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Antagonistic Interactions in Onychomycosis: Antifungal Activity of Extracts from Pure and Mixed Cultures of *Candida parapsilosis* and *Trichophyton* spp.

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Abstract: Onychomycoses are nail infections that require prolonged therapy and have high recurrence rates. Dermatophytes are the main etiological agents of these infections, followed by yeasts and non-dermatophyte filamentous fungi. The limited antifungal arsenal used to treat onychomycosis and the change in the susceptibility profile of these agents contribute to the chronicity and recalcitrant profile of infections. The present study aimed to determine the antifungal activity of extracts obtained from pure and mixed cultures of *Candida parapsilosis*, *Trichophyton mentagrophytes*, and *Trichophyton rubrum*. Additionally, in vivo toxicity tests with *Galleria mellonella* and time-kill assays were carried out. The susceptibility profiles of dermatophytes were determined using a microdilution technique with minimum inhibitory concentrations (MICs) between 250 and 8000 µg/mL. The time-kill assay, compared to growth control, resulted in the death of dermatophytes within 48 h. No toxicity of the extracts was detected in experiments with *Galleria mellonella* larvae under the test conditions. The extracts of pure and mixed cultures of *Candida parapsilosis* and dermatophytes present antifungal activity against *T. mentagrophytes* and *T. rubrum*. Isolating and identifying compounds in the extracts may allow the development of new therapeutic approaches to control fungal infections.

Keywords: onychomycosis; antagonism; dermatophytes; *Candida* spp.; antifungal activity

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1. Introduction

Onychomycoses are infections with worldwide incidence, clinically characterized by discoloration, hyperkeratosis, and onycholysis of the nail plate [1]. Dermatophytes, which account for 70% of cases, are fungi that can degrade keratinized tissues such as skin, hair, and nails. Among the prevalent species, *Trichophyton rubrum* (TR) and members of the *T. mentagrophytes* (TM) complex are the most isolated [2]. Recently, studies indicate an increase in nail infections caused by yeasts of the genus *Candida*, including the species *C. albicans* and *C. parapsilosis* (CP), and non-dermatophyte filamentous fungi such as *Scopulariopsis brevicaulis*, *Acremonium* spp., *Aspergillus* spp., *Fusarium* spp., and *Neoscytalidium* [3–5], occurring before or concomitantly with dermatophytes [6]. In infections of mixed etiology, interactions between species promote the production of various metabolites and the expression of virulence factors, such as hydrolytic enzymes, that are capable of regulating morphogenesis, growth, and biofilm formation. These mechanisms are essential for the survival of microorganisms in hostile environments, helping them to tolerate the

action of antifungal drugs and evade the host's immune response [7,8]. Considering the increase in cases of onychomycosis caused by different species of fungi, the knowledge of the competitive interactions between microorganisms constitutes a challenge, providing a new scenario for novel therapeutic approaches in the treatment of these infections [9,10]. The present study evaluated the antifungal activity of extracts obtained from pure and mixed cultures of CP, TM, and TR against strains of *Trichophyton* spp.

2. Materials and Methods

2.1. Microorganisms

Clinical isolates of CP (1), TM (6), and TR (6) from the collection of the Microbiology Laboratory of the School of Medicine in São José do Rio Preto (FAMERP), Brazil, from the American Type Culture Collection (*T. mentagrophytes* 11481—TMATCC) and from the Central Bureau of Fungal Cultures (Centraalbureau voor Schimmelcultures—*T. rubrum* 118892—TRCBS) were used. All isolates were registered in the National System for the Management of Genetic Heritage and Associated Traditional Knowledge (SisGen) under protocol number AF41CDD.

