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ANALYSIS OF THE ANTIFUNGAL ACTIVITY OF THE ESSENTIAL OIL AND ALCOHOLIC EXTRACT OF *ROSMARINUS OFFICINALIS* LINN (ROSEMARY) LEAVES ON *CANDIDA ALBICANS*

Mariana Martins da Silva
Estácio de Brasília University Center, Taguatinga Sul. DF
http://lattes.cnpq.br/4852562860695589

Erica Araújo Rego
LS University Center, Taguatinga Sul. DF
http://lattes.cnpq.br/7663564577536307

Paulo Henrique Rosa Martins
LS University Center, Taguatinga Sul. DF
http://lattes.cnpq.br/3726131848493586

Mariana Alcântara Araújo Vieira
Estácio de Brasília University Center, Taguatinga Sul. DF
http://lattes.cnpq.br/3945358975489623

Lorena Roberta de Souza Mendes Kawamura
Estácio de Brasília University Center, Taguatinga Sul. DF
http://lattes.cnpq.br/5896990005944857

Thaís Rocha Paes
http://lattes.cnpq.br/5703745422090011

Clemilda Carlos da Silva
http://lattes.cnpq.br/0588132098971245

Jéssica dos Santos Folha
Estácio de Brasília University Center,
Taguatinga Sul. DF
http://lattes.cnpq.br/1624049224269863

Abstract: *Candida* is a yeast-like fungus that is commensal in the microbiota of humans, mainly on the skin and oral, urogenital and gastrointestinal mucous membranes. The main *Candida* species are *C. albicans*, *C. glabrata*, *C. tropicallis*, *C. parapsilosis* and *C. krusei*, with *C. albicans* strains being the most frequent cause of candidemia in hospital settings. Despite the great development of allopathic medicine, its use by the poor still faces many obstacles, and for this and other reasons, there is greater use of medicinal plants in developing countries. One example of a plant with popularly accepted medicinal effects is *Rosmarinus officinalis Linn,* better known as rosemary. Rosemary is a medicinal plant widely used in cooking, but its medicinal properties have been known since antiquity, with its main uses being as a healing, antidiabetic, expectorant, anti-inflammatory and antimicrobial agent. The aim of this study was to evaluate the antifungal potential of the alcoholic extract and essential oil of rosemary against the species *C. albicans*. Two samples (alcoholic extract and essential oil) were analyzed in direct contact with *Candida albicans* colonies using two methods: agar diffusion (disk diffusion) and agar perforation (well technique), in order to identify possible antifungal activity. After incubating the plates, a halo of fungal inhibition was observed in the agar perforation methods of the essential oil plates, but no halo formation was observed in the agar diffusion method of the same plates. With regard to the alcoholic extract, none of the methods used formed an inhibitory halo. Therefore, the antifungal activity of the essential oil was observed in comparison with the positive control with a fluconazole disk.

Keywords: *Candida albicans*. *Rosmarinus officinalis*. Plant extract. Essential oil. Rosemary.

INTRODUCTION

Fungi are eukaryotic microorganisms that can be found naturally in soil, water, food and as commensals in the body cavities of living beings. These fungi surrounding living beings may or may not be capable of causing infections in humans, which range from mild and self-limiting to systemic and fatal, depending on the health status of the host and the type and species of fungus causing the infection. Among the many species of fungi known today, it is known that there are around 50 species that are considered pathological and among these 50 species is *Candida* spp (MADIGAN *et al*., 2016).

Fungal diseases are less common than bacterial infections of medical interest, but mycoses are more complex and difficult to treat and recover from. One of the factors contributing to this is the ability of fungal species to develop mechanisms of resistance to traditional antifungal drugs, such as the production of biofilms, leading to increased failure of conventional antifungal therapies (LÜLLMANN; MOHR; HEIN, 2017; NUCCI, 2021).

Over the years, there has been an increase in traditional clinical procedures using medicinal plants. Despite the great development of allopathic medicine, there are still fundamental obstacles to its use by underprivileged populations, from access to hospital care to obtaining tests and especially medicines. These reasons, together with the easy availability and long tradition passed down through generations of the use of medicinal plants, contribute to their greater use by populations in developing countries (JUNIOR; PINTO; MACIEL, 2005).

