Short Communication

REVISTA PREVENÇÃO DE INFECÇÃO E SAÚDE (REPIS)

Atividade antifúngica *in vitro* do timol e carvacrol sobre espécies de cândida

In vitro antifungal activity of timol and carvacrol on candida species

Actividad antifúngica in vitro del timol y carvacrol sobre especies de cándida

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ABSTRACT

Aim: to evaluate the in vitro action of thymol and carvacrol against the yeasts of *Candida albicans* ATCC10231 and *Candida krusei* ATCC34135. **Method**: A laboratory study was performed to evaluate antifungal activity. The characterization of the Minimal Inhibitory Concentration (MIC) of the thymol essential oil was carried out using the technique where the microdilution is performed, in which a plate containing 96 wells is used. The determination of the Minimum Fungicidal Concentration (MFC) was performed by dripping 10 µL of each of the concentrations evaluated on Sabouraud agar plates. **Results**: The MIC of thymol and carvacrol for *C. albicans* was 40 µg/mL and for *Candida krusei* it did not present antifungal activity. While the MIC of nystatin was 0.03mg for both species with thymol and carvacrol. **Conclusion**: Thymol presented satisfactory antifungal activity against the pathogens studied, but carvacrol did not present antifungal activity.
Keywords: Phytotherapy; Lippia; Thymol.

RESUMÉN

Objetivo: evaluar la acción in vitro del timol y carvacrol frente a las levaduras de *Candida albicans* ATCC10231 y *Candida krusei* ATCC34135. **Método**: Se realizó un estudio de laboratorio para evaluar la actividad antifúngica. La caracterización de la Concentración Inhibitoria Mínima (CIM) del aceite esencial del timol fue efectuada a través de la técnica donde se realiza la microdilución, en ésta se utiliza una placa que contiene 96 cavidades. La determinación de la Concentración Fungicida Mínima (CFM) fue realizada a través de goteo de 10 µl de cada una de las concentraciones evaluadas en placas de agar Sabouraud. **Resultados**: La CIM del timol y carvacrol para *C. albicans* fue de 40 µg/mL y para *Candida krusei* no presentó actividad antifúngica. Mientras que la CIM de la nistatina fue de 0.03mg para ambas especies con timol y carvacrol. **Conclusión**: El timol presentó una actividad antifúngica satisfactoria frente a los patógenos estudiados, pero el carvacrol no presentó actividad antifúngica.
Palabras-clave: Fitoterapia; Lippia; Timol.

RESUMO

Objetivo: avaliar a ação in vitro do timol e carvacrol frente as leveduras de *Candida albicans* ATCC10231 e *Candida krusei* ATCC34135. **Método**: Realizou-se um estudo laboratorial, para avaliação de atividade antifúngica. A caracterização da Concentração Inibitória Mínima (CIM) do óleo essencial do timol foi efetuada através da técnica onde se realiza a microdiluição, nesta é utilizada uma placa que contém 96 cavidades. A determinação da Concentração Fungicida Mínima (CFM) foi realizada através de gotejamento de 10 µL de cada uma das concentrações avaliadas em placas de agar Sabouraud **Resultados**: A CIM do timol e carvacrol para *C. albicans* foi de 40µg/ mL e para *Candida krusei* não apresentou atividade antifúngica. Enquanto que a CIM da nistatina foi de 0.03mg para ambas espécies com timol e carvacrol. **Conclusão**: O timol apresentou uma atividade antifúngica satisfatória frente aos patógenos estudados, porém o carvacrol não apresentou atividade antifúngica.
Palavras-chave: Fitoterapia; Lippia; Timol.

How to cite:

Souza LTR, Oleiveira MT, Costa CC, Gusmão ICCP. In vitro antifungal activity of timol and carvacrol on candida species. Rev Pre Infec e Saúde [Internet]. 2018;4:7673. Available from: http://www.ojs.ufpi.br/index.php/nupcis/article/view/7673 DOI: https://doi.org/10.26694/repis.v4i0.7673

INTRODUCTION

The oral cavity corresponds to a large reservoir of microorganisms, being thus deeply relevant to the health and disease of the host. They can be highlighted as the main microorganisms that compromise oral health, *Streptococcus mutans, Lactobacillus, Candida albicans, Candida krusei* and others[1]. In view of these evidences, the increase in the incidence of fungal infections is more and more noticeable, with emphasis on *Candida albicans* and *Candida krusei*.

Candida is considered an opportunistic microorganism that is present in the human's microbiota, as well as in other parts of the body. It is a fungus with many types of virulence, some of them the ability to adhere to some tissues such as dental surface, the presence of hydrolytic enzymes and morphogenesis[2]. Given these favorable factors, when there is a biological imbalance favoring a fungal growth greater than 106 colony forming units, these fungal species may evolve in a favorable zone for candidiasis or stomatitis by denture, for example. However, it is important to emphasize that these microorganisms do not usually promote infectious processes in individuals without high systemic impairment[1,2].