2.2. Extracts

To obtain the CP culture extract, colonies grown on Sabouraud Dextrose Agar were incubated at 35 °C for 24 h. An inoculum suspension (5 mL) in sterile saline (0.85%), with turbidity corresponding to MacFarland Scale 10, was inoculated into 500 mL of Sabouraud Dextrose Broth (SDB) and incubated at 35 °C for 72 h. For pure culture extracts of TM and TR, dermatophyte colonies with seven days of growth on Potato Dextrose Agar were covered with sterile saline. Subsequently, the mixture of conidia and hyphae in the suspension (5 mL) was transferred to 500 mL of SDB and incubated at 35 °C for 120 h. In preparing mixed culture extracts with CP and TM (CPTM) and with CP and TR (CPTR), 5 mL of inoculum of CP and previously prepared samples of *Trichophyton* spp. were transferred to 500 mL of SDB and incubated at 35 °C for 120 h. The culture medium containing the inoculum was then filtered through a 0.2 µm Millipore membrane and subjected to liquid-liquid extraction with ethyl acetate (250 mL culture medium/50 mL ethyl acetate). The extraction procedure was repeated three times, allowing total extraction of the metabolites in the culture filtrate. The acetate phase was subjected to drying using a rotary evaporator. The mass of the resulting compound was solubilized in a 10% solution of dimethyl sulfoxide (DMSO) with sterile water to carry out subsequent tests.

2.3. Susceptibility Profile of Dermatophyte Strains against Extracts and Antifungal Drugs

The susceptibility profiles of TR strains in respect to extracts and drugs were determined by the microdilution technique following the protocol described in document M38-3rd of the Clinical Laboratory Standard Institute (CLSI) with modifications [11]. The dermatophytes were cultivated on Potato Dextrose Agar at 35 °C for seven days. After this period, the colony surface, covered with 5 mL of sterile saline (0.85%), was scraped with a sterile loop. The inoculum was adjusted with a spectrophotometer (530 nm), followed by dilution at a ratio of 1:50 in RPMI 1640 medium (Sigma-Aldrich®—Rosen Park Media Institute, Saint Louis, MO, USA) with a final concentration of 0.4–5 $\times$ 10^4 cells/mL. Subsequently, 0.1 mL of the suspension was inoculated in 96-well polystyrene plates containing RPMI 1640 medium at different extract concentrations (8000–1.9 µg/mL) and drugs were tested: itraconazole 4–0.16 µg/mL, fluconazole and terbinafine 64–0.001 µg/mL. Sterility control wells (containing 0.2 mL of RPMI) and growth controls (containing inoculum and RPMI only) were prepared. Plates were incubated at 35 °C for 120 h. The MICs of fluconazole and itraconazole were determined as the lowest concentration capable of inhibiting 80% of fungal growth (MIC80); concentrations capable of inhibiting 100% (MIC100) were considered for the extracts and for terbinafine. For the minimum fungicidal concentration (MFC), an aliquot of each well of the plates prepared with extracts and inoculum was transferred to Petri dishes containing Sabouraud Dextrose Agar. The MFC was defined as

the lowest concentration of the extract to inhibit visible growth on a solid medium. All tests were performed in triplicate.

2.4. Time-Kill Assay

The time-kill assay was conducted according to the method described by Klepser et al. with *Trichophyton* spp. The MFC values obtained were used for this assay. The dermatophyte inoculum was prepared as described previously. Suspensions of 0.1 mL of conidia were added to 4.9 mL of SDB to, obtain a final concentration corresponding to 0.4–5 $\times$ 10^4 colony-forming units (CFU/mL). The inoculum after preparation was diluted at a ratio of 1:1 with the extract (0.2 mL of the extract with 0.2 mL of the inoculum). Tubes containing only inoculum were included to evaluate growth control. At predetermined times (8 h, 24 h, 48 h), an aliquot of 30 µL was taken from each sample and transferred to Sabouraud Dextrose Agar (DIFCO®, Detroit, MI, USA) plates using a Drigalski loop and incubated at 35 °C for 120 h. Subsequently, the number of colony-forming units (CFU) was counted. The percentage of inhibition was calculated by comparing with the growth control using the colony counting method [12].

