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Research Article

Synergistic antifungal effectiveness of essential oils from andean plants combined with commercial drugs

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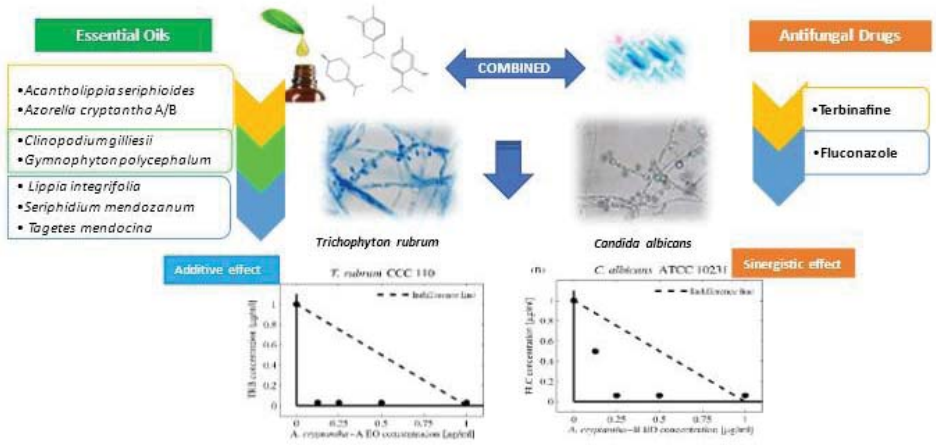
Abstract

The appearance of antifungal resistance promotes the investigation of therapeutic options. There are few studies on the combined effect of antifungal drugs and essential oils (EOs). In the present work, regarding the association of eight EOs Andean plants with antifungal agents against a panel of fungi strains. Combinatorial effects were determined using the Fractional Inhibitory Concentration Index (FICI) and Dose Reduction Index (DRI). A combination of *A. cryptantha*-B EO with fluconazole showed a synergistic effect against *C. Albicans* (FIC = 0.31 and DRI = 16.25). EOs from *A. cryptantha*-A and *L. integrifolia* showed an additive effect (FICI = 0.75) against *C. neoformans*. A combination of EOs from *A. seriphioides* and *A. cryptantha*-A with terbinafine showed an additive effect on *T. rubrum* (FIC = 0.56; DRI = 16) and *M. gypseum* (FICI = 1.03; DRI = 32). In conclusion, combinations between EOs of species from Andean plants and commercial antifungal drugs yielded some interesting findings, as potential antifungal strategies used for treating infections associated with *C. Albicans* and *T. rubrum*.



Graphical Abstract

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Introduction

Around the world, the number of deaths from fungal infections significantly increases every year. The most susceptible age groups are the elderly, premature newborns, and individuals with deficient immune systems. Additionally, prolonged and prophylactic therapeutic use of antifungal drugs promotes the emergence of drug-resistant microorganisms [1]. Fungal diseases cause dermatophytosis, subcutaneous mycoses, systemic mycoses, and other mycoses. In particular, *Candida albicans* fungi are one of the leading causes of superficial infections of the skin, mouth, and mucous membranes, as well as life-threatening systemic infections [2,3].

Another clinically important fungus that affects immunocompromised patients is *Cryptococcus neoformans*. Moreover, cryptococcosis in positive HIV/AIDS patients has become a leading cause of death [4-6]. These infections are treated by drugs that mainly contain fluconazole (FLC) due to its high efficacy and low toxicity, a consequence of its high-water solubility and low affinity for plasma proteins. Nevertheless, its inadequate and long-term use produces multidrug-resistant strains that strongly affect the effciency of commercial drugs [7-9]. On the other hand, dermatophytes are species of fungi that typically infect and invade a living host's skin, hair, and nails. These benign, common infections are caused by the genera *Trichophyton*, *Microsporum*, and *Epidermophyton*, and are usually limited to the stratum corneum or keratinized adnexal structures [10-12].

