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Original Research Article

Impact of Human Serum on Pharmacodynamic Action of Echinocandins against *Aspergillus* spp. : an *in Vitro* Study

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Abstract

Echinocandins represent a class of lipopeptide antifungal agents that possess a high protein binding capacity which could have an impact on their activity. This study was focusing on the role of human serum on the activities of echinocannins (caspofungin, micafungin, and anidulafungin) against *Aspergillus* species (*A.fumigatus*, *A.flavus* and *A.terreus*). The aspergillus growths have been assessed by measuring minimal effective concentrations of each (caspofungin (0.5-1 mg/L), micafungin (0.06-0.12 mg/L) and anidulafungin (0.03-0.09 mg/L). Broth microdilution method was used to study *in vitro* activities based on the Clinical and Laboratory Standards Institute documentation (CLSI M38-A2) by using standard medium (Roswell Park Memorial Institute (RPMI 1640)) with or without 50% human serum. Each drug was diluted ranging from 0.03-4 mg/L, inoculated with $2x10^4$ Colony Forming Units/ml for each isolate, then incubated at 37°C for 10 hrs to *A.fumigatus* and *A.flavus* and 15 hrs to *A.terreus*. The fungal growth was assessed by measuring the galactomannan index for each concentration with ELISA (Platelia Aspergillus); percentage of fungal growth inhibition was measured from galactomannan index and analyzed as maximal growth inhibition (E_{max}) and drug concentration with 50% of growth inhibition (IC_{50}). IC_{50} values of echinocandins were ranged from 0.03-0.06 mg/L without serum and 0.09-0.25 mg/L in presence of serum while E_{max} values were 50(44-56)% for *A.fumigatus*, 70(65-74)% for *A.flavus* and 65(63-66)% for *A.terreus* without serum and 94(92-96)% for *A.fumigatus*, 92(91-95)% for *A.flavus* and 80(75-83)% for *A.terreus* with human serum. The study concluded the effective concentrations of all echinocandins were increased concomitantly with more than 90% fungal growth inhibition with human serum.

Key words: growth inhibition, *Aspergillus*, galactomannan, caspofungin, micafungin, anidulafungin.

الخلاصة

الاتشينوكانديات تمثل احدى أصناف المضادات الفطرية التي تعمل على طريق تثبيط تكوين الجدار الخلوي للفطريات وهذه المضادات تمتلك القدرة العالية على الارتباط بالبروتينات المصلية والتي لها تأثير على نشاطها. تركز هذه الدراسة على دور المصل البشري على تنشيط الاتشينوكانديات ضد أنواع الرشاشيات *A.flavus A.fumigatus* و *A.terreus* وقد تم تقييم النمو لأنواع الرشاشيات عن طريق قياس الحد الأدنى للتركيزات الفعالة (MEC) (للكاسبوفنجين0.5–1) ملغ / لتر وللميكانوفنجين 0.06–0.12 لغ / لتر وللانديلوفنجين 0.03–0.09 ملغ/ لتر وقد تم استخدام طريقة التخفيف لدراسة الأنشطة الزرعية المختبرية للفطريات على أساس CLSI M38–A2 وباستخدام الوسط الزرعي القياسي (RPMI 1640) مع أو بدون ٥٠٪. من المصل البشري وقد اختبر كل دواء بتخفيف تراوح بين ٤–0.03،ملغم / لتر، مع التلقيح بالرشاشات بنسبة $2X10^4$ CFU/ml، ثم حضنت في ٣٧°C لـ١٠ساعات بالنسبة لل *A.fumigatus* و١٥ساعة بالنسبة *A.flavus* و *A.terreus*وقد تم تقييم نمو الفطريات عن طريق قياس مؤشر الجلاكتومانون لكل تركيز بطريقة الالايزة ELISA؛ وقيست نسبة تثبيط نمو الفطريات من مؤشر الجلاكتومانون وتحليلها ع تثبين طريق التثبيط الاقصى للنمو وتركيز الدواء مع ٥٠٪ من تثبيط النمو. ان قيم تركيز الدواء مع ٥٠٪ من تثبيط النمو تراوحت من 0.03–0.06ملغ /لتر من دون المصل وبين 0.09–0.25 ملغ /لتر في وجود المصل بينما كانت قيم التثبيط الاقصى للنمو تتراوح بين (44–56) 50% لل*A.fumigatus* ، (65–74) 70% لل*A.flavus* و ٦٥% (٦٣–٦٦) لل *A.terreus* من دون المصل و ٩٤% (٩٢–٩٦) لل*A.fumigatus* ، (91–95) 92% لل*A.flavus* و ٨٠% (٧٥–٨٣) لل