2.5. Toxicity Test with Galleria mellonella

Toxicity tests of the extracts were performed using *G. mellonella* larvae following the experimental model of toxicity according to the protocol described by Ignasiak and Maxwell. Five *G. mellonella* larvae (275 $\pm$ 25 mg) in the sixth instar of development were separated in Petri dishes (90 $\times$ 15 mm^2) for each condition. Artificial inoculation was achieved by injecting 5 µL of extracts at concentrations of 2000–8000 µg/mL using a Hamilton micro syringe for gas chromatography (model 7000.5 KH: 10 µL). The experimental controls were untouched larvae (naïve), larvae inoculated with 10% ethanol (positive toxicity control), and 10% DMSO (negative toxicity control). Inoculated larvae were deprived of food and direct light, incubated at 37 °C, and scored for viability at 24 h intervals for 5 days. Differences in resulting survival plots were evaluated using the Origin software (pro 9.1) with the Mantel–Cox test (Log-rank method) [13].

3. Results

3.1. Susceptibility Profile of Dermatophyte Strains against Extracts and Antifungal Drugs

The results for the susceptibility profile of dermatophyte strains against antifungal drugs are shown in Table 1. MIC values for fluconazole ranged from 2 to 32 µg/mL, with the highest values (32 µg/mL) being found for the TM strains (TM5094, TM6085, and TM6007) and the lowest (2 µg/mL) for the TR strains (RCBS, TR7604, and TR7984). Itraconazole values ranged from 0.5 to 2 µg/mL, with the highest (2 µg/mL) for the TM5094 strain and the lowest (0.25 µg/mL) for TRCBS. For terbinafine, the highest MIC value (64 µg/mL) found was for the clinical strain TR7259; the other strains had values of 0.03 µg/mL.

The MIC values found for the extracts ranged from 250 to 8000 µg/mL (Table 2). A range of lower values was observed for the CP extracts against TR and CPTM against the TM and TR strains (250 and 500 µg/mL). In contrast, a higher MIC range was observed for CPTR and TR extracts (1000–8000 µg/mL). MFC values followed this trend, with smaller intervals for CPTM (250 and 1000 µg/mL) and CP (500 and 1000 µg/mL); CPTR and TR extracts showed higher MFC values (2000–8000 µg/mL).

Considering MIC and MFC geometric mean values, the CP extract showed the best activity against TR and TM at 885 and 1284 µg/mL, respectively. The TM and CPTM extracts showed the second-best activity, with MIC geometric means of 1557 and 1577 µg/mL, respectively. The lowest antifungal activity was observed for CPTR and TR with values of 3035 and 2883 µg/mL for MICs and 4157 µg/mL for MFCs (Table 3).

Table 1. Minimum inhibitory concentration (MIC) values of the drugs fluconazole, itraconazole, and terbinafine against dermatophyte strains.

	MIC (μg/mL)		
	Fluconazole	**Itraconazole**	**Terbinafine**
TMATCC	4	0.5	0.03
TM5094	32	2	0.03
TM5419	16	1	0.03
TM6007	32	1	0.03
TM6085	32	1	0.03
TM7389	16	1	0.03
TRCBS	2	0.25	0.03
TR6185	4	0.5	0.03
TR6195	4	0.5	0.03
TR6284	4	0.5	0.03
TR7259	32	0.5	64.0
TR7604	2	0.5	0.03
TR7984	2	0.5	0.03

Table 2. Minimum inhibitory concentration (MIC) and minimum fungicidal concentration (MFC) values for fluconazole, itraconazole, and terbinafine against dermatophyte strains.

