Research, Society and Development, v. 10, n. 14, e220101421758, 2021
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Prospecting of anti-*Candida* bioactive in *Origanum vulgare* L. artisanal essential oil

Prospecção de bioativos anti-*Candida* no óleo essencial artesanal de *Origanum vulgare* L.

Prospección de bioactivos anti-*Candida* en el aceite esencial artesanal de *Origanum vulgare* L.

Received: 10/14/2021 | Reviewed: 10/20/2021 | Accept: 10/28/2021| Published: 10/31/2021

André Ricardo Ferreira da Silva Rocha
ORCID: https://orcid.org/0000-0002-0422-4453
Universidade Federal de Viçosa, Brazil
E- mail: andrercrd06@gmail.com
João Victor da Costa Santos
ORCID: https://orcid.org/0000-0002-2240-3498
Universidade Federal de Viçosa, Brazil
E- mail: victorbarrosov@gmail.com
Rodrigo Henrique Moura
ORCID: https://orcid.org/0000-0002-7944-3896
Universidade Federal da Integração Latino Americana, Brazil
E-mail: rodrigoklauss21@gmail.com
Eliana Campelo Lago
ORCID: https://orcid.org/0000-0001-6766-8492
Universidade Estadual do Maranhão, Brazil
E- mail: anaileogal@gmail.com
Francisca Lúcia de Lima
ORCID: https://orcid.org/0000-0003-1664-4903
Universidade Estadual do Piauí, Brazil
E- mail: karnauba@gmail.com

Abstract
Candida albicans is a yeast belonging to the normal microbiota of the human body, considered the most pathogenic species of the genus. It is the main microorganism related to candidiasis. Essential oils of *Origanum vulgare* have phenolic compounds, such as thymol and carvacrol, which have an effective antimycobacterial character at certain concentrations, but little is known about its biological activity in artisanal preparation. Thus, we evaluated the resistance of standard strains of opportunistic yeast *C. albicans* against in vitro antifungal activity of artisanal extract of *O. vulgare*. An inoculum of the challenger was subjected to different concentrations of the fungicidal agent in solidified Muller Hinton and broth followed by incubation at 35°C. The oil was prepared in a manner similar to a possible homemade procedure, and was further sterilized to ensure homogeneity of the indicator. The readings were performed in two days every 24 hours so that there were different possible moments of growth. The tests, which occurred in triplicate, showed that, under experimental conditions, the yeast was resistant to the essential oil compounds at all observed concentrations. Morphological variation was observed in both colonies and yeast cells. Based on analyzes, the artisanal essential oil is incorporated as a promising candidate for the development of antimycotics for clinical use, although in vivo tests are required.
Keywords: Fungicide; Plant extracts; Secondary compounds; Yeast.

Resumo
Candida albicans é uma levedura pertencente a microbiota normal do corpo humano, considerada, sendo a espécie de maior potencial patogênico no gênero. É o principal microrganismo relacionado a candidíase. Óleos essenciais de *Origanum vulgare* possuem compostos fenólicos, como timol e carvacrol, que extraídos possuem carácter antimicobacteriano efetivo em certas concentrações, mas pouco se sabe sobre sua atividade biológica em preparo artesanal. Portanto, neste trabalho avaliamos a resistência de linhagens padrão da levedura oportunista *C. albicans* frente atividade antifúngica *in vitro* do óleo essencial artesanal de *O. vulgare.* Um inóculo do desafiante foi submetido a diferentes concentrações do agente fungicida, em meio *Muller Hinton* solidificado e em caldo, seguido de incubado à 35ºC. O óleo foi preparado de maneira semelhante a um possível procedimento caseiro, sendo ainda esterilizado para garantir a homogeneidade do indicador. As leituras foram realizadas em dois dias à cada 24h para que houvesse diferentes momentos possíveis de crescimento. Os testes, que ocorreram em triplicata, demonstraram que, nas condições de experimentação, a levedura se mostrou resistente aos compostos do óleo essencial em todas as concentrações observadas. Variação morfológica foi verificada tanto nas colônias, quanto nas células das leveduras. Perante análises, incorpora-se o óleo essencial artesanal como um candidato promissor para o desenvolvimento de antimicóticos de uso clínico, ainda sendo necessários testes *in vivo*.
Palavras–chave: Fungicida; Extratos vegetais; Compostos secundários; Leveduras.