In some parts of the world, dermatophytoses are found easy to treat, although recurrent infections such as tinea corporis and cruris need more extensive treatments. Antifungal agents like imidazoles and allylamines, namely terbinafine, are very effective but can cause severe toxicity [13]. This situation demands an understanding of the pharmacokinetic and pharmacodynamic properties of the commercially available effective drugs against dermatophytes and an analysis of the factors that may be contributing to drug resistance [14]. Antifungal combination therapy is one of the strategies used to fight the resistance of fungi, caused by the limited therapeutic efficiency of the existing commercial antifungals [15]. Moreover, different authors emphasize the need of combining commercial drugs with natural bioactive products to reduce their toxicity and decrease the mortality index caused by inadequate and extensive use of antimicrobials [16-18]. Combination therapy produces different effects, such as synergistic, additive, indifferent, and antagonistic results. These can be equal, weaker, or stronger than that of the use of an individual drug *per se* [19]. A recent review showed that essential oils (EOs) have a variety of different properties (antiviral, nematocidal, antimicrobial, insecticidal, and antioxidant), due to the presence of diverse bioactive molecules [20]. Therefore, EOs could become important partners for antifungal drugs [21,22]. The main goal of this study was to evaluate the synergistic antifungal effects (FICI ≤ 0.5) of the combination between eight EOs from *Acantholippia seriphioides* Gray Mold, *Azorella cryptantha* (Clos) Reiche (A and B) *Clinopodium gilliesii* (Benth.) Kuntze, *Gymnophyton polycephalum* Gillies & Hook, Clos., *Lippia integrifolia* Griseb Hieron, *Seriphidium mendozanum* (DC.) K. Bremer & Humphries, and *Tagetes mendocina* Phil, and the commercial drugs fluconazole (FLC) and terbinafine (TRB), on the fungi *C. Albicans*, *C. neoformans*, *M. gypseum*, and *T. rubrum*.

Materials and methods

Chemicals and materials

RPMI-1640 culture medium (Sigma-Aldrich, St. Louis, MO, USA) was buffered to pH 7.0 with 3-(N-morpholino) propane sulfonic acid (MOPS) (Sigma-Aldrich). Fluconazole (FLC) and terbinafine (TRB) were obtained from Sigma-Aldrich. Dimethyl sulfoxide (DMSO) was purchased from Merck (Darmstadt, Germany).

Plant material

The following plant species were used: *Acantholippia seriphioides* (A. Gray) Moldenke (Verbenaceae), voucher number (vn) BT-39; *Azorella cryptantha* (Clos) Reiche (Apiaceae) collected at 2700 m.a.s.l., vn CORD 1193 (collection *A. cryptantha*-A) and collected at 4000 m.a.s.l., vn CORD 1188 (collection *A. cryptantha*-B); *Clinopodium gilliesii* (Benth.) Kuntze (Asteraceae), vn BT-7; *Gymnophyton polycephalum* Gillies & Hook, Clos. (Apiaceae), vn BT-41; *Lippia integrifolia* (Griseb.) Hieron (Verbenaceae), vn BT-40; *Seriphidium mendozanum* (DC.) K. Bremer & Humphries (Asteraceae), vn BT-6; and *Tagetes mendocina* Phil. (Asteraceae), vn BT-5.

Essential oil extraction

Fresh aerial parts (1000 g) were subjected to hydrodistillation (2 h) using a Clevenger-type apparatus according to *The European Pharmacopoeia* [23]. EOs yields (% w/v) were the following: *A. seriphioides* (0.75), *A. cryptantha*-A (1.0), *A. cryptantha*-B (0.4), *Clinopodium gilliesii* (2.40), *G. polycephalum* (1.15), *L. integrifolia* (1.50), *S. mendozanum* (0.62), *T. mendocina* (0.81% w/v). EOs were stored at -18°C until used in the antifungal combinatory studies.

Antifungal activity assay

Microorganisms: *Candida albicans* (ATCC 10231), *Cryptococcus neoformans* (ATCC 32264), *Trichophyton rubrum* CCC110 and *Microsporum gypseum* CCC115, were provided by the Centro de Referencia Micólogica CEREMIC (CCC), Facultad de Ciencias Bioquímicas y Farmacéuticas, Universidad Nacional de Rosario, Argentina. *Minimum inhibitory concentration* (MIC). EOs and commercial drugs were determined by the microdilution technique according to Lima, et al. [24]. The serial dilutions of EOs (final concentration of 1000 to 0.98 µg/ml) and commercial drugs (final concentration of 50 to 0.98 µg/ml) were obtained starting from stock solutions in DMSO to a final concentration ≤1%) and were added to the medium achieving a final volume of 100 µl. Endpoints were defined as the lowest concentration of EOs or commercial drugs resulting in total inhibition (MIC) compared to growth controls (culture media and microorganisms alone) and were determined spectrophotometrically at 405 nm using a VERSA Max microplate reader (Molecular Devices, USA).

