Antifungal Activities of Artesunate, Chloramphenicol, and Co-trimoxazole in Comparison to Standard Antifungal Agents against Basidiobolus Species

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Abstract

Background: Basidiobolomycosis, which is caused by pathogenic Basidiobolus species, is a rare fungal infection affecting the skin and gastrointestinal tract, which is mainly reported from tropical and subtropical regions. There is evidence that 'unconventional' antifungal drugs affect some fungal species. The current study intended to assess the in vitro effect of non-antifungal drugs, namely, artesunate, chloramphenicol, and co-trimoxazole in comparison to amphotericin B, fluconazole, itraconazole, metronidazole, and voriconazoleon in comparison to amphotericin B, fluconazole, itraconazole, metronidazole, and voriconazoleon on representative medically significant fungi.

Methods: *Basidiobolus* species (n =13) isolated from human gastrointestinal basidiobolomycosis and lizards were tested against artesunate, chloramphenicol, and co-trimoxazole. The antimicrobial assay was done using the agar diffusion method. The broth microdilution method was performed with a two-fold serial dilution of each antimicrobial agent to determine the minimum inhibitory concentration (MIC). The nonparametric Wilcoxon Signed Rank Test. analyzed the mean difference between inhibitory measures between the drugs.

Results: The three agents exhibited inhibitory actions against Basidiobolus species comparable to the known antifungals. The combined effects of artesunate + voriconazole and co-trimoxazole + voriconazole have significant synergic effects, p = 0.003 and p = 0.021, respectively.

Conclusions: This study showed that non-antifungal agents were active against Basidiobolus species. These are promising results that enhance the accelerated combined treatment of GIB in humans, particularly the combination of artesunate and voriconazole.

Introduction

Basidiobolomycosis, which is caused by pathogenic *Basidiobolus* species, notably *Basidiobolus ranarum*, is a rare fungal infection affecting the skin and gastrointestinal tract. The disease is mainly reported from tropical and subtropical regions. The genus *Basidiobolus* (family *Basidiobolaceae*) falls withing the order *Basidiobolales*, class *Basidiobolomycotes*, and the phylum *Entomphthoromycota* [1] is a zygomycete filamentous fungus isolated from plant debris, soil, amphibians, reptiles, and insectivorous bats, and lice [2, 3]. One of the severe forms of basidiobolomycosis is human gastrointestinal basidiobolomycosis (GIB). Most of the GIB cases were pediatrics and predominately reported from the south of Saudi Arabia [4–7].

Diagnosis of GIB based on clinical suspicion is challenging and requires histopathological and mycological confirmation. Similarly, treatment is difficult given the nature of the disease, which requires endoscopic examination, early surgical resection of the infected tissue, and prolonged treatment with antifungals. Antifungals such as itraconazole provide suitable treatment options [6]. Many of the azole antifungals, for instance, itraconazole, can produce a range of serious cardiac and fluid-associated undesirable episodes. Dose decrease or cessation usually results in symptomatic improvement or reversal [8].

There is a continuous need for new antifungals because of the limited number of accessible therapeutic drugs for treating fungal infections [9, 10]. Because of the widespread antibiotic resistance [11–14] and lack of novel antibiotic options, studies are needed to discover the possible activities of known antibiotics to act on unconventional pathogens [15]. Several studies have demonstrated a good clinical outcome of the antifungal effects of "non-antifungal drugs" against true pathogenic species [16–18], for example, Trimethoprim/ sulfamethoxazole [15, 16], artesunate [19], and chloramphenicol [18, 20]. Co-trimoxazole is one of these "non-antifungal drugs," which is widely used to treat a variety of bacterial infections. Trimethoprim/ sulfamethoxazole, also known as co-trimoxazole, with Septrin and Bactrim being the common trade names.