	MIC (μg/mL)					
	TMATCC	**TM5094**	**TM5419**	**TM6007**	**TM6085**	**TM7389**
CP	1000–2000	1000–2000	1000–2000	1000–2000	1000–2000	1000–2000
CPTM	250–8000	250–8000	500–8000	250–8000	250–8000	250–8000
CPTR	2000–8000	4000–8000	4000–8000	4000–8000	4000–8000	4000–8000
TM	1000–8000	2000–8000	1000–8000	1000–8000	1000–8000	1000–8000
TR	2000–8000	2000–8000	4000–8000	4000–8000	4000–8000	4000–8000
	MIC (μg/mL)					
	TRCBS	**TR6185**	**TR6195**	**TR6284**	**TR7604**	**TR7984**
CP	500–2000	500–2000	500–2000	250–500	500–2000	250–2000
CPTM	250–8000	250–8000	250–8000	250–1000	500–8000	250–8000
CPTR	2000–4000	2000–8000	2000–4000	1000–2000	2000–4000	2000–4000
TM	1000–4000	1000–8000	2000–8000	250–2000	1000–8000	250–4000
TR	1000–4000	1000–8000	2000–8000	1000	2000–8000	1000–8000
	MFC (μg/mL)					
	TMATCC	**TM5094**	**TM5419**	**TM6007**	**TM6085**	**TM7389**
CP	1000–2000	1000–2000	1000–2000	1000–2000	1000–2000	1000–2000
CPTM	500–8000	500–8000	500–8000	500–8000	1000–8000	500–8000
CPTR	4000–8000	4000–8000	4000–8000	4000–8000	4000–8000	4000–8000
TM	1000–8000	2000–8000	2000–8000	1000–8000	1000–8000	1000–8000
TR	2000–8000	4000–8000	4000–8000	4000–8000	4000–8000	4000–8000
	MFC (μg/mL)					
	TRCBS	**TR6185**	**TR6195**	**TR6284**	**TR7604**	**TR7984**
CP	1000–2000	1000–2000	1000–2000	1000–2000	500–1000	500–2000
CPTM	250–8000	250–8000	500–8000	500–4000	500–8000	500–8000
CPTR	2000–4000	2000–8000	2000–8000	2000	4000	2000–4000
TM	1000–4000	1000–8000	2000–8000	1000–4000	2000–4000	1000–4000
TR	1000–8000	2000–8000	2000–8000	1000–4000	4000–8000	2000–4000

CP: *C. parapsilosis*; CPTR: *C. parapsilosis* and *T. rubrum*; CPTM: *C. parapsilosis* and *T. mentagrophytes*; TR: *T. rubrum*; TM: *T. mentagrophytes*; TRTM: *T. rubrum* and *T. mentagrophytes*.

Table 3. The minimum inhibitory concentration (MIC) and minimum fungicidal concentration (MFC) geometric mean values for the extracts against the dermatophyte strains.

	Geometric Mean (μg/mL)					
	TR		TM		TRTM	
	MIC	MFC	MIC	MFC	MIC	MFC
CP	689	1212	1137	1361	885	1284
CPTM	1414	1714	1759	2619	1577	2119
CPTR	2245	3429	4104	5040	3035	4157
TM	1343	2245	1805	3299	1557	2722
TR	2133	3429	3899	5040	2883	4157

CP: *C. parapsilosis*; CPTR: *C. parapsilosis* and *T. rubrum*; CPTM: *C. parapsilosis* and *T. mentagrophytes*; TR: *T. rubrum*; TM: *T. mentagrophytes* and *T. mentagrophytes*.

3.2. Time-Kill Assay

The time-kill assay was carried out for 48 h, exposing the TRCBS, TR6185, TMATCC, and TM5094 strains to the extracts (Figure 1).