A.terreus مع المصل البشري. استنتج من الدراسة أن زيادة التركيزات الفعالة لجميع الاتشينوكانديات كان متزامنا مع تثبيط نمو الفطريات لأكثر من ٩٠٪ من نموها مع المصل البشري.

Introduction

Echinocandins are regarded one of a new class of antifungal agents that is often used in the combination with other antifungal drugs for the treatment of invasive aspergillosis which is considered as a life threatening disease in prolonging and profound immunocompromised patients [1]. High protein binding capacity of these agents is changed their pharmacological activity. Several studies with antibiotics have demonstrated that the role of free or unbound drug is always available for antibiotic activity [2-5]. Moreover, numerous studies reported that pharmacodynamic effects of echinocandins have been increased in the presence of human serum not correlated with minimal inhibitory concentration (MIC) increases in concomitant with high protein binding capacity of echinocandins (ranging between 96-98%) [6-8].

The endpoints of susceptibility testing for the echinocandins are difficult to determine while the end point for other antifungal classes are easier by using of minimal inhibitory concentration methods [9].

However, a clear *in vitro-in vivo* clinical correlation is still lacking [10]. The fluctuation of drug concentrations on *in vivo* environment does not correlate well with *in vitro* study, where *in vivo* the drugs have interacted with the fungus in addition to the presence of human serum and tissues. Thus, in the presence of serum, the antifungal effects may be changed by using the same agent that were not seen *in vitro* without serum [11,12].

In addition, a clinical study found that the infections with *A. fumigatus* isolates with minimal effective concentrations (MECs) (0.125-8 µg/ml) in patients with haemopoitic stem cell transplantation receiving caspofungin prophylaxis leading to empirical treatment as breakthrough reported [13] while on in vivo study of an animal model with *A. fumigatus* infection suggested decreasing in susceptibility to caspofungin with high doses possibly by upregulation of the level of target enzyme [14].

This study investigated the *in vitro* pharmacodynamic changes of the antifungal activities of echinocandins (caspofungin, micafungin and anidulafungin) in presence of human serum against three clinical isolates *Aspergillus* species (*A. fumigatus*, *A. flavus* and *A. terreus*) were investigated.

Materials and Methods

Antifungal Drugs:

Caspofungin (Merck-USA), Micafungin (Astellas-USA) and Anidulafungin (Pfizer-USA) were prepared as stock solutions and dissolved in either dimethyl sulfoxide for anidulafungin or water for caspofungin and micafungin, and stored at -70^{o}C until use. The Clinical and Laboratory Standards Institute (CLSI M38-A2) method, [15] with serial dilutions and the final concentration were prepared in double-strength test medium (Roswell Park Memorial Institute (RPMI 1640) medium buffered with morpholine propane sulfonic acid (MOPS) [0.165M], pH=7.0).

Isolates and Culture Media:

The clinical isolates of each *A.fumigatus*, *A.flavus* and *A. terreus* were used. Inocula from 5-7 days-old cultures in Sabouraud dextrose agar were prepared in saline or human serum and adjusted twice with Neubauer counting chamber to get the final inoculum (2x10^{4} Colony Forming Units (CFU)/ml).

The pharmacodynamic activities of the echinocandins were tested with a broth microdilution method[15] by using standard medium (RPMI 1640, 0.165 M [MOPS], pH=7.0) either with 50% (v/v) pooled human serum or without.