Data have revealed agents that were previously unknown to be anti-Candida agents, which allows for the design of novel therapies against invasive candidiasis [21]. Quinolones and other antibiotics can enhance the activity of azole and polyene agents; this synergistic action could have a clinically significant effect [22]. Several drugs such as potassium iodide, amphotericin B, ketoconazole, itraconazole, and fluconazole as well as co-trimoxazole have been used successfully in the treatment of infections caused by *B. ranarum* [23]. For its antifungal use, it is acceptable, but the application of co-trimoxazole requires *in vitro* assessment. The in vitro activities of amphotericin B, miconazole, ketoconazole, 5-fluorocytosine, and potassium iodide were studied on human and wild type isolates of Basidiobolus and Conidiobolus species. The study recommended potassium iodide as a favorable treatment as it has a direct influence on these fungi. The study proposed that ketoconazole may be of use in the treatment of

entomophthoromycosis [24]. Treatment of patients with amphotericin B failed even after eight weeks and the isolate when tested in vitro was found resistant to amphotericin B, itraconazole, fluconazole, and flucytosine but susceptible to ketoconazole and miconazole [25].

In view of the limited reports on the effect of "non-antifungal drugs", further *in vitro* verification of these studies is necessary. The present study aimed to evaluate the *in vitro* antifungal effect of co-trimoxazole, artesunate, and chloramphenicol against *Basidiobolus* species. These agents were chosen based on their wide spectrum of activity and from few reports in the literature. The chloramphenicol is commonly used as a supplement in fungal media to suppress bacterial growth and to selectively isolate fungi in lower concentrations (usually about 50–100 mg/L). The supplement has to be considered for selective isolation of fungi with precautions.

Materials & Methods

Isolation of Basidiobolus *species*

Basidiobolus species (n = 16), which have been isolated from human gastrointestinal basidiobolomycosis (GIB) and lizards, were evaluated in this study. Details of the source of the strains are shown in Table 1. Isolation and identification of *Basidiobolus* species has been descried in our previous publication [5]. Briefly, the initial isolation the subsequent subculturing were performed on Sabouraud dextrose agar (SDA; Difco Inc.) incubated at 30◦C for up to 3 weeks. Phenotypic identification of *Basidiobolus* spp. was established according to O'Donnell morphological identification criteria of the genus [26].

The molecular confirmation of isolated fungi was done sequence analysis of the large subunit ribosomal RNA (LSU). DNA amplification and sequencing service were completed by Macrogen Inc. (Seoul, Korea) using the primers LR0R and LR7 for amplification of the partial LSU region [27]. Produced DNA sequences were aligned with additional relative reference sequences obtainable from the GenBank database using BLAST. Data were then analyzed phylogenetically by the neighbor-joining method using MEGA 7 software [28].

Preparation of fungal strains

The strains were obtained from stored stock at 4ºC, then subcultured onto Sabouraud dextrose agar (SDA; Difco, Becton, Dickinson and Company, Sparks, Maryland) and incubated at 25ºC for 10 days. Prior to antimicrobial testing, the viability and purity of each isolate were evaluated by microscopic examination. All procedures were performed within a class II biological safety cabinet in a biosafety level 3 laboratory.

Preparation of antimicrobial agents

The following agents have been used in the study: Co-trimoxazole (800 mg sulfamethoxazole and 160 mg trimethoprim; F Hoffmann-La Roche Ltd, Basel, Switzerland; also known as Bacterim or Septrin and abbreviated as CMX); artesunate (10 mg/ mL, Yashica Pharmaceuticals Private Limited, Thane, Maharashtra, India); chloramphenicol (200 mg/ mL, Cloranfenicol, BioGer Laboratories in Caracas - Venezuela); amphotericin B (100 mg/mL, Sigma, Missouri, USA); fluconazole (2 mg/mL (Diflucan I.V. Roerig/Pfizer Inc., France); itraconazole (10 mg/mL, Sporanox I.V, Janssen Biotech N.V, Belgium); metronidazole (5 mg /mL, PSI Pharmaceutical Co., Jeddah, Saudi Arabia); voriconazole (10 mg/ mL, Vfend, Pfizer Inc.).

