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Antifungal Activity of Phytochemicals against Samples of *Penicillium*

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Authors' contributions

This work was carried out in collaboration between all authors. Authors TBD and EOL did the study design, wrote the protocol and analyses of study. Authors TBD, LSP, CPM, FQSG and JPS did the processing samples while the literature searches were done by authors TBD and SBF. All authors read and approved the final manuscript.

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ABSTRACT

Aims: The incidence of fungal infections has increased over the last ten years and fungi of the genus *Penicillium* can be found in various substrates and affect immunocompromised people, hospitalized patients, many animals and plants, as well as compromise the quality of air indoors. The current situation of indiscriminate use of antibiotics and the consequent resistance of microorganisms to conventional antimicrobial therapy has been stimulating researchers to seek alternative sources of antimicrobial compounds, among them the medicinal plants. The tendency of getting phytochemicals from extracts, fractions, fixed or essential oils obtained from plant species is currently observed. In this context, the present study aims to evaluate the in vitro antifungal activity of seven phytochemicals (geraniol, carvacrol, thymol, linalool, p-cymene, terpinolene and citral) against twelve samples of *Penicillium*.
Place of Study: Laboratory tests were carried out at the Mycology Laboratory Department of Pharmaceutical Sciences, located in the Health Sciences Center (CCS) of the Federal

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University of Paraíba (UFPB).
Methodology: Firstly, screening was carried out to find the phytochemical with the best activity by determining the minimum inhibitory concentration (MIC) by the broth microdilution technique. Following, the tests were proceeded with thymol to determine of minimum fungicidal concentration (MFC).
Results: Through analysis of results, it is observed that carvacrol, thymol and citral showed the best activities of the samples of *Penicillium* studied. The MIC values were 256 mg / mL, for carvacrol and citral, and 128 mg / mL, for thymol (compound showed better results). The thymol had its MIC 90 established at 128 mg / mL, inhibiting, at this concentration, 92% of the tested samples. Analyzing compared to MIC and MFC, it was observed that thymol presented CFM values similar to CIM values for 1/3 of the samples, showing also values greater than 1024 mg / mL for only two samples. The CFM value ranged from 128 µg/ml to 1024 µg/ml.
Conclusion: The thymol is a promising new possibility among the products with antifungal activity against *Penicillium*, however if the performance is needed further studies, deeper, about their mechanism of action and toxicity, as well as in vivo tests, aiming a possible application therapy.

Keywords: Antifungal activity; phytochemicals; Penicillium; thymol.

1. INTRODUCTION

The incidence of fungal infections has increased over the last ten years [1]. Airborne fungi use the atmospheric air as a mean of dispersal [2]. Inhalation of airborne fungi spores by man can in some cases lead to the development of complications in the respiratory tract, such as asthma and rhinitis [3]. In addition, airborne fungi can cause sometimes fatal infections in immunocompromised individuals, which are called opportunistic infections [4,5].

Among airborne fungi, the genus *Penicillium* deserves mention, it is one of that has the largest number of species and it can be found in various substrates [6]. Fungal infections by *Penicillium marneffei* ranks third in Thailand in relation to opportunistic infections in patients with HIV [7].

The indiscriminate use of antibiotics by the general population has led to a complex framework of resistance of microorganisms to conventional antimicrobial therapy, what may be a relevant factor in the emergence of difficult control infectious diseases and has stimulated researchers to seek alternative sources of antimicrobial compounds, among them the medicinal plants [8].

The plants provide an important source of new biologically active compounds [9-11] containing a number of substances that can be used to treat different infectious diseases [12], for which reason they have been intensively studied to find compounds more effective and less toxic.

The term phytochemical relates to chemical compounds, non-nutritive, which naturally occur in plants and exhibit biological activity [13]. Studies involving phytochemicals are of great importance, because they facilitate the utilization of individual components, instead of a mixture like in essential oils, giving more predictability and probably less collateral effects.

Several studies point to the various activities of phytochemicals, which are: antimicrobial, antioxidant, anti-inflammatory, analgesic, cardioprotective, anti-hemorrhagic, hepatoprotective, antitussive, antitumor, immunostimulating, anticancer, antiviral, among other [14-32]. Among these, some studies attribute considerable antimicrobial activity to phytochemicals commonly found in plants belonging to the family Lamiaceae.

These findings prompted the investigation of the antifungal profile of seven phytochemicals commonly found in plants of the Lamiaceae family (geraniol, carvacrol, thymol, linalool, P-cymene, terpinolene and citral) against different samples of *Penicillium* by microbiological screening. The aim of the present work was to investigate the antifungal activity of phytochemicals, choosing among them that which showed the best antifungal profile for an after in-depth study of its fungicidal and/or fungistatic effects against samples of *Penicillium*.

