCF-7 cancer cell lines

Initially, the cytotoxicity of compounds 4a-c was evaluated in vitro using an MCF-7 cell line exposed to concentrations of 6.25-100 µg/mL for 24 and 48 h and compared to a control without synthesized compounds (Figure 2 and Figure 3). According to the results, the 4c compound had the least toxicity to MCF-7 cells among the three synthesized compounds, but its toxicity was higher than that of the control group. Furthermore, the assessment of compound toxicity at various time points (24 and 48 hours) indicates that as the duration increases from 24 to 48 hours, the cell viability rate of MCF-7 cells



	100	50	25	12/5	6/25
4a	8	15/46	30/8	52	65/64
4b	15/45	25/52	58/5	65	75/5
4c	20/4	45/8	60/32	72/15	80/12
Control	100	100	100/4	100/5	100/8

Figure 3. The MCF-7 Cell Viability After 48 h Incubations With Compounds 4a-c

decreases. This trend may be attributed to the increased exposure time, potentially providing more opportunities for the compounds to exert their effects.

Antifungal susceptibility testing

In this study, there is a significant difference between the MIC values of 4a-4b-4c oxindole derivatives of spirocyclopropane and the control drug (itraconazole) against *Aspergillus* species (P<0.001). Comparing the mean MIC of 4a, 4b, and 4c oxindole spirocyclopropane derivatives showed that the 4b derivatives have lower mean MIC for *A. flavus* and *A. fumigatus* isolates. In addition, 4c derivatives had the highest mean MIC for *A. terreus*. According to the MIC_{50} and GM obtained, all three investigated derivatives showed the least antifungal activity against *A. terreus*, so the mean MIC of the three derivatives 4a, 4b, 4c for *A. terreus* was 121.6, 182.4, 409.6 µg/mL, respectively. It is important to note that, in contrast to *A. terreus*, 15 isolates of *A. fumigatus* were more susceptible to derivatives. *A. flavus* and *A. niger* also showed moderate susceptibility to their derivatives (Table 2 and Figure 4).

Discussion

Due to the limitation of antifungal drugs, the increase of invasive fungal diseases, and the emergence of treatment-resistant species, researchers are moving towards using various compounds with suitable chemical structures with anti-inflammatory effects.

The cytotoxicity evaluation results indicated that 4c displayed the lowest toxicity among the three synthesized compounds, albeit with a toxicity level higher than that of the control.

Our study showed that the mean MIC of spirocyclopropane oxindole derivatives (4a-4b-4c) compared with itraconazole significantly differs against *Aspergillus* spp.

Rajaraman et al. synthesized 7 new spirooxindole derivatives via dipolar 1,3-cycloaddition. They evaluated their antifungal activity using disk diffusion and broth microdilution methods on a series of clinical fungal isolates, including *Candida albicans* and *A. niger*, *A. flavus*, *Cryptococcus neoformans*, and *Fusarium oxysporum*, compared to the ketoconazole as a control drug. Derivatives 4a and 4b showed good inhibitory activity against *A. niger* with MIC of 3.125 µg/mL, which was higher MIC compared to the control drug ketoconazole [18]. In the current study, derivative 4b of oxindole spirocyclopropane with a mean MIC of 14.1 µg/mL showed good inhibitory activity against *A. flavus*, with the difference that the inhibitory activity of derivative 4b was lower than itraconazole. The difference in the results may be due to the difference in the type of fungal strains. A previous study evaluated the antifungal activities of new polyheterocyclic spirooxindole derivatives against *Rhizoctonia solani*, *Fusarium semitectum*, *Alternaria solani*, *Valsa mali*, and *Fusarium graminearum*) using the mycelium growth rate method. Among the evaluated derivatives, polyheterocyclic spirooxindole derivative 53 had a better inhibitory effect on *F. graminearum* (31/3 IC_{50}=micromolar) [19]. As in the previous study,