Preparation of fungal suspensions (inocula)

Sterile normal saline was added to each agar slant, and the cultures were gently scraped with cotton swabs. The suspension containing conidia and hyphae was diluted 1:10 with RPMI 1640 medium [16]. The suspension was transferred to a sterile tube and allowed to settle for 5 min. Then the transmittance of the upper homogeneous supernatant was read at 530 nm and adjusted to 95% transmittance (approximately 1 x 10^3 to 5 x 10^3 CFU/mL). The strains were tested against each antimicrobial alone to determine the minimum inhibitory concentrations (MICs). The procedures were repeated at least twice, and each fungal strain was tested in duplicate.

Agar well diffusion method

All strains were screened for their susceptibility to antifungal and non-antifungal drugs shown in Table 1 using the agar well diffusion method [29]. Each strain was tested against all antimicrobials single-handedly to determine its susceptibility. Firstly, the SDA plate was inoculated with a test strain using a cotton swab impregnated with the earlier prepared fungal suspension (approximately 1 x 10^3 to 5 x 10^3 CFU/mL). A 100 µL of each drug with the provided concentration (Table 1) was spotted into wells made in SDA plates. Plates were incubated at 30 ^oC for 3 to 5 days. The inhibition zone was next read, and a strain was recorded sensitive (S) or resistant (R) accordingly.

Broth microdilution method

The broth microdilution method (M38-A2) was as per the Clinical and Laboratory Standards Institute guidelines [30]. A serial two-fold dilution of each antimicrobial agent was performed to determine the minimum inhibitory concentration (MIC). Selected strains were used to determine the MICs. The procedure was repeated three times to allow statistical analysis.

MICs for AMB and azoles were defined as the lowest concentration of the drug at which there was no visible fungal growth [30]. MIC of co-trimoxazole was defined as the lowest drug concentration that caused 80% inhibition of visible fungal growth. MIC for ART and CHL was defined as for co-trimoxazole.

Statistical analysis

To determine the mean difference between inhibitory measures (in triplicate) amongst the subject drugs, the nonparametric Wilcoxon Signed Rank Test was used for the analysis of antimicrobial combinations. *P*-value of < 0.05 was considered significant.

Results

The inhibitory activity of artesunate, co-trimoxazole, chloramphenicol, and in comparison with standard antifungal antibiotics is shown in Table 1. The three tested drugs were found active (*in vitro*) against *Basidiobolus* species and were able to inhibit all Basidiobolus species strains' growth.

Table 1

Basidiobolus species isolated from human gastrointestinal basidiobolomycosis and gecko lizards used in the antimicrobial assay.

SN	Laboratory and (DSM)code	Identity	Source	Sensitive (S) or Resistant (R) and inhibition zone (mm)							
				CTX	ART	CPL	AMB	FLC	ITZ	MTZ	VCZ
1.	Doza	*Basidiobolus haptosporus-like**	Human gastrointestinal basidiobolomycosis (GIB), Aseer region, Saudi Arabia (2013)	S (50)	S (75)	S (28)	R (0)	S (36)	S (65)	R (0)	S (45)
2.	9 – 4	*B. haptosporus-like*	Human GIB, Aseer region, Saudi Arabia (2014)	S (48)	S (72)	S (30)	R (0)	S (32)	S (68)	R (0)	S (40)
3.	F43-5	*B. haptosporus-like*	Human GIB, Aseer region, Saudi Arabia (2016)	S (48)	S (70)	S (30)	R (0)	S (33)	S (60)	R (0)	S (43)
4.	V81 (DSM06014)	*B. haptosporus-like*	Human GIB, Aseer region, Saudi Arabia (2017)	S (49)	S (73)	S (30)	R (0)	S (35)	S (62)	R (0)	S (44)
5.	F15-1	*B. haptosporus-like*	Human GIB, Aseer region, Saudi Arabia (2017)	S (50)	S (72)	S (30)	R (0)	S (34)	S (64)	R (0)	S (43)
6.	F17-5 (DSM06015)	*Basidiobolus* sp.	Human GIB, Aseer region, Saudi Arabia (2017)	S (47)	S (71)	S (30)	R (0)	S (33)	S (65)	R (0)	S (42)
7.	L1 (DSM107663)	*B. haptosporus-like*	Gecko lizard, Muhayil, Aseer region, Saudi Arabia (2017)	S (49)	S (74)	S (30)	R (0)	S (33)	S (62)	R (0)	S (44)
8.	L2 (DSM107662)	*B. haptosporus-like*	Gecko lizard, Muhayil, Aseer region, Saudi Arabia (2017)	S (50)	S (73)	S (29)	R (0)	S (37)	S (64)	R (0)	S (45)
9.	L3 (DSM 05995)	*Basidiobolus* sp.	Gecko lizard, Muhayil, Aseer region, Saudi Arabia (2017)	S (48)	S (74)	S (28)	R (0)	S (36)	S (65)	R (0)	S (42)
10.	L4	*Basidiobolus* sp.	Gecko lizard, Muhayil, Aseer region, Saudi Arabia (2017)	S (48)	S (73)	S (30)	R (0)	S (37)	S (63)	R (0)	S (44)
11.	L6	*Basidiobolus* sp.	Gecko lizard, Muhayil, Aseer region, Saudi Arabia (2017)	S (47)	S (72)	S (30)	R (0)	S (37)	S (62)	R (0)	S (43)
12.	891 – 10	*Basidiobolus* sp.	Human GIB, Aseer region, Saudi Arabia (2018)	S (49)	S (73)	S (28)	R (0)	S (37)	S (63)	R (0)	S (44)
13.	ACH230	*Basidiobolus* sp.	Human GIB, Aseer region, Saudi Arabia (2020)	S (50)	S (74)	S (30)	R (0)	S (35)	S (65)	R (0)	S (43)