Taking into account the high incidence of resistance to antifungal drugs, the severity of infections caused by *Candida* spp and the limitations of the drugs currently used in clinical practice, it is understood that the use of rosemary may be a treatment option with fewer adverse effects, but it is necessary to carry out research to prove its antifungal action (ESTRUZANI; KOZUSNY-ANDREANI, 2022; THIEL; EHRHARDT, 2021; BAIOTTO *et al*., 2021).

Throughout history, rosemary has had various effects in different situations. The hypothesis that rosemary may have antifungal effects guided the aim of this study, which was to evaluate the antifungal potential of the alcoholic extract and essential oil of rosemary against the species *C. albicans*. Thus, producing data that can be used to develop clinical research on the subject, generating the development of herbal products with fewer adverse effects, which are easier to handle and more accessible to the population.

CANDIDA SPP AND CANDIDIASIS

Candida spp is a yeast-like fungus that forms pseudohyphae, belonging to the Ascomycetes group (a large and diverse group of fungi that includes unicellular and filamentous species) that is present in the microbiota of humans, being found mainly on the skin, oral cavity, gastrointestinal tract and urogenital system. Despite being commensal in our bodies, this yeast can behave as an opportunistic pathogen, causing anything from superficial lesions on mucous membranes to serious systemic infections in its host. These infections occur more frequently in immunocompromised patients (HIV/AIDS patients; people undergoing chemotherapy, radiotherapy and immunosuppressant treatments; transplanted and hospitalized patients, among others) and in people with dysregulated normal microbiota (COSTA *et al.,* 2009; MADIGAN *et al.,* 2016; ESTRUZANI; KOZUSNY-ANDREANI, 2022).

The species *Candida albicans*, *Candida glabrata*, *Candida tropicallis*, *Candida parapsilosis* and *Candida krusei* are the main strains isolated from opportunistic infections in

immunosuppressed patients (COSTA, 2009). Since *C. albicans* is the most prevalent species and the main cause of cutaneous and oral candidiasis, the others are often found in hospital environments causing candidemia (THIEL; EHRHARDT, 2021).

C. albicans is a polymorphic microorganism that can present itself in the form of yeast, hyphae or pseudohyphae, and can also form chlamydoconidia. The hyphae have a greater ability to adhere to and penetrate epithelial cells, and are more pathogenic than blastoconidia. However, in yeast form they are only commensal. The pathogenicity of this species depends on the host's immunity and its virulence factors, the main ones being the production of proteases and adhesins that are able to bind to proteins located in the extracellular matrix and to receptors that bind to complement, and they also have the ability to develop biofilm (SOARES *et al.,* 2018).

Vulvovaginal candidiasis stands out among the infections that affect women who seek medical treatment. It is an inflammatory process that presents abnormal secretions from a secondary infection, most of which is caused by the *C. albicans* species, which is responsible for the vast majority of cases. Studies show that at least once in their lives, around 75% of women will experience vulvovaginal candidiasis (SOARES *et al.,* 2018).

TREATMENT

The treatment currently available for candidiasis is classified into three main classes: ergosterol synthesis inhibitors (azoles and the allylamines and benzylamines), echinocandins and polyenes. These are the main choices in the medical clinic as a form of treatment, which are generally divided between therapies for systemic infections and for mycocutaneous infections (BLACK; BLACK, 2021; KATZUNG; VANDERAH, 2023; GOLAN, 2014).

One of the representatives of the pharmacological group of ergosterol synthesis inhibitors are the azoles, synthetic compounds classified as imidazole, when they contain two nitrogen atoms inside the azole ring, and triazole, when they contain three nitrogen atoms. The drugs in this class have a broad spectrum of action, proving useful against *Blastomyces dermatitidis*, *Cryptococcus neoformans*, *Histoplasma* capsulatum, as well as species of *Coccidioides and Paracoccidioides brasiliensis* (KATZUNG; VANDERAH, 2023).

Ergosterol is a component of the plasma membrane of fungal cells and its function is to regulate the integrity of the membrane, so by acting on a cytochrome P450 enzyme called 14α-sterol demethylase, azoles cause the plasma membrane of fungi to rupture (NUCCI, 2021; GOLAN, 2014).

Another class of drugs belonging to the group of ergosterol synthesis inhibitors are allylamines and benzylamines, which act on the cytochrome P450 enzyme squalene epoxidase, affecting the early conversion of lanosterol into ergosterol, thus causing the accumulation of squalene sterol, which is toxic to fungal cells, characterizing the fungistatic action of this class (GOLAN, 2014).

