In vitro contribution of herbal products on the activity of antifungal drugs against clinical *Candida* isolates

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ABSTRACT

Aims: The contribution of natural compounds may provide a significant progress in the discovery of new antifungal drugs. We aimed to investigate the contribution of carvacrol, farnesol, epigallocatechin gallate, ginger, naringenin and curcumin on the activity of antifungals, voriconazole, caspofungin and amphotericin B against clinical *Candida* isolates.
Methodology: Eight clinical *Candida* isolates were included in this study. The MIC values of each herbal compound and each antifungal were determined using reference broth microdilution method. The interactions of herbal compounds and antifungal drugs were assessed by checkerboard microdilution method.
Results: The MIC values ranged from 60 to 160 µg/mL for carvacrol, from 16 to 64 µg/mL for epigallocatechin gallate, from 80 to 320 µg/mL for ginger and 800 µg/mL for curcumin. Any MIC value was not detected for farnesol and naringenin in concentrations tested. Although the most common interaction type was indifference, farnesol, carvacrol and ginger reduced the MICs of all three antifungals against most isolates. Voriconazole and amphotericin B MICs decreased in combinations with epigallocatechin gallate, whereas naringenin and curcumin did not show any conspicuous effect on antifungal drug activities.
Conclusion: We showed that carvacrol, epigallocatechin gallate, curcumin and ginger enhanced the activity of voriconazole, caspofungin and amphotericin B against *Candida* isolates in vitro. These compounds may represent novel agents to be used in combination with available antifungal drugs to lower the doses of antifungals, thus toxic side effects and treatment costs may decrease.

Keywords: Antifungal, Candida, carvacrol, farnesol, epigallocatechin gallate, ginger

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1. INTRODUCTION

Invasive fungal infections (IFIs) are increasingly threats for critically ill patients and significant causes of morbidity and mortality for them. *Candida* spp are one the most common agents of IFIs and still the most common fungi isolated from blood stream infections. Although new antifungal agents are being developed, there is an increasing resistance to standard antifungal therapy, and no new classes of antifungal agents have been approved since 2006 [1]. Currently, three antifungal drug classes including triazoles, polyenes and echinocandins are available to use in treatment of IFIs. However, treatment is often complicated due to their high toxicity, low tolerability, drug interactions and limited spectrums of activities. Moreover, some fungi are intrinsic resistant to these antifungal agents. Therefore, the requirement of new drug or treatment alternatives especially those with a wider spectrum, lower toxicity and cheaper are increasing day by day.

In recent years, the interest to studies related with therapeutic use of natural products is increasing. Essential oils (EOs) are aromatic oily liquids obtained from plant material [2]. There are many studies investigating the antibacterial, antiviral, antifungal and antiparasitic activity of various herbal oils or their components [3-5]. However, the acts of such compounds on the antifungal activity of available drugs in treatment of *Candida* infections have not been researched so much. Carvacrol, farnesol, epigallocatechin gallate, ginger, naringenin and curcumin are herbal products that have been shown to have some antifungal effects; carvacrol alone was almost more effective than fluconazole against oral *Candida* isolates [6], and the combination of carvacrol with voriconazole exhibited synergistic or additive activity against drug-resistant *Candida* spp [7]; the combinations of farnesol with fluconazole and micafungin showed synergistic interactions against *C. albicans* biofilm [8]; epigallocatechin gallate enhanced the activity of miconazole, fluconazole or amphotericin B against *Candida* isolates [9]; antifungal activity of ginger was stronger than those of fluconazole and nystatin against *C. albicans* [10]; synergistic interactions were detected in the combinations of curcumin with azoles and polyenes against clinical *Candida* isolates [11]. In this study, we investigated the contribution of natural compounds carvacrol, farnesol, epigallocatechin gallate, ginger, naringenin and curcumin on the activity of antifungals such as voriconazole, caspofungin and amphotericin B against clinical *Candida* isolates.