Abbreviations: CTX, Co-trimoxazole (800 mg sulfamethoxazole and 160 mg trimethoprim); ART, Artesunate (10 mg/ mL); CPL, Chloramphenicol (100 mg/ mL); AMB, Amphotericin B (50 mg/ mL); FLC, Fluconazole (2 mg/ mL); ITZ, Itraconazole (10 mg/ mL); MTZ, Metronidazole (5 mg/ mL); VCZ, Voriconazole (10 mg/ mL). S, sensitive; R, resistant. DSM, Deutsche Sammlung von. Mikroorganismen und Zellkulturen GmbH, Inhoffenstraße 7B, 38124 Braunschweig, Germany; **Basidiobolus haptosporus*-like was identified in our previous study (6) and other strains were identified based on phenotypic properties and currently underway for full descriptions and designations.

Determination of the minimum inhibitory concentrations

The minimum inhibitory concentrations (MIC) of artesunate, chloramphenicol, co-trimoxazole, and in comparison with voriconazole against *Basidiobolus* species are shown in Table 2. MIC value for artesunate was found to be 20 µg/ mL; for chloramphenicol, 3,130 20 µg/ mL; co-trimoxazole, 160 µg/ mL, and for voriconazole was 80 µg/ mL.

Table 2. Minimum inhibitory concentrations (MIC) of artesunate, chloramphenicol, co-trimoxazole, and in comparison with voriconazole against *Basidiobolus* species Strain V81*.

	Artesunate									
Concentration (mg/mL)	5	2.5	1.25	0.63	0.31	0.16	0.08	0.04	0.02	0.01
Reaction	NG	NG	NG	NG	NG	NG	NG	NG	G	G
	Chloramphenicol									
Concentration (mg/mL)	50	25	12.5	6.25	3.13	1.56	0.78	0.4	0.2	0.1
Reaction	NG	NG	NG	NG	G	G	G	G	G	G
	Co-trimoxazole 5/1									
Concentration (mg/mL)	20/8	10/4	5/ 2	2.5/1	1.25/0.5	0.63/0.25	0.31/0.13	0.16/0.7	0.08/0.35	0.04/0.18
Reaction	NG	NG	NG	NG	NG	NG	NG	G	G	G
	Voriconazole									
Concentration (mg/mL)	5	2.5	1.25	0.63	0.31	0.16	0.08	0.04	0.02	0.01
Reaction	NG	NG	NG	NG	NG	NG	G	G	G	G

*Strain V81, *Basidiobolus haptosporus*-like (DSM06014); NG, no growth; G, growth.