Resumen

Candida albicans es una levadura perteneciente a la microbiota normal del cuerpo humano, considerada la especie con mayor potencial patógeno del género. Es el principal microorganismo relacionado con la candidiasis. Los aceites esenciales de *Origanum vulgare* tienen compuestos fenólicos, como el timol y el carvacrol, que extraídos tienen un carácter antimicobacteriano eficaz en determinadas concentraciones, pero se sabe poco sobre su actividad biológica en la preparación artesanal. Por ello, en este trabajo evaluamos la resistencia de cepas estándar de levadura oportunista *C. albicans* frente a la actividad antifúngica in vitro del aceite esencial artesanal de *O. vulgare*. Se sometió un inóculo del desafiador a diferentes concentraciones del agente fungicida, en medio Muller Hinton solidificado y en caldo, seguido de incubación a 35ºC. El aceite se preparó de forma similar a un posible procedimiento casero, siendo aún esterilizado para garantizar la homogeneidad del indicador. Las lecturas se tomaron dos días cada 24 horas para que hubiera diferentes tiempos posibles de crecimiento. Las pruebas, que se realizaron por triplicado, demostraron que, en las condiciones experimentales, la levadura demostró ser resistente a los compuestos de aceites esenciales en todas las concentraciones observadas. Se verificó variación morfológica tanto en colonias como en células de levadura. A la vista de los análisis, el aceite esencial artesanal se incorpora como un candidato prometedor para el desarrollo de antimicóticos para uso clínico, que aún requieren pruebas in vivo.

Palabras clave: Fungicida; Extractos vegetales; Compuestos secundarios; Levaduras.

1. Introduction

Candidiasis is an infection often caused by the overgrowth of the commensal yeast *Candida albicans* in immunosuppressed individuals (Tortora et al., 2016). In patients receiving prolonged antibiotic therapy, the bacterial microbiota may decrease in the mucous membranes in the genitourinary tract and mouth, making *Candida* sp. the predominant microorganism in the local (Black, 2002; Tortora et al., 2016). *C. albicans* is considered, among the genus, the specie of greatest pathogenic potential.

According to Firmino et al. (2019) and Yaya et al. (2011), substances derived from plant products retain active principles with antifungal properties. Essential or volatile oils, extracted from several species of brazilian plants (Bizzo et al., 2009), have this effective antimycobacterial character (Araújo Ribeiro etal., 2020; Sartorato et al., 2004; Valeriano et al., 2004). The use of these oils is varied and can be used as analgesics, sedatives, antiseptics, diuretics, expectorants, repellents (Novelino et al., 2007) according to present compounds.

Origanum vulgare L. (orégano), is an aromatic herb belonging from Lamiaceae family (Souza & Lorenzi, 2008) that has phenolic compounds in its composition, such as carvacrol, thymol and terpin-4-ol, the last two in large quantities, 38,0 and 33.3% respectively; in addition to other 19 in a lower percentage as α-terpinol: 4.25%, trans-caryophillene: 2.66% and γ-terpine: 1.99% (Sartoratto et al., 2004). Separate, compounds such as thymol and carvacrol have microbial and fungicidal power (Cleff et al., 2008).

The efficacy of industrially synthesized *Origanum vulgare* oils has been discussed in different studies. Bastos Oyarzabal et al. (2011) in his study about the application of *O. vulgare* in the treatment of bovine mastitis, concluded that the compound had a fungicidal effect on some *Candida* sp. strains. Silva et al. (2008), in a study on the prevention and treatment of pathologies, evaluated the antimicrobial activity of dye and infusion prepared with *O. vulgare* on *Candida* sp. strains, and concluded that in test conditions the extract had no significant inhibitory activity.

In addition, essential oils can be extracted in various ways, however, scientific studies involving such techniques and their effectiveness in relation to industrial oil are deficient. In literature, there is a great deal of information on the use of essential oils against opportunistic yeast *C. albicans*. However, given the fungicidal importance of *Origanum vulgare* already described in the literature, it is essential to know the anti-*Candida* of artisanal oil. Thus, we evaluated the sensitivity of yeast strains *Candida albicans* (ATCC 10231) against the *in vitro* antifungal activity of *Origanum vulgare* artisanal essential oil.