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In vitro antifungal combinatory effect between EOs and commercial drug

Checkerboard design: The checkerboard design was used to assess the combinatory effect between EOs and FLC and ITC on yeasts and of EOs and TRB on dermatophytes, as described by Gomez, et al. [25]. The fractional inhibitory concentration (FIC) and fractional inhibitory concentration index (FICI) were calculated using the following equations:

$$FIC_{EO} = MIC_{EO\ alone}/MIC_{EOalone} \quad (1)$$

$$FIC_{ANT} = MIC_{ANT\ comb}/MIC_{ANTalone} \quad (2)$$

$$FICI = FIC_{EO}+FIC_{ANT} \quad (3)$$

The results were interpreted as follows: synergistic effect (FICI ≤ 0.5), additive effect (0.5 > FICI< 2.0), no interaction or indifference (2 ≥ FICI < 4), and antagonistic effect (FICI > 4) as described by Nikolić, et al. [26].

Dose Reduction Index (DRI)

The dose reduction index (DRI) is a measure of how many times the dose of each antifungal drug in a synergistic combination may be reduced to a given effect level compared to the doses of each individual substance. A higher value of DRI (>1) indicates a greater dose reduction for a given effect level [27,28]. The DRI value for each corresponding drug is calculated as follows:

$$DRI = MIC\ x\ alone\ /\ MIC\ x\ in\ the\ comb \quad (4)$$

Isobolograms

The potential antifungal combinatory effects between EOs and commercial drugs are presented in normalized isobolograms based on the results of the checkerboard design (**Supplementary Figure 1**). In the isobologram, the x-axis and y-axis represent EOs and commercial antifungals, respectively. The MIC value of EOs alone is located on the x-axis and the MIC value of the antifungal drug alone is on the y-axis. The line connecting these two points represents the line of no interaction (indifference line). The MIC values obtained below the line indicate a synergistic effect (FICI ≤ 0.5), whereas those above the line are explained as an antagonistic effect (FICI > 4) [29].

Results and discussion

An increase in microbial resistance to existing drugs has sparked a surge of global interest in antimicrobial natural products originating from plants such as EOs. Aljaafari, et al. [30] reported the effect of EOs combined with other available antifungal agents is a promising strategy for increasing antifungal efficacy and/or decreasing the toxicity of commercial antifungal agents.

Antifungal activity

Table 1 shows the antifungal effect of the EOs of Andean plants from *A. seriphioides*, *A. cryptantha*-A, *A. cryptantha*-B, and *Clinopodium gilliesii* showed the greatest activity against *C. Albicans* with MIC values between 250-500 µg/ml. The *C. neoformans* strain was strongly inhibited by the EO of *A. cryptantha*-B with a MIC value of 125 µg/ml, but lower effects were observed with *A. cryptantha*-A and *Clinopodium gilliesii* EOs (MIC = 250 µg/ml).

Table 1: Antifungal effect of EOs and commercial antifungals. Values are shown as MIC.

EOs Species	MIC: Minimum inhibitory concentration (µg/ml)			
	C. albicans	*C. neoformans*	*M. gypseum*	*T. rubrum*
A. seriphioides	250	>1000	250	250
A. cryptantha-A	500	250	500	250
A. cryptantha-B	500	125	500	250
C. gilliessi	500	250	500	1000
G. polycephalum	>1000	>1000	>1000	>1000
L. integrifolia	>1000	500	250	1000
S. mendozanum	>1000	1000	500	250
T. mendocina	>1000	>1000	250	500
Antifungal Drug				
Fluconazole	1.95	50	-	-
Terbinafine	-	-	0.016	0.004

Regarding dermatophytes, *M. gypseum* was sensitive to EOs from *A. cryptantha*-A, *A. seriphioides*, *L. integrifolia*, and *T. mendocina* (MIC = 250 µg/ml), whereas *T. rubrum* was inhibited by EOs from *S. mendozanum*, *A. seriphioides*, *A. cryptantha* (A and B), *Clinopodium gilliesii* and *T. mendocina* EOs with MIC values between 250-500 µg/ml. Chemically, EOs are a mixture of terpenes, phenylpropanoids, and terpenoids. Their reactivity against different pathogens is based on the functional groups they possess [20,30]. EOs of *A. seriphioides*, *S. mendozanum*, and both collections of *A. cryptantha* showed the greatest combinatory antifungal effect with the selected commercial drugs. Previous studies reported the chemical composition of the EOs from these species collected in the central Andes region of Argentina [24,31]. The main components of the EO from *A. seriphioides* are *p*-cymene, γ-terpinene, and a high content of bioactive oxygenated monoterpenes such as thymol (27.61%) and carvacrol (13.24%) [24]. Thymol is a naturally occurring phenol monoterpene derived from cymene and an isomer of carvacrol.