The combined effects of voriconazole with artesunate, co-trimoxazole, chloramphenicol on Basidiobolus *species*

The combined effects of voriconazole with artesunate, co-trimoxazole, and chloramphenicol on *Basidiobolus* species are shown in Figs. 1 and 2.

The triplicate reading of the effect of voriconazole alone was 40-50 mm ±2.89. The effect of artesunate was 50-60 mm ± 2.89 compared to artesunate + voriconazole, 87-90 mm ± 1.45 ($p = 0.003$). The effect of co-trimoxazole was 40-50 mm ± 2.89 compared to co-trimoxazole + voriconazole, 60-64 mm ± 1.15 ($p = 0.021$). The effect of chloramphenicol was 18-22 mm ± 1.15 compared to chloramphenicol + voriconazole, 53-57 mm ± 1.15 ($p = 0.109$).

Both artesunate and co-trimoxazole were as effective as voriconazole showing no significant difference between them, $p = 0.074$ and 1.0, respectively. Whereas, chloramphenicol was less active against *Basidiobolus* sp. ($p= 0.009$) (Fig. 2).

Discussion

The results of our current study revealed the potential *in vitro* effect of artesunate, chloramphenicol, and co-trimoxazole as antifungal, in particular against *Basidiobolus* spp., the etiological agent of basidiobolomycosis. This is not completely new since few studies have suggested this idea and but most trials have been carried out on a clinical-based treatment course of therapy [17, 31]. Nonetheless, *in vitro* evaluation of antifungal drugs and sulfamethoxazole-trimethoprim against clinical isolates of *Conidiobolus lamprauges* has been done [32]. *Conidiobolus lamprauges* is a member of the order *Entomophthorales,* a species phylogentically related to *Basidiobolus ranarum*, *Basidiobolus haptosporus* or *Conidiobolus coronatus*, the later species are agents implicated in skin and abdominal basidiobolomycosis [23, 33].

Several conventional antifungal drugs, for example, potassium iodide, co-trimoxazole, amphotericin B, ketoconazole, and itraconazole, have been tried for the treatment of entomophthoromycosis due to *Conidiobolus coronatus* or basidiobolomycosis due to *Basidiobolus ranarum* or *Basidiobolus haptosporus*-like fungi with variable results [34]. Comparably, information derived from a number of investigations indicated that non-antifungal compounds supplement the action of conventional antifungal agents either

through the elimination of natural resistance or through exhibiting in some way activity antagonizing certain fungal species [21, 22, 31].

These results confirm the hypothesis that co-trimoxazole has an antifungal inhibitory, as reported previously [16]. This substantiating that folic acid blockade may be a potential antifungal target for *Basidiobolus* species. This report of the antifungal potential of co-trimoxazole drug against this fungal pathogen support earlier one e [15, 16]. Potassium iodide and co-trimoxazole were found to be simple and effective for basidiobolomycosis treatment [35]. While there is no consensus, African physicians prefer to use potassium iodide or trimethoprim-sulfamethoxazole in the treatment of tropical mycosis infections caused by either *Basidiobolus ranarum, Basidiobolus haptosporus* or *Conidiobolus coronatus* or others [36]. Experience lead some physicians to adopt septrin (trimethoprim-sulfamethoxazole) as the drug of choice in the management of entomophthorosis due to *Conidiobolus coronatus* [37]. Another study suggested using the combination of itraconazole and fluconazole as an additional option for the treatment of this mycosis, acting better than sulfamethoxazole plus trimethoprim for 2 months [38]. A case of rhinophycomycosis entomophthora was successfully treated with a combination of bacteria, potassium iodide, and steroids were reported [39]. Clinical isolates of *Conidiobolus lamprauges* (entomophthoromycosis cases) showed synergistic interactions of 100% for the sulfamethoxazole-trimethoprim combination, 71% for the terbinafine-azole antifungal combination, and 29% for the terbinafine-micafungin combination. All other interactions were indifferent [32].