2. MATERIAL AND METHODS

2.1 Isolates and Media.

Clinical *Candida* isolates, *C. albicans* (n=3), *C. parapsilosis* (n=3) and *C. glabrata* (n=2), were used in this study. *C. krusei* ATCC 6258 and *C. parapsilosis* ATCC 22019 were used as quality-control isolates for antifungal susceptibility testing. All isolates were subcultured onto Sabouraud dextrose agar (SDA, Merck, Darmstadt, Germany) at 37°C for 24-48 h prior to testing. RPMI-1640 medium (Merck, Darmstadt, Germany) buffered to pH 7.0 with MOPS (3-N-morpholinopropanesulfonic acid) was used for broth microdilution testing and checkerboard method.

2.2 Antifungals and herbal compounds

Stock solutions of voriconazole (VOR) and amphotericin B (AmB) (Sigma Chemical Co., St Louis, MO, USA) in dimethyl sulfoxide (DMSO), caspofungin (CAS, Sigma Chemical Co, St Louis, MO, USA) in distilled water were prepared at the concentrations of 6400 µg/mL and 1600 µg/mL, respectively. Antifungal stock solutions were dispensed into 1 mL tubes and stored at -70 °C until they were used. Carvacrol (CRV), naringenin (NAR), epigallocatechin

gallate (EPG), curcumin (CUR), ginger (GGR) and farnesol (FAR) were commercially obtained (Sigma Chemical Co.) and solved in DMSO, to be at concentrations at least 100 times higher than the desired highest test concentration, prior to each experiment [12].

2.3 Determination of minimum inhibitory concentration (MIC)

The MIC values for each of antifungal drugs and herbal compounds were determined against all *Candida* isolates using broth microdilution method according to Clinical and Laboratory Standards Institute (CLSI) M27-A3 guideline [12]. For MIC testing, U bottom 96 well microplates were used and serial twofold dilutions ranging 0.0313 to 16 µg/mL for VOR and AmB, 0.015 to 8 µg/mL for CAS, 10 to 1280 µg/mL for CRV, 3.12 to 1600 µg/mL for NAR, 0.06 to 64 µg/mL for EPG, 1.56 to 1600 µg/mL for CUR, 12.5 to 6400 µg/mL for GGR and 6 to 6000 µM for FAR were prepared in RPMI 1640 medium and stored at -70°C until use. A standard 0.5 McFarland fungal suspension was prepared with sterile 0.85% saline by a spectrophotometer at 530 nm wavelength. This procedure yielded a yeast stock suspension of 1-5 × 10^6 cells per mL. It was diluted with RPMI 1640 broth medium to obtain a starting inoculum of 1-5 × 10^3 cells per mL. Microplates were inoculated and incubated at 35°C. The MICs were visually read after both 24 and 48 h. Endpoints for azoles, echinocandins and herbal compounds were defined as the lowest concentration of drug that resulted in a prominent reduction (approximately 50% inhibition) of growth compared with the growth control wells, and MICs of AmB were defined as the lowest concentration of drug which resulted in total inhibition of growth [12].

2.4 Checkerboard microdilution tests

The interaction of each herbal compound with each of all three antifungals was assessed against all isolates by the checkerboard method using CLSI document M27-A3. The concentrations of antifungal agents ranged from 1/32 to 8xMIC and herbal compounds ranged from 1/8 to 8xMIC. Antifungal and herbal compound dilutions were prepared in RPMI 1640 medium to be 4-fold of the final concentrations in the microplates. Each of them, 50 µL were dispensed to be antifungal in rows and compound in columns of 96-well microplate. Fungal inoculums were prepared and inoculated to all wells as described in antifungal susceptibility testing. After incubation at 35°C, results were visually read at 24 h and when the absence of adequate growth in growth control well, the incubation was extended more 24 h. The interactions of drug and herbal compound were evaluated based on the fractional inhibitor concentration index (FICI). The FICI was obtained by summing the FIC values of each drug; the FIC was calculated for each agent by dividing the inhibitory concentration of each antifungal or compound when used in combination by its MIC. Synergy was defined as a FICI of ≤ 0.5; no interaction was defined as a FICI > 0.5 but < 4; and antagonism was defined as a FICI ≥ 4 [13]. Off-scale MIC values were converted to the next highest two-fold concentration.

3. RESULTS AND DISCUSSION

Susceptibility testing results of all antifungal agents and herbal compounds were summarized in Table 1. Any MIC value was not detected for FAR and NAR in concentrations tested against *Candida* isolates in this study. However, the MIC values ranged from 60 to 160 µg/mL for CRV, from 16 to 64 µg/mL for EPG, from 80 to 320 µg/mL for GGR and 800 µg/mL for CUR. The lowest MICs were obtained with EPG.