As in any other area favoring microbial growth, the dental surface also consists of an 'ideal' site for the installation of several species of microorganisms. The accumulation of microorganism on the surface of the tooth is called a dental biofilm. In this sense, a disordered growth of fungi and /r bacteria may occur in the face of specific factors, such as resistance to antifungal drugs and refractory biofilm formation after long periods of exposure to antibiotic therapy, thus promoting some pathologies such as candidiasis oral[3,4].

In the treatment of these oral diseases, the main intention is to neutralize the local pH due to the fact that the metabolism of the microorganism leaves the medium relatively acidic. Usually, conventional antifungal agents, such as nystatin, are used. However, there are lines of research that demonstrate how effective some formulas are prepared through the extraction of substances in Brazilian native plants[5].

Thymol and carvacrol, substances obtained through the *Lippia sidoides* plant, popularly known as rosemary pepper, are then used. They are considered phenolic compounds present in some essential oils such as oregano. Thus there is a growing interest in the use of these substances, due to its antioxidant and antimicrobial properties[5].

Based on the assumption that in the dentistry one must use devices to keep the oral cavity as free of pathogenic microorganisms as possible, the topical use of thymol and carvacrol in patients with a high risk of development of pathologies related to fungus accumulation can be installed, avoiding possible damages.

In view of the above, the objective was to evaluate the antifungal activity of phytochemicals Timol and Carvacrol (Sigma-Aldrich,St. Louis, United States) in face of the *Cândida albicans* and *Cândida krusei*.

METHODS

This is an experimental research carried out at the Laboratory of Microbiology of FAINOR -

Faculdade Independente do Nordeste, state of Bahia.

For the experiment the strains of *Candida albicans* ATCC 10231 and *Cândida krusei* ATCC 34135 were obtained from Oswaldo Cruz Institute of Rio de Janeiro. For reactivation of the yeasts 5mL aliquots of each yeast in stock were added and 5mL of Saburaud broth (Sigma-Aldrich, St. Louis, USA) was incubated at 37°C to 48hs. After that, the adjustment was made to 0.5% MacFarland scale with 0.85% NaCl saline solution.

The result was a suspension that was inserted into the vortex agitator for 15 seconds and the cell density adjusted, with a spectrophotometer aiming to obtain transmittance similar to a standard solution of the McFarland 0.5 scale at wavelength 530nm. This step generated a standard suspension of fungus with approximately 1.5x108 CFU/mL colony forming units (CFU) per mL.

The microdilution technique with the use of the 96 "U" shaped base cavities in triplicate was chosen to determine the CIMA6. 100µL of the Sabouraud Broth liquid medium, 20µg/mL of the inoculum of the fungal suspension in saline was added to each well of the plate. The plate was filled respecting the numerical order and then concentrations of thymol and carvacrol ranging from 15 µg/mL to 400 µg/mL were added. The concentrations were diluted previously, starting with the first well of each plate column, at this time it was performed serial microdilution. The latter cavity was separated for growth control of the microorganisms. This material was taken to the stove for 48 h at 37 °C.

After the appropriate incubation time, 35µg/mL of resarzurin (0.01%, 10 mg diluted in 80 mL) (Sigma-Aldrich, St Louis, United States) was added a colorimetric oxy-reduction indicator. The plates were sealed with plastic film and reincubated for another 1 hour for later reading. Therefore, the last blue well (from left to right) was the one with MIC. After 48 h of incubation at 37°C, the concentration with less Thymol and Carvacrol able to prevent visible growth of the subculture was considered as CFM (Minimum Fungicidal Concentration).

Therefore, the lowest concentration of Thymol and Carvacrol capable of inhibiting the growth of the microorganism tested was established as MIC. Visually this definition was achieved by an imutation of the staining of the indicator dye.

CFM determination was performed by dripping 10µL of each of the concentrations evaluated on Sabouraud agar plates. The reading was done after incubation of the plates at 37°C for 48h. CFM concentration was considered to be no growth of subculture microorganism. The tests were produced in triplicate.

RESULTS

Table 1 shows growth inhibition results for the tests using thymol and carvacrol extracts and nystatin as antimicrobials. It is observed that the preparations presented antifungal capacity for *Candida albicans*. While for *Candida krusei*, carvacrol did not show antimicrobial activity. There was no contamination of broth or extracts tested in the negative control. Nystatin inhibited yeasts by 0.03 µg/mL (Table 1).

The observation of the results in this study made it possible to verify that the thymol extracts presented a fungiostatic and fungicidal effect on the *C. albicans* strain, whereas for *C.krusei* there was no antimicrobial activity. The evaluation of (CFM) confirmed that the concentration of 40µg/mL did not obtain microbial growth.