The mode of action of these three drugs is known against their conventional target organisms. It is, however, that the effect on fungi is still unknown. The inhibition of folic acid synthesis by *Coccidioides posadasii* has been speculated as a likely antifungal target [16]. *Saccharomyces cerevisiae* in response to treatment with arsenic has revealed a complex response that influences signaling pathways, including protein kinases such as the mitogen-active protein kinase and target of the rapamycin complex 1 system [40]. The modes of action of artesunate continue to be unclear and debatable [41]. On the other hand, chloramphenicol is a bacteriostatic that acts via inhibiting protein synthesis. It prevents protein chain elongation by inhibiting the peptidyl transferase activity of the bacterial ribosome [42]. However, it not known how it might affect the eukaryotic fungi.

Our data support the fact that artesunate is a potential antifungal as well as its primary antimalarial activity [19]. Data have suggested the enhancement of artesunate with miconazole in antagonizing *Candida's* biofilm. These refer to the potential mixture treatment of *Candida albicans* biofilm-related infections. The results of our current study support the use of "non-antifungal antibacterial drugs as effective against *Basidiobolus* strains, which support their earlier antifungal effects [18, 20]. The combined effect of artesunate + voriconazole and that of co-trimoxazole + voriconazole have significant synergic effects, $p = 0.003$ and $p = 0.021$, respectively. These are hopeful results for the treatment of GIB in humans, which need in vivo application and determining the clinical implications.

In conclusion, the results of the present study demonstrated a clear inhibitory antagonism of artesunate, co-trimoxazole, and a less significant effect in the case of chloramphenicol, against strains of *Basidiobolus*. These are encouraging results for the *in vivo* application on human or animal models. The *in vivo* application is necessary to be combined with standard antifungal drugs rather than a single therapy since our results have indicated a synergistic effect between co-trimoxazole and voriconazole, with a lesser effect between chloramphenicol and voriconazole, but markedly between artesunate and voriconazole. The study recommends the application of artesunate + voriconazole or co-trimoxazole + voriconazole in a clinical trial.

Declarations

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Data availability statement:

The datasets used and/or analysed during the current study are available from the corresponding author and study investigators on reasonable request.

Consent for publication

Not applicable.

Conflict of interest

The authors declare that they have no competing interests.

Author Contribution

S.M.A.-Q and M.R.P.J. conceived and designed the study, and data analysis; A.M.A, A.A.A, A.A.S, A.A.S and A.M. supervised and reviewed the data analysis; A.A.-B , S.A. and (M.E.H. mentored in the elaboration of the manuscript. All authors contributed to the writing of the final manuscript. All authors read and approved the final manuscript.

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Figures

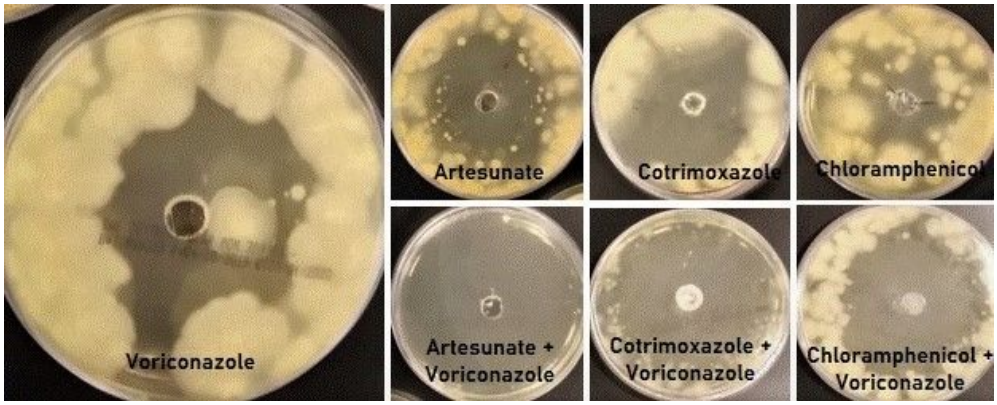

Figure 1

Zone of inhibition of the “non-antifungal" drugs on *Basidiobolus* sp. and in comparison with voriconazole.