Human Serum Preparation:

Whole human blood samples were centrifuged at 4,000 X g for 10 min, and the supernatant serum was taken and then incubated in a water bath at 56°C for 30 min with constant swirling. The serum sample was stored at -70°C that should be used within 7 to 10 days [16].

Susceptibility Testing:

Tested plates were prepared in advance by dispensing 100 µl of twofold serially diluted drugs ranging from 4 to 0.03 mg/L into 96-well flat-bottom microdilution plates and stored at -70°C. On day of testing, plates were thawed and inoculated with $2x10^4$ CFU/ml in saline or human serum. The plates were incubated at 37°C for 10 hrs for *A.fumigatus* and *A.flavus* and 15 hrs for *A. terreus* [17]. The fungal growth was assessed by measuring the galactomannan index (GI) for each drug concentration with ELISA (Platelia Aspergillus; Bio-Rad-USA) [18] by taking 5 µl from each well of microplate and diluted in 5 ml of normal saline (1:1000) then measured GI by ELISA. GI was inversely proportional with percentage (%) of fungal growth inhibition.

Statistical Analysis

The % of fungal growth inhibition was analyzed by using nonlinear regression analysis with GraphPad Prism 5.0 Software. The sigmoidal slope of E_{max} model was described by drug concentration corresponding to 50% of growth inhibition (IC_{50}).

The E_{max} and IC_{50} values were tested for each drug concentration and isolate without human serum and compared the results with those with human serum by plotting the $\log_{10}$ for each concentration of each echinocandins against the % of growth inhibition for each species of *Aspergillus* [Figure 1], [Figure 2], [Figure 3].

Results

IC_{50s} values of the *in vitro* activities of three Echinocandins :

The IC_{50} values of all three echinocandins were 0.05 (0.03-0.06) mg/L without serum and 0.21 (0.09-0.5) mg/L with human serum showing a significant mean increase [Table 1]. The addition of human serum increased the IC_{50} of echinocandins by 0.16 (0.09-0.19) mg/L for *A.fumigatus* [Figure 1], 0.24 (0.06-0.44) mg/L for *A.flavus* [Figure 2] and 0.1 (0.03-0.21) mg/L for *A. terreus* [Figure 3]. The largest increase in IC_{50s} values was found with *A.flavus* and anidulafungin (8.0-fold) and the smaller with *A. terreus* and caspofungin (one-fold) [Table1].

E_{max} Values of The *In Vitro* Activities of Three Echinocandins:

The E_{max} values of all three echinocandins were 50 (44-56)% for *A.fumigatus* [Figure 1], 70 (65-74)% for *A.flavus* [Figure 2] and 65(63-66)% for *A. terreus* [Figure 3] without serum and 94 (92-96) % for *A.fumigatus* [Figure 1], 92(91-95)% for *A.flavus* [Figure 2] and 80 (75-83)% for *A. terreus* [Figure 3] with serum [Table 2]. The addition of human serum increased the E_{max} of echinocandins by 44 (36-50)% for *A.fumigatus* [Figure 1], 22 (18-30)% for *A.flavus* [Figure 2] and 15 (9-21)% for *A. terreus* [Figure 3], resulting complete growth inhibition at concentrations 1-4 mg/L [Table 2]. The largest increase in E_{max} value was found with *A.fumigatus* and micafungin (1.6-fold) [Figure 1-B] while the smaller with *A. terreus* and anidulafungin (0.12-fold) [Figure 3-C].

Discussion

Echinocandins are used as salvage therapy against invasive aspergillosis, but some recent studies suggested their effectiveness as a first line therapy in the treatment of the invasive Aspergillosis [19]. This increase in effectiveness may be due to synergy between antimicrobial factors present in serum and echinocandins. Serum has a direct effect on antimicrobial activity by altering their ability to interact with the target tissues; in addition, the inhibition of glucan synthase by echinocandins could be predicted by the percentage of their protein binding [8].