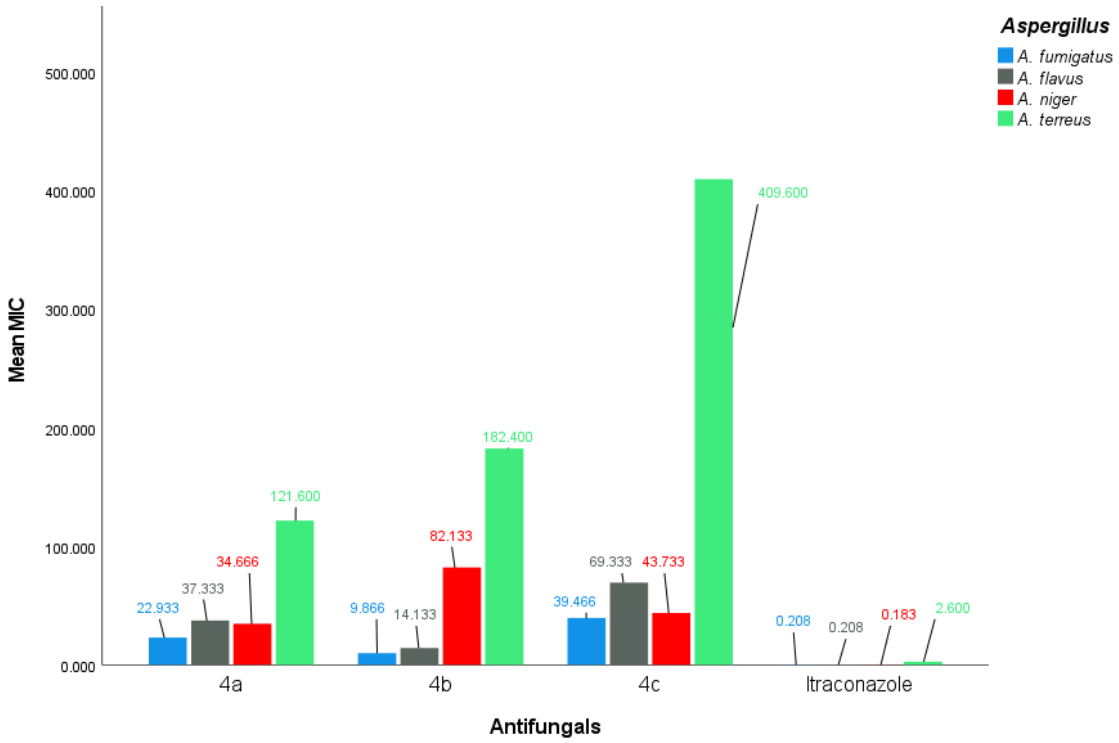


Figure 4. Minimum Inhibitory Concentration (MIC) Mean of Itraconazole and Spirocyclopropane Oxindole Derivatives Against Aspergillus Spp. Isolates

the antifungal effects of spirooxindole derivatives were confirmed in our study. However, the differences in the results can be due to the type of strain investigated, the substitution of oxindole spirocyclopropane derivatives tested, and the antifungal susceptibility evaluation method. In addition to these few studies that confirmed the antifungal effects of various spirooxindole derivatives, other studies were also conducted during the years 2017 to 2023 on various spirooxindole derivatives and their results showed that these compounds have antibacterial effects on various bacteria, including *Mycobacterium tuberculosis*, *Vibrio cholera*, *Escherichiacoli*, *Bacillus Subtleties*, *Bacillus Licheniformis, Pseudomonas Fluorescece, Salmonella enterica*, *Shigella Flexner*, and *Shigella Boydi* [20-23]. In general, the results obtained from the studies conducted in this field show the antifungal, antimicrobial, and antiparasitic effects of derivatives with spirooxindole scaffolds, in some of which the derivatives have better effects than the standard drugs used [24]. These promising results give hope that a drug with better antifungal effects can be obtained from compounds with a spirooxindole scaffold. Therefore, by substituting chlorine in ortho, meta, or other positions, the antifungal properties of these compounds can be increased compared to the standard antifungal drug. It should be noted that, unlike our study, none of the previous studies evaluated the toxicity of spirocyclopropane oxindole derivatives (4a-4b-4c) on cells. Investigation of the toxicity of these and similar compounds may help to clarify the path of research into the unique properties of these derivatives.

Conclusion

Although the antifungal effects of derivatives (4a-4b-4c) oxindole spirocyclopropane were confirmed on clinical isolates of *Aspergillus*, their antifungal properties were lower than itraconazole, but, based on the confirmation of the antimicrobial effects of these derivatives in many other studies, further investigation can be carried out and, by making constructive changes to the structure of the spirooxindole, compounds with good antifungal properties can be prepared.

Ethical Considerations

Compliance with ethical guidelines

This study was approved by the Research Ethics Committee of Babol University of Medical Sciences, Babol, Iran (Code: IR.MUBABOL.HRI.REC.1402.042).

Funding

The Research Vice-Chancellor of Babol University of Medical Sciences has provided financial support for this study (Grant No.: 724135034).

Authors contribution's

Conceptualization, supervision, funding administration, review and editing: Mojtaba Taghizadeh Armaki and Asieh Khalilpour; Writing the original draft: Amirreza Ardebilifard, Firoozeh Kermani, and Akbar Hoseinnejad; Methodology: Amirreza Ardebilifard, Jalal Jafarzade, and Aryan Yousefifard; Software: Akbar Hoseinnejad; Data analysis: Akbar Hoseinnejad and Jalal Jafarzade; Investigation: Aida Rajabnia Baboli, Aryan Yousefifard, Nava Hajizadeh Jouybari.

Conflict of interest

The authors declared no conflict of interest.

Acknowledgements

The authors would like to thank Babol University of Medical Sciences for funding this study.

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