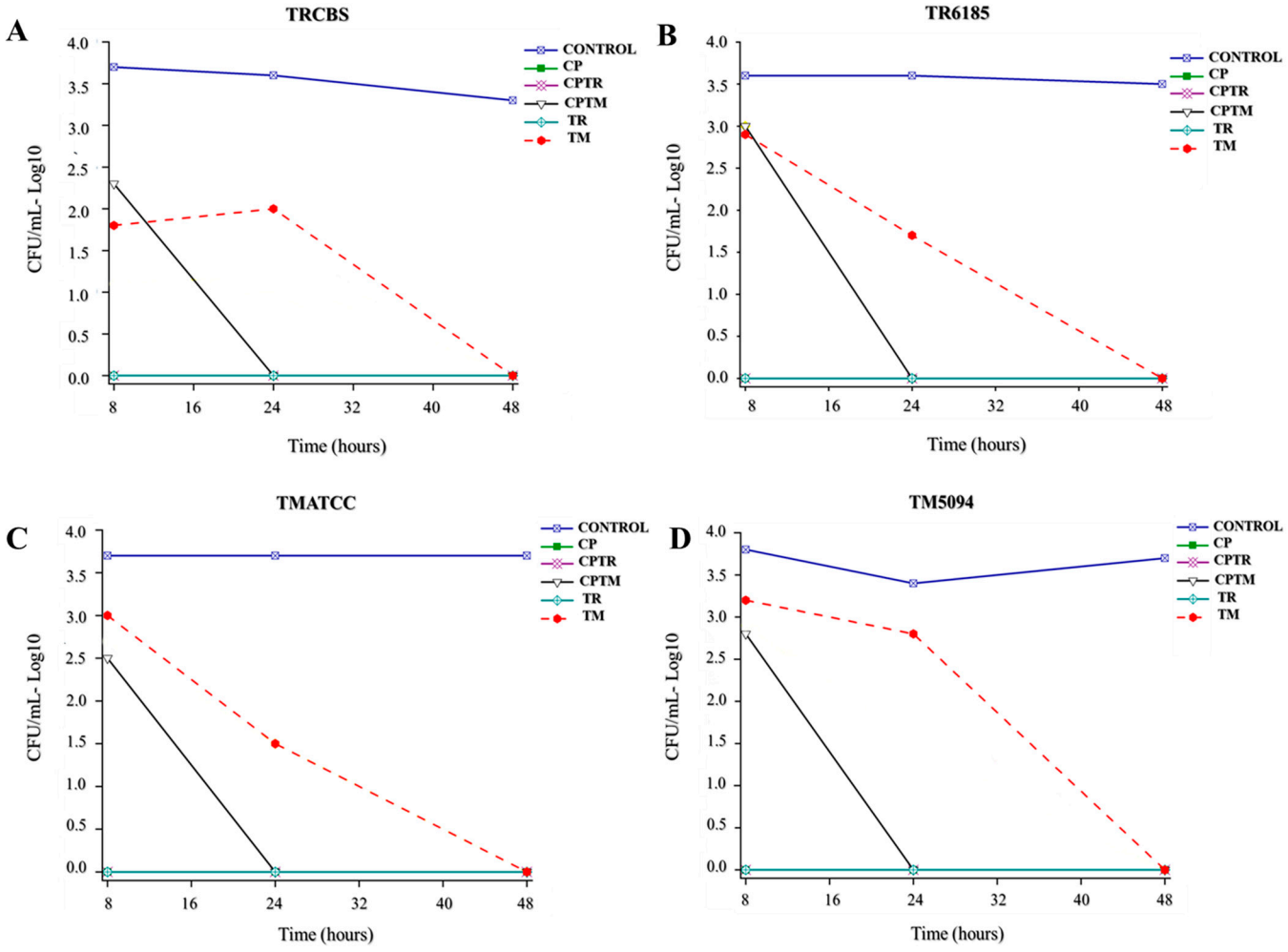


Figure 1. Graphical representation of the dermatophyte death kinetics in contact with the following extracts: (**A**) TRCBS; (**B**) TR6185; (**C**) TMATCC; (**D**) TM5094. CP: *C. parapsilosis*; CPTR: *C. parapsilosis* and *T. rubrum*; CPTM: *C. parapsilosis* and *T. mentagrophytes*; TR: *T. rubrum*; TM: *T. mentagrophytes*.