Indeed, the addition of the serum enhanced the echinocandins' action to a 45%, 22% and 18% of their *in vitro* inhibitory activities against *A.fumigatus*, *A.flavus* and *A. terreus* respectively [Table 2] with concs. higher than the MEC and complete growth inhibition with no paradoxical effects; these results are supported by the results obtained by Odabasi *et al* [7] who tested echinocandins activities against only *A.fumigatus* with 50% enhancement in presence of serum. Other researchs showed that caspofungin has *in vivo* activity similar to that of anidulafungin, [20] while *in vitro* IC_{50} without serum indicated potency three times less than with serum [Table 1]. Moreover, animal studies showed that 5 mg/kg of caspofungin or micafungin resulted in similar *in vivo* outcomes (spleen and kidney fungal burden and survival) when caspofungin concentrations were two-times higher than those of micafungin *in vitro* [21].

Moreover, micafungin showed the greatest increase of E_{max} activity against only 4-fold increase in IC_{50} (0.03 versus 0.12 mg/L) in the presence of 50% human serum especially against *A.fumigatus* [Figure 1-B] [22] while anidulafungin exhibited the smallest increase in E_{max} activity against more than 8-fold differences (0.06 versus 0.25 mg/L) in the presence of 50% human serum, especially against *A.flavus* and *A. terreus* [Figure 2-C; Figure 3-C] [23].

Furthermore, the current study exhibited that the action of echinocandins was low in the presence of 50% human serum at lower near-MEC concentrations (<0.5 mg/L) in comparison with absence of human serum. This decrease of activity could be explained by the free-drug hypothesis, considering that echinocandins are highly protein-bound drugs.

Conclusion

The maximum activity of echinocandins was enhanced in presence of 50% human serum leading to the complete inhibition of fungal growth at concentrations ranging from 1 to 4 mg/L oppositely to less than 60% fungal growth inhibition without human serum with same concentration.

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Table 1: The IC_{50}s in presence and absence of human serum calculated for all echinocandins against *Aspergillus* spp.

Species (Isolates)	Echinocandin	Mean IC_{50} (mg/l) without serum	Mean IC_{50} (mg/l) with serum	Increase of IC_{50}
Aspergillus fumigatus	Caspofungin	0.06	0.25	0.19
	Micafungin	0.03	0.12	0.09
	Anidulafungin	0.06	0.25	0.19
Aspergillus flavus	Caspofungin	0.03	0.25	0.21
	Micafungin	0.06	0.12	0.06
	Anidulafungin	0.06	0.5	0.44
Aspergillus terreus	Caspofungin	0.06	0.09	0.03
	Micafungin	0.06	0.12	0.06
	Anidulafungin	0.03	0.25	0.21

Table 2: The E_{max} values in presence and absence of human serum calculated for all echinocandins against *Aspergillus* spp.

Species (Isolates)	Echinocandin	Mean E_{max} (%) without serum	Mean E_{max} (%) with serum	Increase of E_{max} (%)
Aspergillus fumigatus	Caspofungin	56	92	36
	Micafungin	44	94	50
	Anidulafungin	49	96	47
				44
Aspergillus flavus	Caspofungin	74	92	18
	Micafungin	65	95	30
	Anidulafungin	73	91	18
				22
Aspergillus terreus	Caspofungin	66	80	14
	Micafungin	63	84	21
	Anidulafungin	66	75	9
				18