The results of interactions between antifungal drugs and herbal compounds were exhibited in Table 2. The most obvious positive interaction was observed between FAR and all three antifungal drugs. FAR caused a prominent decreasing in the MICs of antifungal drugs (Table

3). For other combinations, although the most common interaction type was no interaction, CRV and GGR reduced the MICs of all three antifungals against most isolates (Table 3). VOR and AmB MICs decreased in combinations with EPG, whereas NAR and CUR did not show any conspicuous effect on antifungal drug activities.

Table 1. The antifungal susceptibility testing results for all antifungals and herbal compounds

Isolates	MIC values (µg/mL; µM for FAR)								
	VOR	CAS	AmB	CRV	FAR	NAR	EPG	CUR	GGR
C. albicans-1	0.03	0.125	1	120	>6000	1600	32	800	320
C. albicans-2	0.015	0.125	1	120	>6000	>1600	64	800	240
C. albicans-3	0.015	0.125	0.5	120	>6000	>1600	32	800	160
C. glabrata-1	0.25	0.125	2	120	>6000	>1600	32	800	160
C. glabrata-2	0.03	0.125	2	160	>6000	>1600	64	800	120
C. parapsilosis-1	0.06	0.5	1	60	>6000	>1600	32	800	80
C. parapsilosis-2	0.03	0.5	0.5	60	>6000	>1600	16	800	320
C. parapsilosis-3	0.5	0.5	1	60	>6000	>1600	32	800	160

VOR, voriconazole; CAS, caspofungin; AmB, amphotericin B; CRV, carvacrol; FAR, farnesol; NAR, naringenin; EPG, epigallocatechin gallate; CUR, curcumin; GGR, ginger.

Although there are several classes of antifungal drugs at the present time, treatment and prophylaxis of invasive fungal infections continue to be a significant clinical problem. Because, treatment is often complicated due to their high toxicity, low tolerability, drug interactions and limited spectrums of activities, alongside with higher treatment costs. Moreover, some fungi are intrinsic resistant to these antifungal agents. Therefore, new drug or treatment alternatives especially those with a wider spectrum, lower toxicity and cheaper are needed. The most important challenge to developing a new antifungal drug is the eukaryotic nature of fungal cell similar to mammalian host cell. Natural products are unique chemicals with different biological activities and the potential antimicrobial effects of certain natural compounds have attracted serious attention within the scientific area. Therefore, it has been estimated that significant progress may be observed in the discovery of new antifungal drugs with the contribution of inexpensive, natural, nontoxic and easily accessible natural compounds. This study aimed to investigate the antifungal activities of several herbal products that are widely consumed in the diet worldwide and their contribution on the efficacy of antifungal drugs against *Candida* spp.

Carvacrol is a monoterpenoic phenol derivative extracted from the herb thyme (Thymus) and its many pharmacological properties, including the antimicrobial activity, have been investigated [2, 3, 6, 14]. It has been shown that CRV is almost more effective than fluconazole against oral *Candida* isolates; the range of MICs was 0.03-0.5% at 24 h [6]. There are many studies evaluating the antifungal activity of CRV, its activity in combination with antifungal drugs or its contribution on the activity of antifungal drugs has not been investigated adequately. Recently, Sharifzadeh et al. [7] investigated the interaction of CRV and VOR against drug-resistant *Candida* spp. They reported that MIC values of CRV were ≤ 125 µg/mL for all isolates and they detected synergistic or additive interactions in this combination against all *Candida* isolates; FICI values were ≤ 0.853 and no antagonistic activity was seen in the strains tested [7]. In other two studies, CRV MICs were ≤ 100 µg/mL and synergic interactions were reported in combination of CRV with fluconazole [14, 15]. The MIC values of CRV were 60-160 µg/mL against all *Candida* isolates in our study. Although we didn't detect any prominent synergistic interaction in combinations with CRV and VOR, CAS or AmB (FICI=0.5-2, 0.58-2, 0.75-1, respectively), CRV caused significant reductions in

MICs of all three antifungal drugs; usually four-fold reduction. Although the exact mechanism is still unclear, it has been reported that the action of CRV based on the inhibition of ergosterol biosynthesis and the disruption of fungal cell membrane integrity similarly to azoles and polyenes [16]. The contribution of CRV on the MICs of antifungals may be explained by; i) these compounds affect simultaneously the same target on fungal cell resulting with enhanced strength in effectiveness; ii) these compounds show sequentially effects on the different targets on fungal cell and the disruption of cell wall by a drug (i.e. an echinocandin) makes it easy to reach of other compound the membrane target. As a result, simultaneously or sequentially effects of antifungals with herbal compounds can provide the reduction in the dose of the administered antifungal.