The multicomponent composition of EOs (usually comprised of twenty to sixty constituents in varying quantities), generates simultaneous activities and presents advantages compared to commercial drugs. The reactivity against different pathogens is related to their functional groups of them [30]. These complex mixed mechanisms of action make it more difficult to develop fungal resistance compared with a single target therapy. EOs deploys effects against pathogen fungi, interrupting cell communication and mycotoxin synthesis which in turn debilitates fungal growth [32,33]. Ahmad, et al. [34] reported interactions between the components of essential oils that resulted in synergistic combinations. Thymol and carvacrol alter permeability, causing the release of cellular components from the cells. In addition, these terpenoids modify cellular processes such as DNA transcription, protein synthesis, and enzyme activity, affecting, for example, the enzymes producing ATP and thus reducing intracellular ATP levels. γ-terpinene

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promotes the entry of other molecules via the membrane, which then act on their respective targets. The combination of terpinenes with terpenoids increases the amount and size of the pores through a synergistic interaction between them [35,36]. Thymol was found to have a fungicidal effect on *Candida* species and exhibited a synergistic effect combined with nystatin (FICI = 0.25) [37]. Nazzaro, et al. [33] reported that the combination of carvacrol and fluconazole exhibited a significant synergistic effect, inhibiting the over-expression of efflux-pump drug-resistance genes CDR1 (*Candida* drug resistance) and MDR1 (multidrug resistance) in *C. Albicans* which inhibited efflux by 70% - 90%, thus evidencing a great potential to block drug transporter pumps. Also, Braga, et al. [38] evaluated the synergistic effect of eugenol and thymol and their mechanism of action showed morphological alterations in the membrane of *C. Albicans.* Jafri and Ahmad [39] reported that antifungal drug activity is greatly increased in the presence of thymol on *C. Albicans* and *C. tropicalis* strains. In addition, thymol resulted in an alternative agent for the treatment of biofilm-associated with *C. Albicans* and *C. tropicalis* infections. Shaban, et al. [40] showed evidence of enhanced antifungal activity for a combination of oxygenated compounds with antifungal drugs against *Candida Auris*, where the interaction of carvacrol was evidenced as antifungal and anti-virulent. Also, carvacrol effectively reduced the gene expression of secreted aspartyl proteinase (SAP), generating a higher effect against *C. Albicans* isolates [41]. Previous reports described the chemical composition of the EOs of *S. mendozanum*, in which the main actives terpenes are camphor, borneol, astemizole, and artemisia alcohol [42]. On the other hand, although the chemical profile of sesquiterpene hydrocarbons in the EOs of both collections of *A. cryptantha* was similar, there were variations in the relative proportion of the main components such as the content of oxygenated monoterpenes (3.0% total) as cis-β-terpineol, isoborneol, and borneol present in *A. cryptantha*-B EO [43]. The antifungal effect of borneol and its derivates against *C. Albicans* yeast has been previously reported [44]. Also, borneol, thymol, camphor, and isoborneol are responsible for the anti-dermatophyte efficacy of EOs [45,46].

In vitro antifungal combinatory effect between EOs and antifungal drugs

The antifungal combinatory effect (determination of FIC and FICI) between FLC and selected EOs against yeasts is shown in Table 2 and Figures 1,2. The EOs of *A. cryptantha*-B with FLC showed a synergistic effect on *C. Albicans*, (FICI = 0.31, Equations 1-3; DRI = 16.25, Equation 4), while the EO of *A. cryptantha*-A and *A. seriphioides* elicited an additive effect (FICI = 1.03; DRI = 32.50 and FICI = 1.49). The results represented in the normalized isobolograms (Figure 1A-C) agree with those obtained by the checkerboard analysis. The combination of *A. cryptantha*-B EO and FLC evidenced a synergistic effect on *C. Albicans*, as shown by the resulting values of the experimental combinations which are all below the line of indifference (Figure 1A). On the other hand, an additive effect was observed for the EOs from *A. cryptantha*-A and *A. seriphioides* EOs evaluated against this strain (Figure 1B, C).