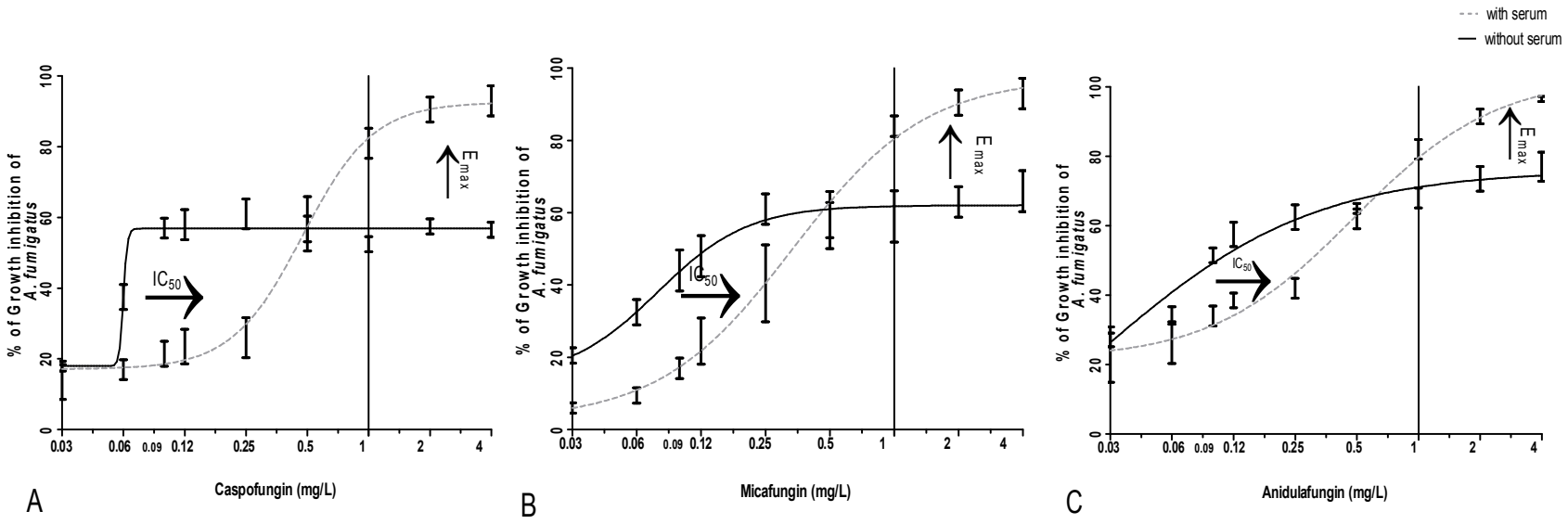

Figure 1: The activity of all three echinocandins (Caspofungin (A), Micafungin (B) & Anidulafungin (C) against *Aspergillus fumigatus* by measuring the IC_{50} and E_{max} for each drug against *Aspergillus fumigatus* in the presence and absence of 50% human serum.