2. Material and Methods

2.1 Test culture preparation

The sample pattern of *Candida albicans* (ATCC 10231), assigned by Oswaldo Cruz Foundation, was subjected to a regeneration process, which was inoculated in different culture media, in order to ensure the purity and viability of the microorganism.

A quantity of 100 µl of the test microorganism was inoculated in sterile Tryptona Soy Broth (TSB) containing 0.5g of yeast extract, with the help of micropipettor in unidirectional flow cabin. Afterwards, in a greenhouse type B.O.D. (Biologic Oxygen Demand) for incubation at 35°C for 40h. After the growth time, an aliquot of 100µL of yeast was submitted to 5mL of BBL ™ (Brucella Broth) and incubated in a B.O.D. oven at 35°C for 24h. In a third rush, 100µl of the concentration was again inoculated into sterile BBL™ and incubated at 35°C for 24h. Method for determination of Gram was performed to confirm the purity and viability of strain used in the work.

A total of 100 µl of yeast was picked in duplicate in the middle Tryptone Soy Agar (TSA) containing 1.0g of yeast extract, followed by incubating the plates in an incubator at 35°C for 72h. After growth, it has been inoculated edge of the colony fragments in 5.0ml MHB (Muller Hinton Broth) in triplicate and incubated for 24h in an environmental chamber at 35°C. This inoculum was used as the final indicator in the work at 1.0 on McFarland scale.

2.2 Obtaining plant material and preparation of artisanal essential oil (AEO)

It was used for the production of the artisanal essential oil dehydrated material of *Origanum vulgare* (popular oregano) in commercial form acquired in supermarket in the northern region of the city of Teresina in Piauí (Brazil). The extraction of essential oil followed the methodology of artisanal productions, which are adopted by the general population.

A 30g quantity of the dried material was rehydrated with 250ml of distilled water in Erlenmeyer so that the material was completely covered by the water, then sealed with cotton plugs and vortexed. The mixture was kept for 30 min in contact with the leaves, without replacing the lost volume. Stored for 24h. The solution was filtered through a fine sieve so that all solid residues were removed, and then 10 mL were distributed into sterile tubes, which remained at rest for two weeks. It was then stored in a dark glass container and stored in a dark environment.

2.3 Anti-Candida test

For susceptibility testing and Minimum Inhibitory Concentration (MIC) of yeast to artisanal essential oil, 10 test tubes containing 5.0 mL of MHB were each inoculated into the unidirectional flow cabin in a proportion of 100µl of inoculum per tube. Subsequently, the resulting contents were submitted to different concentrations, in triplicate, of *O. vulgare* oil: 2.5 µL, 5.0 µL, 10 µL, 25 µL, 50 µL 100 µL, 250 µL, 500 µL, 750 µL, 1000 µL. And, incubated in stove BOD at 35°C for 24 h.

A quantity of 100 µl of the resulting contents of each tube was inoculated with micropipettor onto MHA (Muller Hinton Agar) plates sterile to obtain the CFU/mL (Colony Forming Unit) of each concentration under test. The plates were incubated in a B.O.D. oven at 35°C and read at 24 and 48 h. As a negative control, the above-mentioned culture medium was used in different extract concentrations with no yeast. The positive control was verified with the presence of yeast *Candida albicans*, in sterile culture medium, without extract, in order to ensure the viability of the microorganism.

2.4 Determination of Total Phenolic Content

Two ml of the sample was centrifuged at 8,000 rpm for 5 min; then, 1.0 ml of the supernatant was diluted in ultrapure water. The content of total phenolic compounds in the artisan oil was determined in the supernatant resulting from centrifugation, according to Goyal, et al. (2021). For this, in microplates, 30 µL of the sample, 150 µL of 10% Folin-Ciocalteu

phenol reagent (1:10, v/v) were added to each well, and the mixture was kept at rest for 5 minutes in dark environment, followed by addition of 120 µL of sodium carbonate solution (Na_2CO_3, 7.5%) and standing for 30 min in dark environment. The absorbance of the resulting solution was measured at 765 nm in a microplate reader. As a control, distilled water was used. A standard curve was prepared using gallic acid to calculate the phenolic content, which was expressed as µg of gallic acid equivalents per mL of sample (µg EAG/mL).