The maximum reduction in CFU/mL for the dermatophytes was observed at 48 h, with an inhibition rate of 99.9% compared to the growth control. CP, TR, and CPTR extracts showed the fastest reduction time for all dermatophyte strains (8 h), followed by CPTM (24 h—Table 4).

Table 4. The colony-forming unit (CFU/mL) of dermatophyte strains exposed to extracts for different times.

	TRCBS				**TR6185**		
	8 h	**24 h**	**48 h**		**8 h**	**24 h**	**48 h**
CONTROL	5083	3812	1955	CONTROL	3957	4258	3450
CP	0	0	0	CP	0	0	0
CPTR	0	0	0	CPTR	0	0	0
CPTM	183	0	0	CPTM	1117	0	0
TR	0	0	0	TR	0	0	0
TM	67	100	1	TM	817	54	1
	TMATCC				**TM5094**		
	8 h	**24 h**	**48 h**		**8 h**	**24 h**	**48 h**
CONTROL	5173	5465	5217	CONTROL	5971	2745	4753
CP	0	0	0	CP	0	0	0
CPTR	0	0	0	CPTR	0	0	0
CPTM	283	0	0	CPTM	667	0	0
TR	0	0	0	TR	0	0	0
TM	1000	33	0	TM	1533	633	0

CP: *C. parapsilosis*; CPTR: *C. parapsilosis* and *T. rubrum*; CPTM: *C. parapsilosis* and *T. mentagrophytes*; TR: *T. rubrum*; TM: *T. mentagrophytes*.

3.3. Toxicity Test with Galleria mellonella

The toxicity tests using *G. mellonella* show the non-toxicity of the extracts at concentrations of 2000–8000 µg/mL with the survival rate of 80–100% of the larvae injected with the compounds. Toxicity tests using *G. mellonella* showed that the CP, TR1, and CPTM extracts tested were not toxic after five days, with a 100% survival rate of the injected larvae. Similarly, TM and CPTR extracts were not considered toxic, with an 80% survival rate (Figure 2).

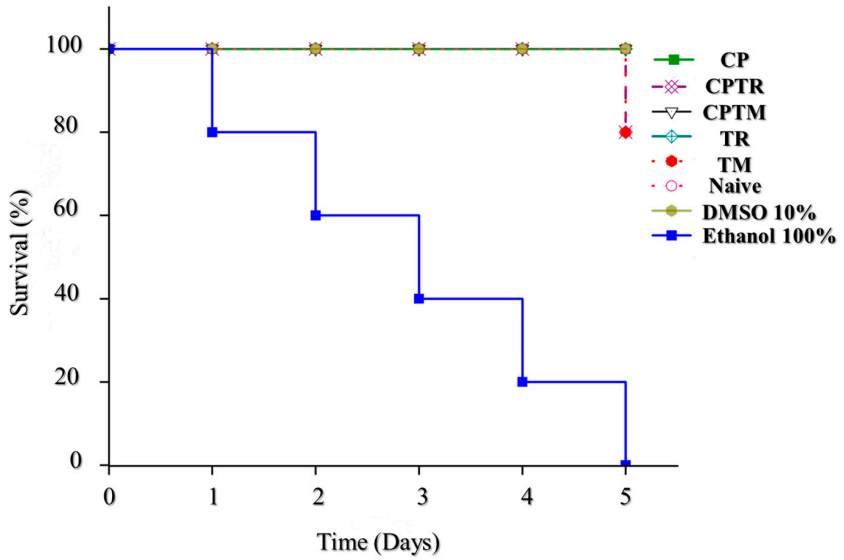


Figure 2. Survival (%) of *Galleria mellonella* larvae exposed to extracts (2000–8000 µg/mL) with monitoring at 24 h intervals for five days. CP: *C. parapsilosis*; CPTR: *C. parapsilosis* and *T. rubrum*; CPTM: *C. parapsilosis* and *T. mentagrophytes*; TR: *T. rubrum*; TM: *T. mentagrophytes*.