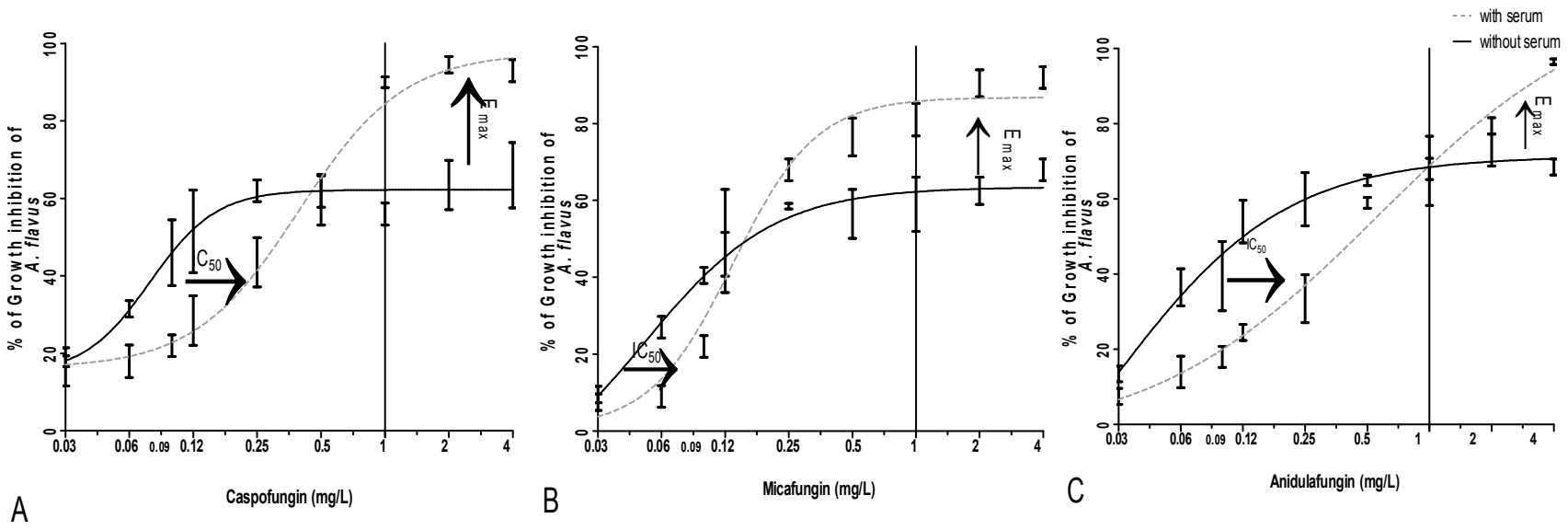

Figure 2: The activity of all three echinocandins (Caspofungin (A), Micafungin (B) & Anidulafungin(C) against the three species of *Aspergillus flavus* by measuring the IC_{50} and E_{max} for each drug against *Aspergillus flavus* in the presence and absence of 50% human serum.

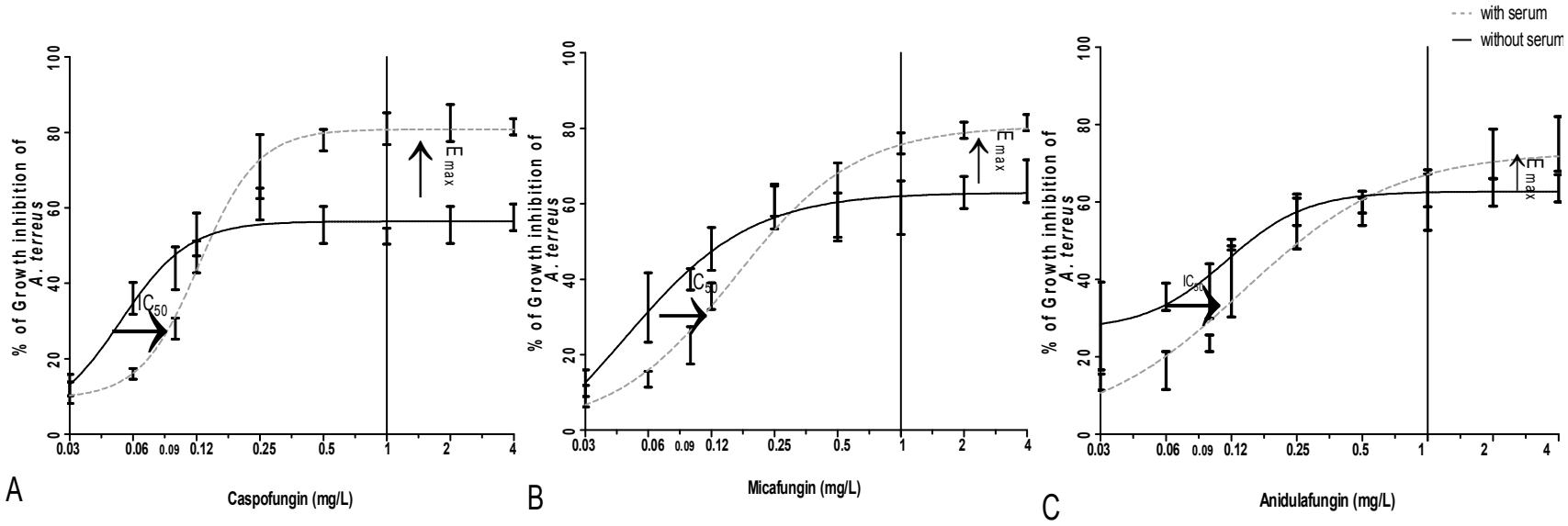

Figure 3: The activity of all three echinocandins (Caspofungin (A), Micafungin (B) & Anidulafungin (C) against the three species of *Aspergillus terreus* by measuring the IC_{50} and E_{max} for each drug against *Aspergillus terreus* in the presence and absence of 50% human serum.