Regarding the effects on *C. neoformans*, the combination between *S. mendozanum* EO and FLC showed an additive effect (FICI = 0.625; DRI = 2), (Figure 2A-D). A similar effect was observed with the EOs of *A. cryptantha*-A, *L. integrifolia* (FICI = 0.75; DRI = 4), and *A. cryptantha*-B (FICI = 1; DRI = 2). Only the EOs from *A. cryptantha* A and B showed additive interactions as shown by the experimental values of FICI which are all below the line of indifference Figures 2B, D.

Table 3 shows the combinatory effect (FIC and FICI) of TRB and EOs against dermatophytes *M. gypseum* and *T. rubrum*. Additive interactions were observed against *M. gypseum* (FICI = 1.03-1.06). The significant decrease observed in the dose of TRB (DRI = 16-32) could be largely due to the adjuvant property of the compounds present in the EOs coinciding with the available literature [26]. Regarding *T. rubrum*, additive interactions were observed when combining the EOs of *A. seriphioides*, and *A. cryptantha*-B and TRB, obtaining a "borderline case of synergism" (FICI = 0.56, DRI = 16). The five EOs extracted from *S. mendozanum*, *A. cryptantha*-A, *L. integrifolia*, *C. gilliessi*, and *T. mendocina* displayed additive activity (FICI = 1.03-1.06). Moreover, a pronounced decrease in the individual MIC value of TRB was observed (DRI = 16-32).

Table 2: The combinatory effect of EOs and FLC on *C. Albicans* and *C. neoformans*. Results are shown as MIC_{comb} (expressed in µg/ml), FIC, FICI, and DRI values.

EOs species/ FLC	*C. albicans*					*C. neoformans*				
	$MIC_{in\ comb}$	FIC	FICI	DRI	Effect	MIC_{comb}	FIC	FICI	DRI	Effect
A. seriphioides	250	1	1.49	16.25	Add	-	-	nd	-	-
FLC	0.12	0.49				-	-			
A. cryptantha-A	500	1	1.03	32.5	Add	125	0.50	0.75	4	Add
FLC	0.12	0.03				6.25	0.25			
A. cryptantha-B	250	0.25	0.31	16.25	Syn	62.5	0.50	1	2	Add
FLC	0.12	0.06				25	0.50			
C. gilliessi	250	0.5	1.5	1	Add	125	0.5	1	2	Add
FLC	3.9	1				12.5	0.5			
L. integrifolia	-	-	nd	-	-	250	0.5	0.75	4	Add
FLC	-	-				12.5	0.25			
S. mendozanum	-	-	nd	-	-	500	0.50	1	2	Add
FLC	-	-				4	0.50			
T. mendocina	-	-	nd	-	-	62.5	0.125	2.03	0.5	Ind
FLC	-	-				16	2			

EOs: Essential oils; FLC: Fluconazole; MIC_{comb}: Minimum inhibitory concentration in combination; FIC: Fractional Inhibitory Concentration; FICI: Fractional Inhibitory Concentration Index; DRI: Dose Reduction Index; Syn: Synergism, Additive: Add; Ind: Indifference; nd: not determined for MIC >1000µg/ml.

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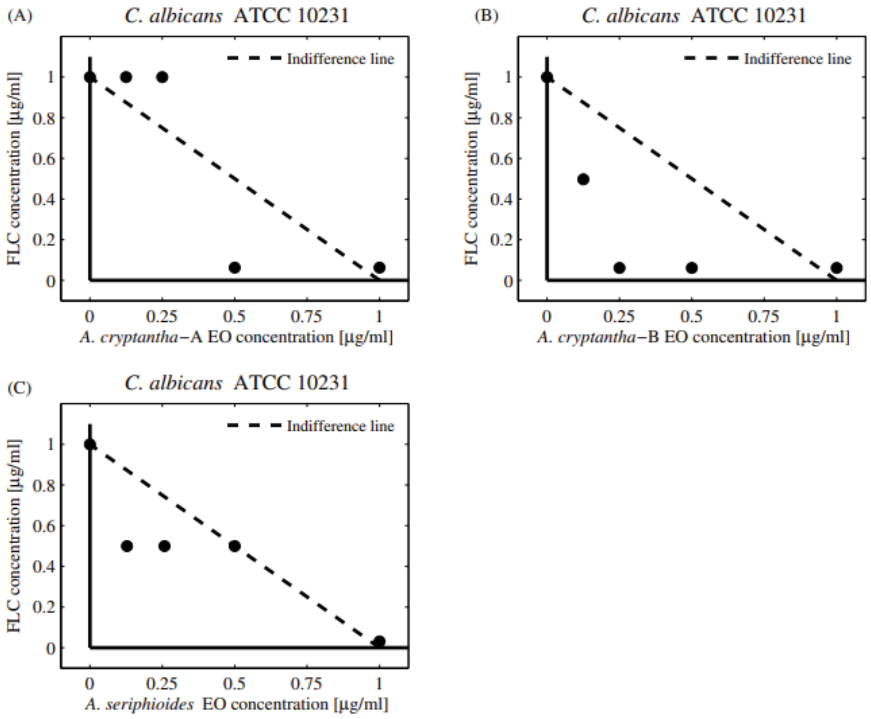