4. Discussion

Clinical laboratory case reports of onychomycosis caused by *Candida*, filamentous non-dermatophytes and *Trichophyton* spp. have been common in recent years. Performing an analysis by direct examination often identifies fungi with two or more different structures (e.g., hyphae and yeast); however, fungal growth is often observed only for a single species [14,15].

The biological interference between the dermatophytes TM and TR with *Candida* when cultivated in a solid medium was demonstrated by Lemes et al. The anti-Trichophyton activity of extracts produced from pure cultures of *Candida* spp. corroborates the microbial growth interference [16]. The current study shows that mixed culture extracts also have antifungal activity against dermatophytes demonstrating the presence of antifungal compounds in these cultures. The individual biological characteristics of each strain justify the differences in MICs since the strains originate from different sources of infection. The unstable environment causes adaptation and response with biological changes as a defense or tolerance mechanism.

In the nails, mixed interactions between microorganisms promote the production of essential metabolites for survival, with a variety of virulence factors such as proteases, lipases, and biofilm formation, in addition to resistance to host immune responses or the action of drugs. In this context, Mohammadi et al., analyzing the virulence profile of *Candida* species isolated from nails, observed significant correlations between the MICs of fluconazole and itraconazole and biofilm production [17]. One study coordinated by Oliver et al. on chemical profiles of metabolites showed a variety of molecules produced by species of *Candida* spp. isolated from different sources of infection. In this study, 66 different metabolites were identified. These metabolic pathways are mainly related to energy production and virulence mechanisms [18].

A metabolomics analysis of TR by Ciesielska et al. identified compounds involved in amino acid metabolism, carbohydrate metabolism related to glycolysis, the tricarboxylic acid (TCA) cycle, and nucleotide and energy metabolisms. Due to the limited availability of nutrients, an increased production of substances and specific protective mechanisms such as metabolites that act as energy carriers (GTP), adenosine triphosphate (ATP), and uridine-5′-triphosphate (UTP) were detected in the control medium [19]. In the present study, it was not possible to identify the metabolites produced in pure and mixed cultures. Therefore, investigations are necessary to identify these compounds and better understand the interactions. Although the compounds in the extracts have not been identified, the antifungal activity described here opens possibilities for new therapeutic approaches to manage onychomycosis.

Considering the confluence of the species mentioned above, such as dermatophytes and *Candida* spp., in the present study, the data support the assertion that the synthesis of biologically derived compounds may be altered under growth conditions, whether isolated or mixed, given that the original environments harbor antagonistic or synergistic interferents. This phenomenon elucidates the variation in MIC values of the extracts when originating from mixed cultures.

The kinetics of the time-kill assay provides information on the microbicidal action dynamics of the tested compounds, an essential tool in the antimicrobial analysis of potential antifungal compounds [20]. The ability of extracts to inhibit fungal growth shows fungicidal characteristics against TM and TR strains, with maximum inhibition within 48 h.

The use of *G. mellonella* for toxicity tests has gained attention from the scientific community in recent years. Compared to traditional mammalian models, *G. mellonella* have certain advantages, such as being easy to obtain for large-scale experiments, simple and easy to handle without the need for special equipment, and being exempt from approval by research ethics committees [21]. The similarity with the innate immune response of mammals such as having cellular and humoral defenses should also be highlighted. Additionally, it is possible to establish the dosage of potential drug candidates in mammals [22].

5. Conclusions

The extracts of pure and mixed cultures of CP and dermatophytes present antifungal activity against TM and TR, which may influence the laboratory diagnosis of mixed infections. These results can explain cases of mixed fungal infections where only one of the etiologic agents grows in culture. Furthermore, isolating and identifying the compounds in extracts may allow new therapeutic approaches to control fungal infections.

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Data Availability Statement: The datasets generated during and/or analyzed during the current study are available from the corresponding author on reasonable request.

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