Table 1: MIC of the thymol and carvacrol extracts on standardized strains of *Candida albicans* and *Candida krusei*

Strain (µg / mL)	CIM (Timol) (µg / mL)	CIM(Carvacrol) (µg / mL)	Nistatina (µg / mL)
Candida albicans	40	40	0.03
Candida krusei	-	-	0.03

- No antimicrobial activity

DISCUSSION

The current increase in resistance to antimicrobials encourages research by new agents capable of preventing the growth of microorganisms and is an important strategy in alternative treatments against fungal infections. Therefore, several researchers have increased their interest in the research of extracts supplied by plants as an antimicrobial potential[7,8]. These researches have increased against microorganisms present in the oral cavity.

Candida albicans is present in approximately 30 to 50% of people's oral cavity, being the most virulent species, capable of producing phospholipases and proteases that disrupt host tissue. *Candida krusei* is also an important pathogen that contributes to the disease, the species is more resistant to therapeutic agents, increasing the number of people immunocompromised and thus the frequency of antifungal agents[9].

Antifungal therapy for Candida infections is challenging, since treatment is hindered by the eukaryotic characteristic of the cells of these microorganisms, which are similar to host cells. In addition, there are few antifungal agents available to treat infections caused by these fungi[10]. Currently, the class of azole antifungal agents that are unfortunately fungistatic rather than fungicidal are used in large scale and their prolonged use contributes to the development of resistant strains[11].

The present study showed a therapeutic possibility with the thymol for the candidiasis developed by *C. albicans* which is the most

prevalent in the oral microbiota. An interesting perspective would be the addition of the thymol extract in buccal mouthwashes potentiating its antimicrobial effects for candidiasis.

In this sense, this study investigated by in vitro tests, thymol and carvacrol activity against strains of *C. albicans* and *C.krusei*. Therefore, thymol and carvacrol were found to have excellent antifungal activity, with MIC and CFM of 40 µg/mL and nystatin MIC of 0.03 µg/mL for both strains, whereas the same phytochemicals showed no antifungal activity for *C .krusei* and nystatin presented 0.03 µg/mL (Table1).

Lima et al. (2013)[1], in their similar study, obtained a value between 256 and 512 µL/mL of carvacrol in *Candida albicans*. In another study conducted by Manohar et al. (2001)[2], the carvacrol MFC for *C.albicans* ATCC-48274 was 500 µL/mL. Concentrations of MIC and CFM were low, with a fungicidal effect.

It is understood, therefore, that the results of this research corroborate with studies carried out previously in the literature, which shows that compounds of vegetable origin to possess strong/good antifungal activity, need that their MICs are at least between 50-500µg/Ml[12].

The phytoconstituents of carvacrol and thymol inhibit cell growth by altering the functioning of the cell wall and membrane, as stated by Lee et al. (1999)[4], reducing permeability. Studies have shown that essential oil containing thymol and carvacrol as components have antimicrobial activity. Thus, Pozzart et al. (2010)[5] demonstrated an antimicrobial effect with oregano essential oil containing 58% carvacrol and a MIC of less than 800 µL/mL against *Candida*.

Thus, the route is to optimize the use of carvacrol and thymol in combination with conventional drugs. Associations have been extensively studied in in vitro methods. The " checkerboard " is a widely used technique[13,14].

Thus, in view of the results the phytoconstituents carvacrol and thymol may possibly be used in combination with conventional drugs against candidiasis. However, further research is needed to clarify the mechanism of toxicity of extracts.

This study has limitations. It was evidenced the logistic difficulty and shortage of certain materials that would expand the range of the research. These difficulties made impossible, for example, possible biofilm dental tests. This possibility would be of great significance, since the oral microbiota forms a biofilm.

CONCLUSION

After presenting the results, we conclude that the phytochemicals thymol and carvacrol presented antifungal activity quite satisfactory for *Candida albicans*, which is the main yeast present in the oral cavity, but for *Candida krusei* it was not effective. It is suggested that thymol analyzed exerts a potential for the production of constituents with antifungal activity against strains of *C. albicans*, thus, showing a therapeutic alternative for fungal infections treatments and the discovery of new drugs.

Further studies are required to verify plant toxicity and to isolate the active compounds responsible for biological activity.

However, more extensive research and pharmacological tests should be developed, taking into account the physiology of the human body as well as interference of systemic factors in the oral microbiota.

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Submitted: 2018-10-21
Accepted: 2018-11-26
Published: 2018-12-30

COLLABORATIONS

LTRS: Substantial contributions in the design or design of the work; in the collection, analysis and interpretation of data and in the writing of the article or in its critical review; COM: contribution in the collection, analysis and interpretation of the data and in the writing of the article or in its critical revision; CCC: contribution in the collection, analysis and interpretation of the data and in the writing of the article or in its critical revision and final version to be published; ICCPG: Substantial contributions in the design or drawing of the work and final version to be published.

CONFLICTS OF INTEREST

The authors declare no conflicts of interest.

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