This group of drugs has a broad spectrum of action and is generally used to treat onychomycosis. They are more effective than azoles against topical dermatophytes, but less effective in treating skin infections caused by *Candida* spp. Even though they are considered less toxic than azoles, they often cause adverse effects such as gastrointestinal discomfort, headache and hepatotoxicity. The main representative of this class, terbinafine, has a great affinity for fatty tissues, as well as skin and nails, which causes this drug to accumulate in these places and explains its long half-life of 200 to 400 hours. Therefore, these drugs have important contraindications and cannot be administered to patients with kidney and liver

disorders (KATZUNG; VANDERAH, 2023; WHALEN; FINKELL; PANAVELIL, 2016).

Echinocandins are another group of drugs used in the treatment of candidiasis, but unlike the others mentioned above, they have a narrow spectrum of action and are used against *Candida* strains resistant to azoles, which are considered the treatment of first choice in cases of invasive *Candida* and *Aspergillus* infections, but show an unsatisfactory effect for other fungi. The drugs in this class are made up of amphiphilic cyclic hexapeptides linked to a long fatty acid molecule and act by inhibiting the synthesis of β(1-3)-glycan, which results in the breakdown of the fungal cell wall. As this class of drugs acts on the cell wall, they cause fewer adverse effects. Oral bioavailability is low, so treatment is carried out intravenously in daily doses (KATZUNG; VANDERAH, 2023; GOLAN, 2014; NUCCI, 2021; WHALEN; FINKELL; PANAVELIL, 2016).

Another class of drugs available for the treatment of *Candida* spp are polyenes, which act by binding to ergosterol present in the plasma membrane of fungi, causing pores to open and resulting in fungal death. This class is of bacterial origin and is produced by the bacterium *Streptomyces nodosus* during its fermentation process (LÜLLMANN; MOHR; HEIN, 2017; NUCCI, 2021).

This group of drugs includes amphotericin B and nystatin and were the first to be used in clinical practice. For a long time, they were the first choice for most invasive fungal infections. They are still widely used today because they have a broad spectrum of action, but they are not well absorbed orally, which is why they are administered orally in cases of intestinal *Candida*. The clinical use of amphotericin B, the main drug in this class, although broad, needs to be limited because its use is associated with many adverse reactions at a systemic and hematological level and serious renal effects. As for nystatin, its clinical use is topical for cutaneous, oral and vaginal candidiasis, and it is administered orally as a suspension for oropharyngeal candidiasis, as it does not have sufficient gastrointestinal absorption for systemic action. Both drugs are capable of causing fungal resistance, but with a low incidence (LÜLLMANN; MOHR; HEIN, 2017; NUCCI, 2021; GOLAN, 2014).

MEDICINAL PLANTS AND THEIR CHARACTERISTICS

Currently, most medicinal plants are sold in pharmacies and health food stores, where you can find plant preparations with an industrial label. To be considered medicinal, a plant must have chemical substances with therapeutic properties, i.e. compounds that have an effect on the human or animal organism and can be used to treat or prevent diseases or health conditions. These substances can be present in different parts of the plant such as leaves, roots, flowers, fruit or seeds (THIEL; EHRHARDT, 2021).

In addition, it is important that the plant is safe for human or animal use and that it has no significant side or toxic effects. Scientific studies must therefore be carried out to assess the plant's safety and efficacy as a medicine. It is important to emphasize that the use of medicinal plants must have the attention and guidance of a qualified health professional who can assess the indication, dose and form of use appropriate for each patient. Inappropriate use of medicinal plants can lead to unwanted side effects and harm the patient's health (THIEL; EHRHARDT, 2021).

Rosmarinus officinalis is a medicinal plant native to the Mediterranean region and widely cultivated throughout the world. Famous for being an aromatic herb widely used in cooking, it has medicinal properties known since ancient times (SOUSA *et al.*, 2019). In South America and Europe, rosemary tea is popularly used for various therapeutic purposes, the most fre-

quent uses being as a healing, antidiabetic, expectorant, anti-inflammatory and antimicrobial agent (AMARAL *et al.,* 2021).

Scientific studies have shown that rosemary has antioxidant, anti-inflammatory, antimicrobial and antifungal properties, as well as positive effects on the nervous system. Rosemary can be used in the form of tea, tincture, essential oil or dry extract in capsules. It is generally indicated for treating headaches, digestive problems, muscle pain, as well as having healing and anti-inflammatory properties, and can also help treat skin diseases. Rosemary is also known for its positive effects on memory and concentration and is widely used in aromatherapy to improve cognitive performance and mental alertness (SOUSA *et al*., 2019).