Isolates	Fractional Inhibitory Concentration (FIC) index (interaction)																	
	CRV			FAR			NAR			EPG			CUR			GGR		
	VOR	CAS	AmB	VOR	CAS	AmB	VOR	CAS	AmB	VOR	CAS	AmB	VOR	CAS	AmB	VOR	CAS	AmB
C. albicans-1	0.91 (I)	0.58 (I)	0.75 (I)	0.5 (S)	0.26 (S)	0.25 (S)	2 (I)	2 (I)	2 (I)	0.5 (S)	2 (I)	1.25 (I)	2 (I)	2 (I)	2 (I)	0.49 (S)	1.12 (I)	0.75 (I)
C. albicans-2	0.75 (I)	2 (I)	0.91 (I)	0.5 (S)	0.09 (S)	0.26 (S)	1 (I)	1.5 (I)	1.5 (I)	0.38 (S)	2 (I)	0.75 (I)	2 (I)	2 (I)	3 (I)	0.75 (I)	0.9 (I)	0.58 (I)
C. albicans-3	0.75 (I)	0.78 (I)	0.91 (I)	0.51 (I)	0.38 (S)	0.25 (S)	1 (I)	1.5 (I)	0.75 (I)	0.75 (I)	2 (I)	0.75 (I)	2 (I)	2 (I)	2 (I)	1.25 (I)	0.62 (I)	0.75 (I)

Table 2. The combination results of antifungal drugs and herbal compounds

CRV, carvacrol; FAR, farnesol; NAR, naringenin; EPG, epigallocatechin gallate; CUR, curcumin; GGR, ginger; VOR, voriconazol; CAS, caspofungin; AmB, amphotericin B; S, synergic interaction; I, indifference; A, antagonistic interaction.

C. glabrata-1	0.75 (I)	0.71 (I)	1 (I)	0.14 (S)	0.75 (I)	0.25 (S)	1.5 (I)	1.5 (I)	1.5 (I)	2 (I)	2 (I)	0.75 (I)	2 (I)	3 (I)	2 (I)	0.75 (I)	0.74 (I)	0.75 (I)
C. glabrata-2	0.5 (S)	0.78 (I)	0.75 (I)	0.5 (S)	0.27 (S)	0.25 (S)	1.5 (I)	1.5 (I)	1.5 (I)	0.37 (S)	2 (I)	0.75 (I)	2 (I)	2 (I)	2 (I)	0.58 (I)	0.58 (I)	1 (I)
C. parapsilosis-1	2 (I)	1.25 (I)	1 (I)	0.05 (S)	0.09 (S)	0.26 (S)	1.5 (I)	1.5 (I)	1.5 (I)	0.75 (I)	2 (I)	0.75 (I)	2 (I)	2 (I)	2 (I)	2 (I)	0.5 (S)	0.53 (I)
C. parapsilosis-2	0.75 (I)	2 (I)	0.75 (I)	0.14 (S)	2 (I)	0.27 (S)	1.5 (I)	1.5 (I)	1.5 (I)	0.63 (I)	2 (I)	2 (I)	2 (I)	2 (I)	3 (I)	1.5 (I)	1.03 (I)	0.75 (I)
C. parapsilosis-3	0.67 (I)	2 (I)	0.91 (I)	0.13 (S)	2 (I)	0.26 (S)	1.5 (I)	0.75 (I)	1.5 (I)	2 (I)	2 (I)	1.5 (I)	2 (I)	2 (I)	2 (I)	0.75 (I)	1 (I)	0.38 (S)