2.5 Determination of Total Flavonoid Content

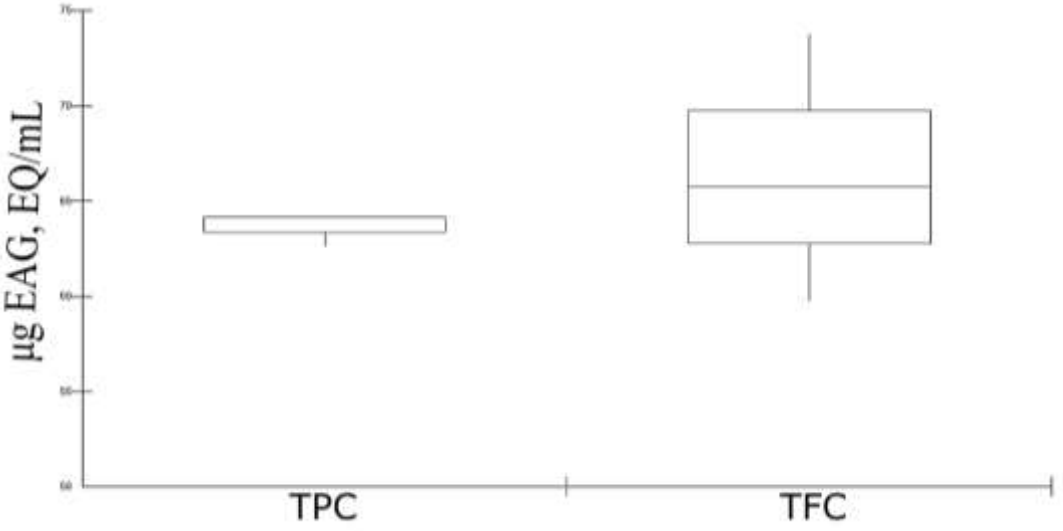
Flavonoid content was determined according to Goyal et al. (2021), by the colorimetric aluminum chloride test. 160 µL of distilled water, 40 µL of artisanal oil sample and 15 µL of sodium nitrite ($NaNO_2$, 5%) were added to a 1 mL Eppendorf tube, the solution was kept at rest for 5 min; soon after, 24 µL of aluminum chloride 6-hydrate ($AlCl_3$.6 H2O, 10%) was added and the mixture was incubated for 6 min. Then, 80 µL of sodium hydroxide (NaOH, 1M), 80 µL of distilled water were added and homogenized. From this solution, 250 µL were transferred to microplate. The absorbance of the supernatant was measured at 510 nm. The control was prepared by replacing the sample with distilled water. Flavonoid content was calculated from the calibration curve prepared with quercentin, and the results were expressed in µg of quercentin equivalents per mL (µg EQ/mL).

3. Results and Discussion

The tests demonstrated that, under the experimental conditions, the opportunistic yeast *Candida albicans* was resistant to antifungal compounds of essential oil extracted in all the 10 concentrations verified, however, this extract interferes significantly in the micro and macromorphological pathogen structure. Gram staining confirmed the purity and viability of C. *albicans* strain used for susceptibility / resistance testing. We emphasize that obtaining the essential oil for this study follows the artisanal production protocol, which directly infers the contents of compounds and bioactive potential, normally attributed to *Origanum vulgare* oil in the literature (Barbosa et al., 2020; Simirgiotis et al., 2020).

The analysis of determination of phenolic compounds (TPC) and total flavonoids (TFC) showed that although the artisanal oil production technique differs from the steam technique, these bioactive compounds have high contents in the final extraction product, with 63.36 ± 0 0.7 µg EAG/ml of TPC and 62.73 ± 3.0 µg EQ/ml of TFC (Figure 1). While the essential oil obtained by steam mainly contains phenolic monoterpenes, such as thymol and carvacrol (Brondani et al., 2018), the artisan oil is predominantly rich in phenolic compounds, mainly flavonoids. The similarity between artisanal production and aqueous extraction, widely discussed in the literature, is also verified in the bioactive composition of the product, which presents a similar profile for these two techniques (Pandey et al., 2019).