Like tea, essential oils are also widely used in folk medicine and can be extracted from the leaves to the roots of the plant of interest. Analyzing the composition of essential oils reveals the presence of terpenes and phenylpropanoids. The main constituents of the essential oil extracted from rosemary are α-pinene, camphor, verbenone, 1,8-cineole, borneol and piperitone, and they have antioxidant, antimicrobial, anti-inflammatory and anticarcinogenic activity, which has been demonstrated in several scientific studies (BAIOTTO *et al.,* 2021; AMARAL *et al*., 2021; CLEFF *et al.,* 2012).

METHODOLOGY

PLANT MATERIAL

The plant material was purchased from a local supermarket in packages of the brand Só Pureza Comercial De Alimentos Ltda (CNPJ: 05.834.853/0001-42, lot 060) containing packets of 10 grams of dried *R. officinalis* (rosemary) leaves.

ALCOHOLIC EXTRACT

The alcoholic extract of rosemary was prepared in the Pharmacognosy laboratory of the Faculty of Health Sciences at the University of Brasilia (UnB), using 50 grams of rosemary to 500 ml of ethyl acetate (ethanol). The rosemary and ethyl acetate were placed in an Erlenmeyer flask, which was then extracted using ultrasound for 2 hours at 45°C. The sample was filtered and concentrated in a rotaevaporator at 45°C, thus obtaining the dry alcoholic extract. The sample was removed from the volumetric flask with the addition of methanol and left in a laminar flow cabinet until the final solvent had completely dried.

After drying, 3 grams of the sample were weighed and separated and resuspended in 3 ml of 100% ethanol to obtain the final alcoholic extract.

ESSENTIAL OIL

The essential oil used in the study was from the company Natz CNPJ 54.603.618/0001-75 at 100% pure concentration.

ANTIFUNGAL ANALYSIS

For the antifungal analysis (Figure 1), the *C. albicans* ATCC 10231 strain was used. Before carrying out the experiments, the *C. albicans* sample was sown in solid Sabouraud (SAB) medium (10g/L peptone, D-Glucose 40g/L, Agar 12g/L) in a bacteriological oven at 30° for around 2 to 3 days to obtain the colonies, and then the fungal count was carried out in a Neubauer chamber and the 10^4 and 10^5 titration adjustment used in the study. Sterile 0.9% saline solution was used for the fungal cell count, dilution and titration adjustment.

Fungal solutions of *C. albicans* were prepared at concentrations of 10^4 and 10^5 in sterile test tubes. The solutions were inoculated onto plates containing solid SAB using a sterile swab. Two plates were titrated at 10^4 (one plate for the alcoholic extract and

one plate for the essential oil) and two plates were titrated at 10^5 (one plate for the alcoholic extract and one plate for the essential oil).

Two samples determined as A (Alcoholic extract), B (Essential oil) were analyzed, all in technical duplicate. Two techniques were used for the analysis: agar diffusion (disk diffusion) and agar perforation (well technique). After inoculation, two wells were made in each plate using a sterile tip and 50µl of each extract were pipetted in. The discs were made using filter paper and sterilized in an autoclave. For preparation, they were moistened with 25 µl of each extract and placed on the medium. Immediately after preparation, all the plates were placed in an oven at 30°C for 48 hours.

The alcoholic extract and essential oil were used in the well technique at a concentration of 50µg/µl and in the agar diffusion technique at 25µg/µl. When preparing the disc, the amount of extract/oil used at a concentration of 50µg/µl led to the disc becoming waterlogged, so the concentration of 25µg/µl was standardized.

CONTROLS

Fungal solutions of *C. albicans* were prepared at concentrations of 10^4 and 10^5 in sterile test tubes. The suspensions were inoculated onto plates containing solid SAB using a sterile swab. Two plates were titrated with 10^4 (one plate for ethanol and one plate for sterile distilled water) and the other two were titrated with 10^5 (one plate for ethanol and one plate for sterile distilled water).

A negative control was 100% ethanol at a concentration and volume of 50µg/µl for the well technique and 25µg/µl for the agar diffusion technique. A 25mcg fluconazole disk was used as the positive control. Both tests were carried out in duplicate. Ethanol was used as a negative control for the alcoholic extract and the essential oil, since ethanol is used as a solvent to produce the volatile oils (essential oils) and the alcoholic extract.