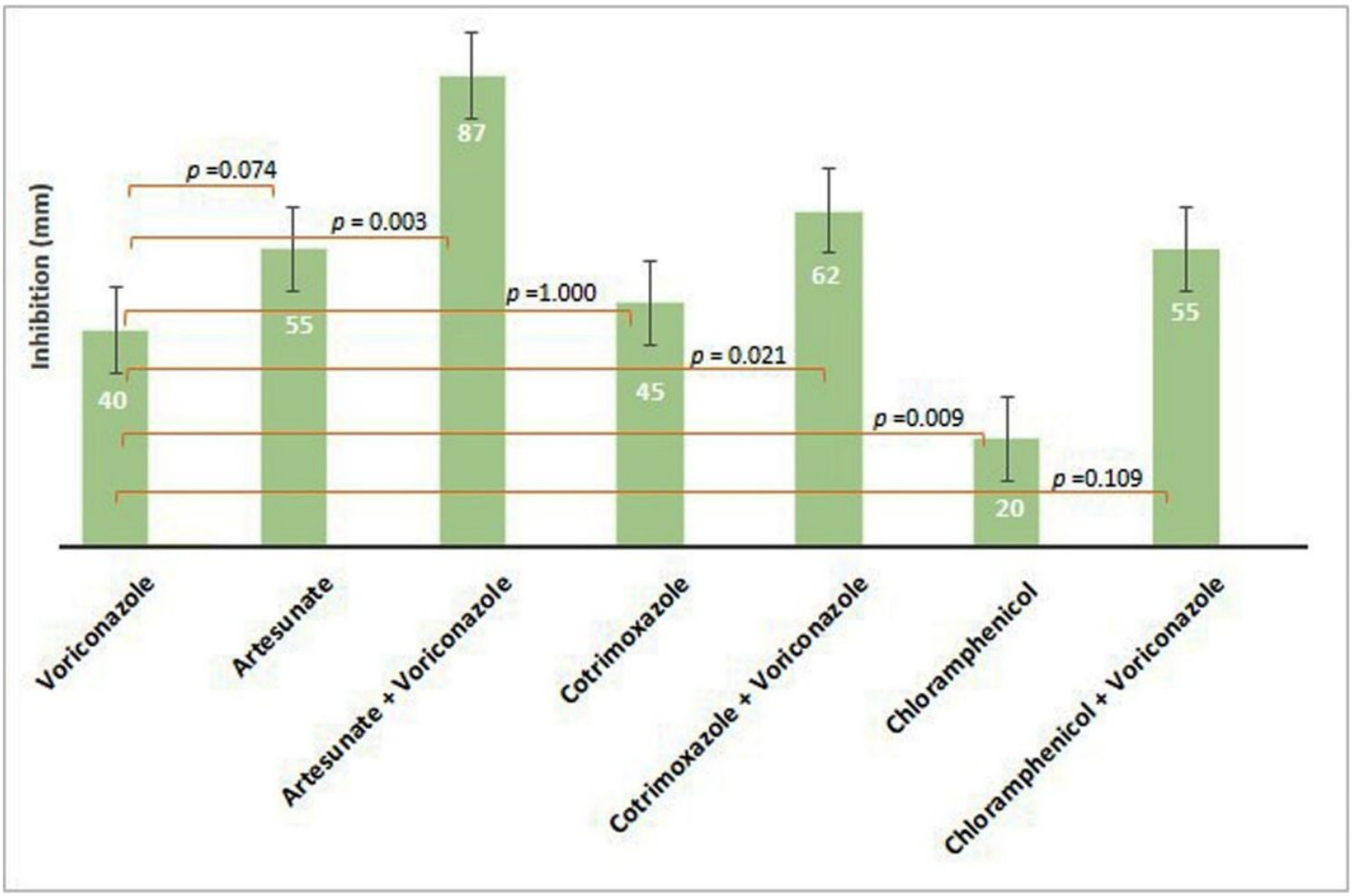

Figure 2

Comparison of the *in vitro* effect of voriconazole with “non-antifungal" drugs on *Basidiobolus* sp. (p <0.5 is considered significant).