Figure 1: Synergistic effect of the *A. cryptantha*-B (B), additive effect of *A. cryptantha*-A and *A. seriphioides* EOs (A, C) EOs with FLC on *C. albicans.*

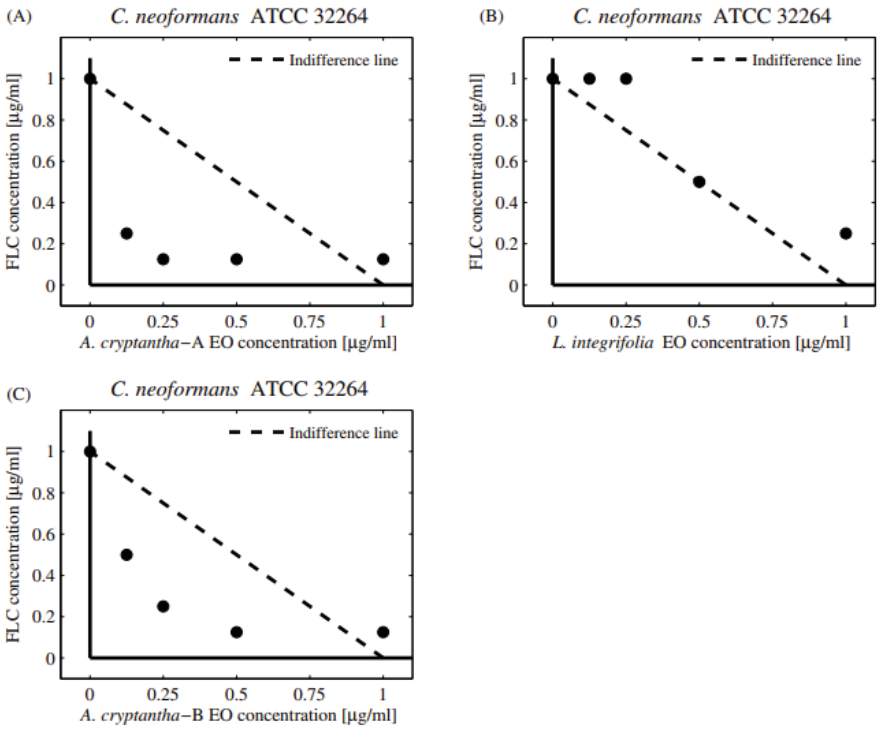

Figure 2: Additive effect of the EOs from *A. cryptantha*-A, (A) *L. integrifolia* (B), and *A. cryptantha*-B (C) with FLC on *C. neoformans*.

Additive activity can be observed in the normalized isobolograms (Figures 3A-D and 4A-D) with combination points below (both collections of *A. cryptantha* EOs) and above the indifference line against both dermatophytes strains tested. The dermatophytes showed low sensitivity to the combinations tested.

Conclusion

The highest antifungal properties reported for carvacrol, and thymol, indicate that these main components in the EOs assayed, and especially to some combinations EOs selected with commercial antifungals provided benefits, including

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Table 3: The combinatory effect of EOs and TER on dermatophytes. Results are shown as MIC and MIC_{Comb} (expressed in µg/ml), FIC, FICI, and *DRI* values.

EOs species/TRB	*M. gypseum*					*T. rubrum*				
	$MIC_{in\ comb}$	FIC	FICI	DRI	Effect	$MIC_{in\ comb}$	FIC	FICI	DRI	Effect
A. seriphioides	250	1	1.03	32	Add	125	0.5	0.56	16	Add
TRB	0.002	0.03				0.0005	0.06			
A. cryptantha-A	250	1	1.03	32	Add	500	1	1.06	16	Add
TRB	0.002	0.03				0.0005	0.06			
A. cryptantha-B	1000	1	1.06	16	Add	250	0.5	0.56	16	Add
TRB	0.002	0.06				0.0005	0.06			
C. gilliessi	250	1	2	1	Ind	500	1	1.03	32	Add
TRB	0.064	1				0.0005	0.03			
L. integrifolia	250	1	1.06	16	Add	1000	1	1.03	32	Add
TRB	0.002	0.06				0.0005	0.03			
S. mendozanum	500	1	1.06	16	Add	250	1	1.06	16	Add
TRB	0.002	0.06				0.002	0.06			
T. mendocina	1000	1	1.06	16	Add	500	1	1.06	16	Add
TRB	0.002	0.06				0.002	0.06			