Farnesol is an extracellular quorum-sensing molecule producing by *C. albicans* and inhibits the yeast-to-hypha transition in *C. albicans* and consequently blocks biofilm formation [17]. FAR is also a sesquiterpene alcohol existing in many herbal products. It has been shown that exogenously FAR inhibits the conidiation in *Aspergillus niger* and the germination of macroconidia in *Fusarium graminearum* [18, 19]. However, the number of studies assessing the antifungal efficacy of FAR with standardised methods is limited. In a study evaluating the combinations of FAR with fluconazole, micafungin and AmB agaist *C. albicans* biofilm, synergic interactions were observed for FAR with fluconazole and micafungin combinations, and no interaction for FAR with AmB combination according to FIC indexes [8]. Cordeiro et al. [20] evaluated the antifungal activity of farnesol and its interaction with fluconazole, itraconazole, AmB and CAS against drug-resistant strains of *Candida* species (n=45); the MICs of FAR ranged 4.68-150 µM and FAR significantly reduced the MICs of all antifungals against all isolates. Furthermore, they observed significant rates of synergic interactions without any antagonistic interactions in all combinations [20]. We didn't detect any MIC value in concentrations tested for FAR against the isolates in this study. However, synergistic interactions and MIC reductions in antifungal MICs were conspicuous in all three combinations with FAR. The action of FAR on the fungal cell is largely unclear, probably; it is effective with several mechanisms including growth-inhibitory and apoptosis-promoting effects [8].

Epigallocatechin 3-O-gallate, the main polyphenol component of green tea, has been extensively investigated for antioxidant, anticancer, antibacterial and antiviral effects [21-23]. In a study evaluating many different teas, although EPG didn't have any effect against *C. krusei, C. tropicalis*, or *A. fumigatus* at the concentrations tested, the MICs of EPG were 0.3125 µg/mL against *C. glabrata*, and 5.0 µg/mL against *C. albicans* and *C. parapsilosis* [24]. Ning et al. [9] reported that synergistic interaction was observed between EPG and miconazole, fluconazole or amphotericin B against most of the planktonic and biofilm cells of seven *Candida* isolates and EPG enhanced the activity of these antifungals. In our study, EPG alone had the MICs between 16-64 µg/mL, the combinations of EPG with antifungals were frequently resulted indifference, however EPG caused the reductions at MICs of VOR and AmB. Navarro-Martinez et al. [25] reported that the combination of EPG with azoles showed synergistic interactions against *C. albicans* and the mechanism of this effect could be explained by disturbing the folate metabolism and inhibiting of ergosterol production, because EPG affects the folic acid metabolism by inhibiting dihydrofolate reductase.

GGR is used as a spice derived from the roots of ginger all over the world and it contains more than 60 active compounds [26]. In a study evaluated the antibacterial, antifungal and anti-biofilm activity of GGR, MICs of GGR were 20–40 µg/mL for bacteria, 5 µg/mL for *C. krusei* and 10 µg/mL for *C. albicans*. Antifungal activity of GGR was stronger than those of fluconazole and nystatin against *C. albicans* [10]. Whereas, Soares et al. [27] did not detect

any inhibitory effect on the growth of *C. glabrata* isolates at the tested concentrations (GGR MIC > 3200 µg/mL). We detected MICs ≤ 320 µg/mL for GGR against our *Candida* isolates,

Isolates	CRV			FAR			NAR			EPG			CUR			GGR		
	VOR	CAS	AmB	VOR	CAS	AmB	VOR	CAS	AmB	VOR	CAS	AmB	VOR	CAS	AmB	VOR	CAS	AmB
C. albicans-1	4 ↓	4 ↓	4 ↓	2 ↓	4 ↓	4 ↓	1 ↔	1 ↔	1 ↔	4 ↓	1 ↔	4 ↓	1 ↔	1 ↔	1 ↔	4 ↓	8 ↓	4 ↓

while we didn't observe significant synergy, GGR reduced the MICs of antifungals for especially CAS and AmB in combination tests.

Naringenin is a flavonoid derivative from citrus fruits. It has been showed that although NAR and its derivatives had significant antimicrobial activity against *Staphylococcus aureus*, it had no antimicrobial efficacy against Gram-negative bacteria and fungi including *Alternaria* sp., *Rhodotorula rubra* and *C. albicans* even at the high concentrations [28]. Similarly, we detected any activity of NAR neither alone nor in combination against our *Candida* isolates; NAR did not exhibit any decreasing effect on the antifungal MICs.