2. MATERIALS AND METHODS

2.1 Phytochemicals and Synthetic Antifungal

The phytochemicals (geraniol, carvacrol, thymol, linalool, P-cymene, terpinolene and citral) and

amphotericin B (standard drug) were acquired from Sigma-Aldrich®. All them were dissolved in 2% Tween 80 (INLAB®) and up to 10% dimethyl sulfoxide – DMSO (MERCK®) in sterile distilled water to obtain 1,024 µg/mL solutions [33].

2.2 *Penicillium* Samples

For testing of antifungal activity were selected and used twelve samples of *Penicillium* previously isolated from the ambient air of a food dry industry and morphologically identified by the Mycology Laboratory of the Department of Pharmaceutical Sciences, Health Sciences Center, Federal University of Paraiba. The samples were maintained on Sabouraud Dextrose Agar - SDA (DIFCO®) at room temperature (28° to 30°C) and under refrigeration (4°C).

2.3 Inoculum

Stock inoculums of samples of *Penicillium* were prepared from 10-day cultures in SDA at 28°C to induce sporulation. Fungal colonies were covered with 5 mL of sterile saline solution (NaCl 0.85% w/v), the surface gently scraped with a sterile loop and the resultant mixture of fungal units was then transferred to a sterile tube. After heavy particles were allowed to settle for 3 to 5 min, the upper homogeneous suspension was collected and vortexed for 15 s. The turbidity of the final inoculum was standardized according to a McFarland scale 0.5 tube and adjusted to a fungal population of 106 colony former units (CFU). The confirmation of inoculum quantification was done by plating 0.01 mL of inoculum suspension in SDA. The dishes were incubated at 28°C and examined daily for the presence of fungal colonies which were counted as soon as growth became visible [34,35].

2.4 Antifungal Activity Screening

Initially, microbiological screening was performed with the phytochemicals (geraniol, carvacrol, thymol, linalool, P-cymene, terpinolene and citral) at a concentration of 1,024 µg/mL. Microbiological screening was performed based on the broth microdilution technique [33,36,37,38].

Sterile 96-U-shaped-well microplates were used and each well of the plates contained 100 µL of Sabouraud dextrose broth - SDB (DIFCO®). Then, 100 µL of the products (1,024 µg/mL) were individually added to each line of wells, so that each line of wells corresponded to a phytochemical tested. Finally, 10 µL of fungal inoculum of each strain of *Penicillium* were added to wells, so that each column corresponded to a strain. The microplates were incubated at 28°C being selected those phytochemicals who showed better inhibition profile visual growth of microorganisms after seven days incubation. Negative control (without drugs) was performed to confirm the viability of the sporangiospores. Sensitivity control for Tween 80 and DMSO was also performed.

Phytochemicals that showed better activity were subsequently selected, which were evaluated for their potential antifungal activity from a concentration of 1,024 to 4 µg/mL, in order to determine their MIC [39].

2.5 Determination of MIC and MFC

Broth microdilution bioassay was used to determine MIC of thymol, carvacrol, citral and amphotericin B against samples of *Penicillium* [40,41]. For this, sterile 96-U-shaped-well microplates and caps were used. The 96-well dishes were prepared by dispensing 100 µL of double concentrated Sabouraud dextrose broth-SDB (Difco®) into each well. A 100 µL volume of double concentrated products was placed into the first wells, to obtain a concentration of 1,024 µg/mL in them. Next, serial twofold dilutions in culture medium were prepared to obtain concentrations ranging from 1,024 to 4 µg/mL. The last well contained 100 µL of broth inoculated with fungal inoculums to confirm cell viability (viability control). Finally, 10 µL of fungal inoculum of each strain were added to wells, so that each column corresponded to one of the twelve samples. All the dishes were aseptically sealed then mixed on a plate shaker (300 rpm) for 30 seconds, incubated at 28°C and read after 5 days of incubation. The MIC values were determined by visual inspection of the growth inhibition of each well compared with that of the control (without drugs) well. Sensitivity control for Tween 80 and DMSO was also performed. The MIC was determined from three independent experiments and was defined as the lowest drug concentration that showed absence of growth or complete fungal growth inhibition (100% inhibition) compared with that of the control (without drugs) well.

Under the same conditions, it was also used the product chlorine sanitizer Tecsa Clor®- TC (SERQUIMICO LTDA., São Paulo, SP, Brazil),

composed of stabilized chlorine dioxide 5%. The TC was prepared in the same concentrations used to the phytochemicals (1,024-4 µg/mL) and furthermore in the concentration recommended by the manufacturer for obtaining the fungicide effect (50 ppm).