EOs: Essential oils; TRB: Terbinafine; MIC_{comb}: Minimum Inhibitory Concentration in Combination; FIC: Fractional Inhibitory Concentration; FICI: Fractional Inhibitory Concentration Index; DRI: Dose Reduction Index; Add: Additive; Ind: Indifference

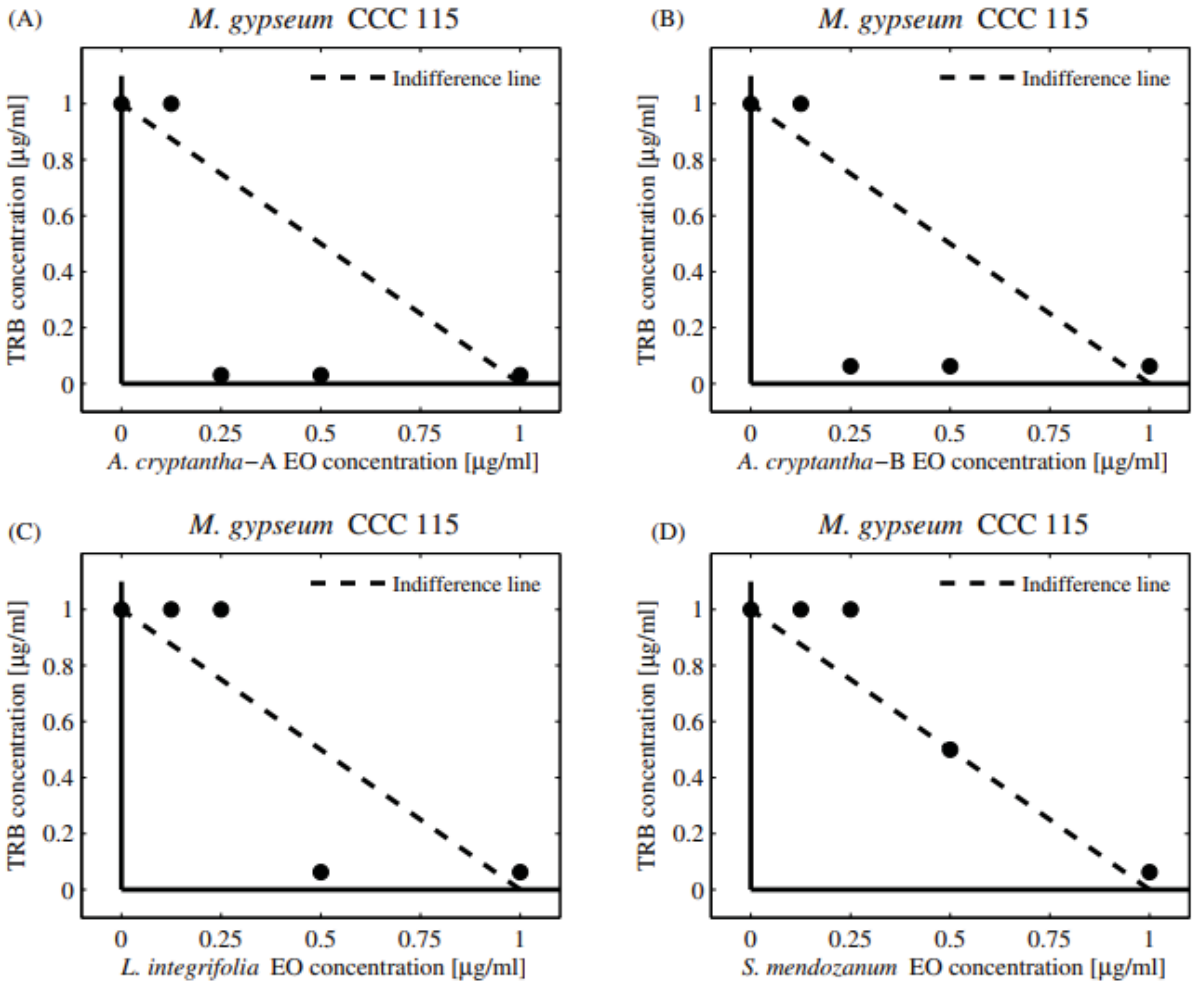

Figure 3: Additive effect of the EOs from *A. cryptantha*-A (A), *A. cryptantha*-B (B), and *L. integrifolia* (C) and *S. mendozanum* (D) with TRB on *M. gypseum*.