CUR, known as Indian saffron in Asia, is a polyphenolic compound and cause of the golden color in turmeric [29]. Martins et al. [29] detected the strong antifungal activity of CUR; it was a more potent antifungal than fluconazole against *Paracoccidioides brasiliensis* and 2.5-fold more potent than fluconazole at inhibition of the adhesion to buccal epithelial cells of *C. albicans* or *C. parapsilosis* [29]. Neelofar et al. [30] observed antifungal activity of CUR against 14 *Candida* strains, with high MICs varying 250-2000 µg/mL, but CUR was less effective than fluconazole. Sharma et al. [11] detected synergistic interactions along with a 10-35-fold reductions in the MIC80 values of drugs in combinations of CUR with azoles and polyenes against clinical *Candida* isolates. In the present study, CUR had high MICs against *Candida* isolates tested, and no positive interaction was observed causing decreases in the MIC values of antifungals against our isolates.

Table 3. The effects of herbal compounds on the antifungal MIC values

C. albicans-2	4 ↓	1 ↔	4 ↓	2 ↓	32 ↓	4 ↓	1 ↔	2 ↓	1 ↔	4 ↓	1 ↔	4 ↓	1 ↔	1 ↔	2 ↑	2 ↓	4 ↓	4 ↓
C. albicans-3	4 ↓	8 ↓	4 ↓	2 ↓	4 ↓	4 ↓	1 ↔	2 ↓	4 ↓	2 ↓	1 ↔	4 ↓	1 ↔	1 ↔	1 ↔	1 ↔	8 ↓	4 ↓
C. glabrata-1	4 ↓	36 ↓	4 ↓	8 ↓	4 ↓	4 ↓	1 ↔	1 ↔	1 ↔	1 ↔	1 ↔	4 ↓	1 ↔	2 ↑	1 ↔	4 ↓	4 ↓	4 ↓
C. glabrata-2	4 ↓	8 ↓	4 ↓	2 ↓	4 ↓	4 ↓	1 ↔	1 ↔	1 ↔	8 ↓	1 ↔	4 ↓	1 ↔	1 ↔	1 ↔	4 ↓	4 ↓	4 ↓
C. parapsilosis-1	1 ↔	4 ↓	4 ↓	16 ↓	32 ↓	4 ↓	1 ↔	1 ↔	1 ↔	4 ↓	1 ↔	4 ↓	1 ↔	1 ↔	1 ↔	1 ↔	4 ↓	32 ↓
C. parapsilosis-2	4 ↓	1 ↔	4 ↓	4 ↓	1 ↔	4 ↓	1 ↔	1 ↔	1 ↔	2 ↓	1 ↔	1 ↔	1 ↔	1 ↔	2 ↑	1 ↔	32 ↓	2 ↓
C. parapsilosis-3	140 ↓	1 ↔	4 ↓	16 ↓	1 ↔	4 ↓	4 ↓	1 ↔	1 ↔	1 ↔	1 ↔	1 ↔	1 ↔	1 ↔	1 ↔	4 ↓	4 ↓	4 ↓

CRV, carvacrol; FAR, farnesol; NAR, naringenin; EPG, epigallotectin gallate; CUR, curcumin; GGR, ginger; VOR, voriconazol; CAS, caspofungin; AmB, amphotericin B; ↑, fold increase of MIC; ↓, fold decrease of MIC; ↔, no change of MIC.

4. CONCLUSION

We showed that some natural products such as carvacrol, epigallocatechin gallate, curcumin, and ginger, acting like an adjuvant, enhanced the in vitro antifungal effects of voriconazole, caspofungin and amphotericin B against *Candida* isolates. Although the exact mechanism of action of these products is not clear, these compounds may represent novel agents to be used in combination with available antifungal drugs to lower doses of antifungal, thus toxic side effects may decrease and treatment costs may reduce. However, further studies are necessary to clarify the mechanisms of action and also to prove in vivo efficacies and present study may be a guide for them.

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

AUTHORS' CONTRIBUTIONS

All authors together designed the study, performed the tests, analyzed the results, and managed the literature searches. Oz Y wrote the first draft of the manuscript. All authors read and approved the final manuscript.

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