The MFC was determined for the phytochemical that showed stronger antifungal activity and for amphotericin B. After determining the MIC, 10 µL were subcultured from each well that showed complete inhibition (100% or an optically clear well) on SDA plates. The plates were incubated at 28°C for five days, and the MFC was the lowest concentration that showed either no growth or fewer than three colonies [42-45]. The MFC was determined from two independent experiments on different occasions.

2.6 Statistical Analysis

The results are expressed as mean ± S.E.M. Statistical analysis for the study of the effects of the phytochemical on mycelial growth and spore germination was performed to determine statistically significant differences ($P < 0.05$) employing analysis of variance (one-way ANOVA), followed by the Bonferroni post-test. For this, the implementation of statistical analysis was performed using GraphPad Prism version 5.0 for Windows, San Diego, California, USA.

3. RESULTS AND DISCUSSION

At 1,024 µg/mL, the concentration of phytochemicals used in the screening, it was found that carvacrol, citral and thymol showed the best activity on the investigated samples of *Penicillium* (Table 1), having been observed, for all three phytochemicals, growth of only one strain among those tested (Pen 9).

After initial screening, in which carvacrol, thymol and citral showed better results in inhibiting the growth of samples of *Penicillium*, we proceeded to a new screening, this time with the MIC determination of selected phytochemicals. To this end, carvacrol, thymol and citral were tested at concentrations ranging from 1024 to 4 µg/mL, with concomitant control of viability of samples, sterility of broth and interference of solvents DMSO and Tween 80 in the results.

The MIC values found were 256 µg/mL, for carvacrol and citral, and 128 µg/mL, for thymol, what triggered interest in further study of the antifungal action of thymol. These results were compatible with the findings of other authors [16,18,46,47,48].

Studies with microencapsulation of thymol and carvacrol to evaluate the potential use of these agents as antimicrobials in food packaging showed the action of thymol and carvacrol was

Table 1. Antifungal activity of phytochemicals against samples of *Penicillium*- microdilution technique

Microorganisms	Phytochemicals (1,024 µg/mL)							
	geraniol	carvacrol	thymol	linalool	p-cymene	citral	terpinolene	Strain control
Penicillium Pen 2	-	-	-	+	+	-	+	+
Penicillium Pen 3	-	-	-	+	+	-	+	+
Penicillium Pen 7	-	-	-	+	+	-	+	+
Penicillium Pen 8	+	-	-	+	+	-	+	+
Penicillium Pen 9	+	+	+	+	+	+	+	+
Penicillium Pen 10	-	-	-	+	+	-	+	+
Penicillium Pen 11	-	-	-	+	+	-	+	+
Penicillium Pen 12	-	-	-	+	+	-	+	+
Penicillium Pen 13	-	-	-	+	+	-	+	+
Penicillium LM 28	-	-	-	+	+	-	+	+
Penicillium LM 63	-	-	-	+	+	-	+	+
Penicillium LM 120	-	-	-	+	+	-	+	+

(+): Microbial growth in culture medium (-): Absence of microbial growth

evaluated against Escherichia coli, Staphylococcus aureus, Listeria innocua, Saccharomyces cerevisiae and *Aspergillus niger*, yielding MICs ranging from 125 to 250 µg/mL for thymol and 75-375 µg/mL for carvacrol [46]. Trials with various essential oils against *Botrytis cinerea, Fusarium sp*. and *Clavibacter michiganensis subsp. Michiganensis* showed that the growth of these microorganisms was completely inhibited by oils of oregano, thyme and marjoram in relatively low concentrations, 85-300 µg/mL, thymol being pointed out as the main component of oregano oil and carvacrol as a major component of oils of thyme and marjoram [47].

In a study using the disk diffusion method, thymol showed antimicrobial activity against 23 different genera of bacteria with inhibition zones ranging from 25.8 to 53.1 mm [48]. In other study designed to evaluate the inhibitory activity of thymol on the formation and maturation of biofilms of *Candida albicans,* thymol significantly interfered by reducing the formation and maturation of the biofilm [18]. In partial agreement with this study, using the methods of broth dilution, disk diffusion and microatmosfera, found in his work that thymol showed the best antifungal activity against *Aspergillus niger*, among the phytochemicals tested (thymol, carvacrol and eugenol), based on the study of MICs [16].

Given that thymol showed the best result in microbiological screening, it is justified the choice to continue the investigation of its antifungal activity. After determination of the MIC, the fungicidal effect of thymol was investigated. The MFC values of thymol varied from 128 to 1,024 µg/mL and the MFCs of this phytochemical corresponded to MIC or 2 × MIC for a half of the samples of *Penicillium* (Table 2). The Amphotericin B, standard antifungal used as control, showed MIC of 128 µg/mL, concentration in which 100% of samples were inhibited. Whereas the sanitizing control Tecsa clor®, showed MIC greater than 1024 µg/mL for 75% of samples of *Penicillium*, that were resistant to the product at tested concentrations.