synergistic effect and reduced toxic effects, showing their potential to treat infections associated to *C. Albicans* and *T. rubrum*. The present study shows the antifungal effect of EOs from Andean plants, containing the active ingredients borneol, carvacrol, and thymol, against relevant fungal strains such as yeasts (*Candida albicans*, *Cryptococcus neoformans*) and dermatophytes (*T. rubrum* and *M. gypseum*). Both collections of *A. cryptantha* (A and B) and *A. seriphioides* EOs yielded some interesting findings; they were the most promising combinations that show efficacy against pathogenic fungi. Finally, these results confirm the importance of carrying out further studies on combined strategies using natural products

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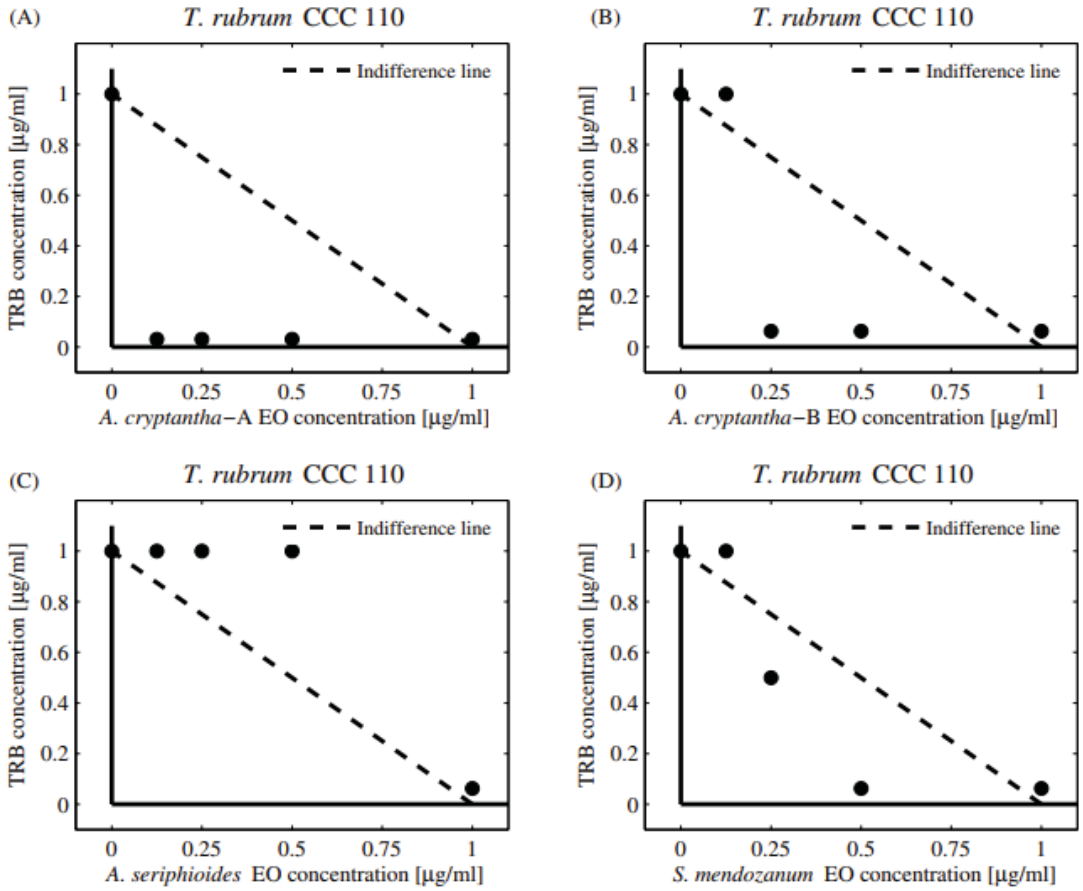

Figure 4: Additive effect of the EOs of *A. cryptantha*-A (A) *A. cryptantha*-B (B), *A. seriphioides* (C); and *S. mendozanum* (D) with TRB, on *T. rubrum*.

and commercial drugs to improve the increasing problem of antibiotic resistance.

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