RESULTS AND DISCUSSION

After the incubation period in the oven for 48 hours at 30°C, it was possible to observe the formation of a small halo of inhibition in the agar perforation technique on the plates containing the essential oil sample. The halo was observed on both plates with inoculums of 10^4 measuring approximately 18 mm and 10^5 measuring approximately 14 mm (table 1 and figure 2). The studies by CASTRO and LIMA (2011) corroborate the results of this research, as they observed the sensitivity of species of the *Candida* genus tested with rosemary and sassafras essential oil.

In the disk diffusion technique, no inhibition halo was formed (Figure 3). One possible cause of the negative result in the agar diffusion analysis of the essential oil is the concentration used to produce the disks, which is 50% lower than that used in the well technique, causing a change in the expected final effect. This leads to the hypothesis that the essential oil only has an inhibitory effect on *C. albicans* when used in 100% pure concentration.

The difference in the size of the inhibition halos between the concentrations can be explained by the difference in the amount of fungus sown, since the concentration of 10^4 has a 10 times smaller amount of *C. albicans* when compared to the concentration of 10^5 . This therefore facilitates the antifungal action of rosemary, as fungal dissemination depends on the virulence of the specimen and the amount inoculated.

When analyzing the plates containing the alcoholic extract, no satisfactory results were observed, i.e. no inhibition halos were formed in any of the techniques (Figure 4). The resistance of the *C. albicans* strain to the alcoholic extract of rosemary can be explained by the physical-chemical characteristics and the method of preparation of the extract, which can significantly interfere with the results.

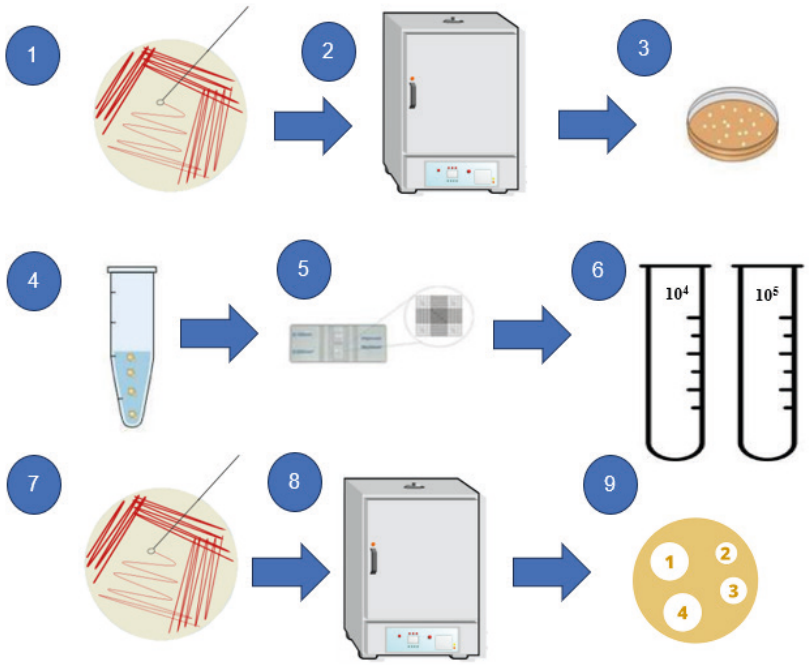

Figure 1- Representative diagram of the preparation of the *C.albicans* ATCC 10231 strain for the antifungal analysis experiments with rosemary alcohol extract and essential oil.

1) Sowing the *C. albicans* ATCC 10231 strain **2)** Fungal growth on plates of solid SAB medium for 2 to 3 days in an oven at 30°C **3)** Obtaining the isolated colonies of *C. albicans* **4)** Preparing the *C. albicans* sample in sterile 0.9% saline solution 5) Counting the cells in a Neubauer chamber and diluting for the appropriate cell titrations **6)** Preparing the fungal solutions of *C. albicans* at concentrations of 10 and 10 in sterile test tubes 7) Sowing the fungal solutions of *C. albicans* at concentrations of 10 and 10 in sterile test tubes *albicans* at concentrations of 10^4 and 10^5 in sterile test tubes **7)** Sowing *of C. albicans* at concentrations of 10^4 and 10 ,^{5} with the aid of a sterile swab, on plates with solid SAB and application of the alcoholic extract and essential oil using the well and disk diffusion techniques **8)** Growth of the tested samples for 2 to 3 days in an oven at 30°C **9)** Analysis of the antifungal potential by the presence/absence of an inhibition halo.