Research indicate a compound with MIC ranging from 50 to 500 µg/mL as having optimal antimicrobial activity, and attribute moderate activity to compounds which have a variation of 500 to 1500 µg/mL in MIC. Thus, according to these parameters, it can be stated that thymol showed an optimal antimicrobial activity [49].

Other authors have tested the antimicrobial activity of thymol, finding satisfactory results, consistent with the findings of this study. Thymol was tested tested against strains of *E. coli, S. aureus, B. cereus* and *L. monocytogenes,* finding MIC values ranging between 300 and 500 µg/mL [50]. Previous studies reported MIC values of 62-250 µg/mL for thymol on strains of Streptococcus mutans, Staphylococcus aureus, Bacillus subtilis, Staphylococcus epidermidis and Escherichia coli [51]. Another study found thymol MICs values

Table 2. Minimal inhibitory concentration (MIC) of thymol, amphotericin B and Tecsa clor® and minimal fungicidal concentration (MFC) of thymol and amphotericin B in samples of *Penicillium sp*.

Strain *Penicillium*	Thymol (µg/mL)		Amphotericin B (µg/mL)		Tecsa clor® (µg/mL)	Control of strain	Sterility control
	MIC	MFC	MIC	MFC	MIC		
Pen 2	128	1024	128	1024	NF*	+	-
Pen 3	128	1024	128	NF*	NF*	+	-
Pen 7	128	128	128	128	512	+	-
Pen 8	128	128	128	128	NF*	+	-
Pen 9	256	NF*	128	128	NF*	+	-
Pen 10	128	128	128	128	1024	+	-
Pen 11	128	512	128	128	1024	+	-
Pen 12	128	1024	128	128	NF*	+	-
Pen 13	128	128	128	256	NF*	+	-
LM28	128	NF*	128	NF*	NF*	+	-
LM63	128	256	128	512	NF*	+	-
LM120	128	256	128	512	NF*	+	-

*Note: *NF: not found- the value is greater than the highest concentration tested, 1024µg/mL; +, fungal growth*

of 15-250 µg/mL on strains of *Staphylococcus aureus, Bacillus cereus, Listeria monocytogenes, Proteus mirabilis, Escherichia coli, Klebsiella pneumoniae* and *Pseudomonas aeruginosa* [52].

In another study that evaluated the activity of thymol nanospheres against *Staphylococcus aureus, Escherichia coli* and *Pseudomonas aeruginosa* strains and observed that free thymol showed a MIC ranging from 10 to 156 µg/mL and MBC ranging 156 - 313 µg/mL, whereas the encapsulated thymol was less effective with MIC values of 10-313 µg/mL and MBC values of 156-1250 µg/mL [53]. Researchers Studies evaluated the activity of thymol on seven species of fish pathogenic bacteria (*Aeromonas salmonicida subsp. masoucida, A. salmonicida subsp. salmonicida, A. hydrophila, Edwardsiella tarda, Vibrio vulnificus, V. parahaemolyticus* and *V. anguillarum)* and found that thymol showed MIC and MBC values ranging from 10 to 320 µg/mL [54]. While other investigation used the essential oil (EO) of *Thymus vulgaris* and its constituents thymol and p-cymene, in order to determine their antifungal activity against *Rhizopus oryzae*, this study revealed that the MIC of thymol and EO ranged 128-512 µg/mL, since the MFC ranged from 512-1024 µg/mL for the EO and 128-1024 µg/mL for thymol [55]. Studied the activity of thymol on various bacteria (*Escherichia coli, Pseudomonas aeruginosa, Salmonella typhimurium, Proteus mirabilis, Listeria monocytogenes, Bacillus cereus, Micrococcus flavus* and *Staphylococcus aureus*) and fungi (*Aspergillus flavus, Aspergillus fumigates, Aspergillus niger, Aspergillus ochraceus, Penicillium funiculosum, Penicillium ochrochloron, Trichoderma viride* and *Candida albicans*), getting through microdilution broth and MIC, MBC/MFC values and found that for bacteria MIC values ranged from 10-100 µg/mL and MBC ranged from 50 to 150 µg/mL, as for fungi MIC and MFC values ranged from 10 to 50 µg/mL [56].

Macromolecules whose functionality is related to growth, survival, virulence or cellular morphogenesis are pointed out as promising targets for new antifungal agents [57]. Thus, thymol is considerate as having a promising antifungal activity.

4. CONCLUSION

Among the tested phytochemicals, thymol showed the best results. The thymol is a promising new possibility among the products with antifungal activity against *Penicillium*, however if the performance is needed further studies, deeper, about their mechanism of action and toxicity, as well as in vivo tests, aiming a possible application therapy.

CONSENT

All the authors declare that no consent was obtained for this study.

ETHICAL APPROVAL

It is not applicable.

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

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