ARANTES et al. (2020), using the microdilution plate methodology, observed an inhibitory effect of rosemary hydroalcoholic extract on strains *of C. albicans* ATCC 10231. The divergence between the results of the alcoholic extract in this study and those of ARANTES et al. (2020) can be explained by the differences in the methods used, both in handling the extracts and in carrying out the tests. The experiment carried out by Arantes et al. used fresh rosemary leaves macerated for 48 hours, in contrast to the present study, which used rosemary alcohol extract prepared using an ultrasound process for 2 hours and subsequent drying and concentration by rotoevaporation.

In a study using an aqueous extract of the stems and leaves of *R. officinalis*, THIEL and EHRHARDT (2021) observed the inhibitory activity of rosemary in natura on strains *of Candida spp*, thus demonstrating the plant's antifungal action and providing a theoretical basis for further research into the use of the aqueous extract in the treatment of fungal infections. The study used a different methodology, where samples of fresh rosemary leaves and stems were handled and another type of solvent was used. What may have interfered in this study was the use of dried leaves, with a possible loss in the components needed for a positive response in the two methods used.

Analysis of the positive control using a 25 mcg fluconazole disk showed a halo measuring around 30 mm (Table 2 and Figure 5). No halo of inhibition was observed on the negative control plates using ethanol (Table 2 and Figure 6).

	Zone of inhibition - Halo (mm)	
	Alcoholic extract	Essential oil
10^4	-	18
10^5	-	14

Table 1 - Inhibition of the *C. albicans* strain by the well technique.

Control	Positive	Negative
10^4	30 mm	-
10^5	30 mm	-

Table 2 - Positive and negative controls used in the tests.

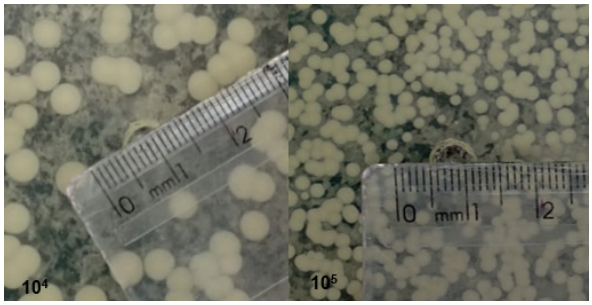

Figure 2 - Antifungal activity of rosemary essential oil in well techniques

Source: Own authorship, 2023.

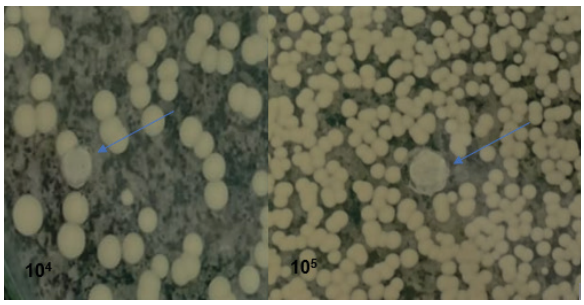

Figure 3 - Absence of antifungal activity of rosemary essential oil in the agar diffusion technique (disk diffusion).

Source: Own authorship, 2023.

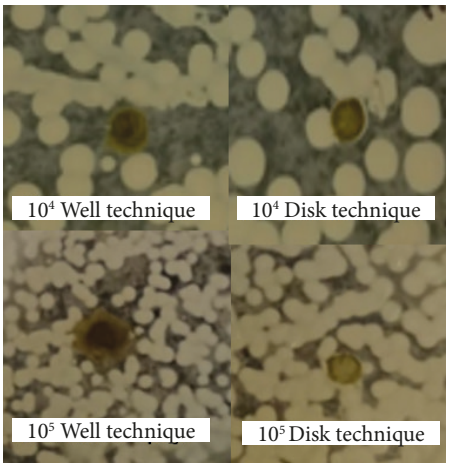

Figure 4 - Absence of antifungal activity of the rosemary alcoholic extract in the agar diffusion (disk diffusion) and agar perforation (well technique) techniques.

Source: Own authorship, 2023.

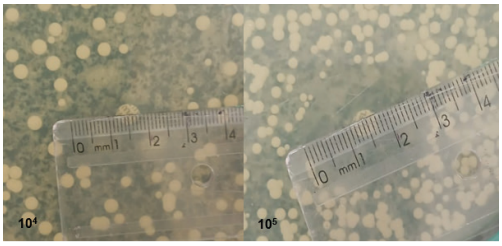

Figure 5 - Positive control with fluconazole disk

Source: Own authorship, 2023.

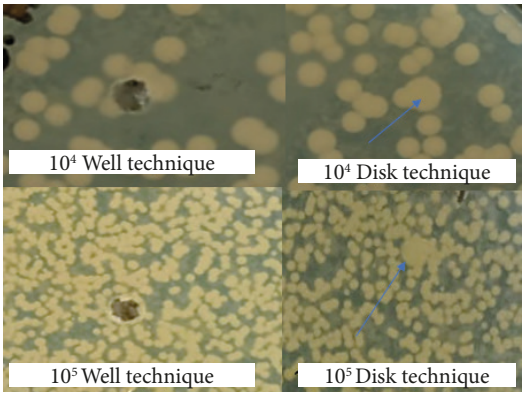

Figure 6 - Negative control with ethanol in the agar diffusion (disk diffusion) and agar perforation (well technique) techniques.

Source: Own authorship, 2023.

Rosemary extracts contain carnosic acid and rosmarinic acid, carnasol, α-pinene, bornyl acetate, 1,8-cineol, camphor, among others, which are related to the plant's therapeutic activities (AMARAL *et al.*, 2021).

It has been observed in other studies that the action of the essential oil in combating microorganisms is more effective against bacteria, especially gram-positive bacteria, because gram-negative bacteria have a lipid bilayer made up of lipopolysaccharides, endotoxins, porins and lipoproteins, which makes it difficult for the essential oil to break down these bacteria. It is difficult to attribute the inhibitory effect of the essential oil to its components that have a higher concentration, such as 1,8-cineole and α-pinene in isolation, so it is understood that its inhibitory activity is the result of a synergistic effect of the total components (SOUSA *et al.,* 2019).

Studies claim that the antimicrobial action of essential oils may be related to their fat-soluble composition, which would cause changes in the permeability of the cell membrane and, consequently, the cell death of pathogenic microorganisms (BAIOTTO *et al.,* 2021).

When analyzing the theoretical framework available for this study, there was a theoretical gap in experiments using solid culture media and alcoholic extracts, given that most of the articles referenced used methodologies in liquid media.

A number of health problems are increasingly recurring in our daily lives, which is why there has been a large increase in the indiscriminate use of antimicrobials and antifungals without a doctor's prescription, a fact which can lead to the possible resistance of various microorganisms to the drugs used to treat these diseases. Considering that phytotherapy has been developing for many years, various plant species that have had their therapeutic properties positively proven can be an aid alongside allopathic medicines in the fight against infections (THIEL; EHRHARDT, 2021).

Various studies investigating the antifungal action of rosemary derivatives, whether extract or essential oil, have presented divergent results in terms of their efficacy, as the methods used varied. Some studies using the maceration method to obtain the extract have had satisfactory results in terms of inhibiting *C. albicans*, especially when a longer extraction time is used, obtaining a significant amount of secondary metabolites and consequently increasing their inhibitory effect (ESTRUZANI; KOZUSNY-ANDREANI, 2022).

LIMITATIONS AND PROSPECTS

The preparation of the aqueous extract was started, however, due to the methodological characteristic of preparing this extract taking longer to produce, it was not possible to test it on the *C. albicans* strain studied.

Future research is planned to determine the minimum inhibitory concentration of the extracts used, as well as testing the aqueous extract of rosemary produced in order to confirm the plant's action in combating *C. albicans*. The analysis of the alcoholic and aqueous extracts and essential oil of rosemary on other types of microorganisms, and a larger and more representative number of strains, should be tested in future studies. In addition, it would be interesting to identify and separate the plant's constituents by means of phytochemical analysis and chromatography, and to test the antifungal action of each constituent.

CONCLUSION

After carrying out the experiment, it was possible to verify the ineffectiveness of the concentration used for the alcoholic extract of rosemary in the two methods used in direct contact with the *C. albicans* strain. On the other hand, the essential oil sample showed a satisfactory result with the formation of a small halo of inhibition in the agar perforation method, with a negative result in the disk diffusion method. Therefore, the antifungal activity of the essential oil was observed in comparison with the positive control with fluconazole disk.

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