Figure 1. Determination of bioactive compounds (total phenolics and total flavonoids) in the artisanal essential oil of *Origanum vulgare*.



Results are expressed as mean ± standard deviation of triplicate extractions. TPC, total phenolic content; TFC, total flavonoid content; EAG, gallic acid equivalents; EQ, quecentine equivalents. Source: Authors.

For the antimicrobial assay, in the results of the first observation, carried out still in test tubes, after 24 h of incubation, the great resistance of the pathogen can be verified in concentrations investigated in this study, showing variable turbidity for the different concentrations according to the McFarland scale. Results similar to those verified after 48h, demonstrating that the compounds remain low in the medium (Table 1).

Table 1. Turbidity and CFU/mL for the systems containing the *Origanum vulgare* artisanal oil in different concentrations.

Fractures	***McFarland***		**CFU/mL**	
	24 h	48 h	24 h	48 h
2.5 µl	0.25	0.5	> 300 x 10^{-5}	> 300 x 10^{-5}
5.0 µl	0.5	0.5	> 300 x 10^{-5}	> 300 x 10^{-5}
10.0 µl	0.5	1	> 300 x 10^{-5}	> 300 x 10^{-5}
25.0 µl	1	1	> 300 x 10^{-5}	> 300 x 10^{-5}
50.0 µl	1	1	> 300 x 10^{-5}	> 300 x 10^{-5}
100 µl	0.5	1	> 300 x 10^{-5}	> 300 x 10^{-5}
250 µl	1	1	> 300 x 10^{-5}	> 300 x 10^{-5}
500 µl	1	1	> 300 x 10^{-5}	> 300 x 10^{-5}
750 µl	1	1	> 300 x 10^{-5}	> 300 x 10^{-5}
1000µl	1	1	> 300 x 10^{-5}	> 300 x 10^{-5}
Cont +	1	1	> 300 x 10^{-5}	> 300 x 10^{-5}
Cont -	0.25	0.25	-	-

Source: Authors.

Similar results are highlighted by Cleff (2008), who used the same technique of extraction in eight samples of oregano essential oil, with different concentrations of antifungal compounds, against *C. albicans* isolates and standards, in vitro, and observed that there were between the MIC of the different extracts tested, in a range of 0.65 to 10.0 µL/mL. The results of the concentrations capable of inhibiting yeast growth for this author were similar to those for the artisanal extracted essential oil.

With the incorporation of the artisanal essential oil (AEO) in Muller Hinton Broth (MHB) containing yeast *C. albicans*, high turbidity was observed in all the samples, occurring between 0.25 and 1.0 in the used scale, in 2.5 and 1000 μL concentration respectively. The lowest observed turbidity appeared once, while the highest turbidity was present in 60% of the samples, and even more frequently in the highest concentrations, showing a proportional increase among these factors.

However, it is noted that the size of the colonies was reduced compared to the standard in this type of culture medium, and therefore, there were considerable morphological variations in relation to that described for viable colonies to infections and candidiasis. It was observed a gradual intervention in the metric structure of these colonies, while the vegetable oil becomes more concentrated, tends to have a diminution of its size in 24 h; post-48 h exhibit no variation, showing more activity in the first few hours. Ratios above 10.0 μL are more efficient, whereas the higher cellular deformation comes from the extract at 1000 μL.

According to Naves et al. (2013), changes in colonies indicate interference in the ability to cause candidiasis. In this study, it was evaluated the antifungal activity of *Origanum vulgare* essential oil obtained by hydrodetilation with Clevenger apparatus, and it was observed that *Candida* species may vary in resistance to the compounds extracted from the plant, from susceptible to resistant, depending on the lot, brand and concentration of samples tested. The susceptibility range of *C. albicans* for this author was 1.012.5 μg/mL at concentrations higher than 8,100 μg/mL, well above the concentrations observed in this study.

Cleff (2008) found that for different isolates, animals, humans and the environment in the southern region of Rio Grande do Sul, there is variation in resistance to essential oil, where concentrations below 0.25% may or may not inhibit the growth of the pathogen, under the test conditions. It was also observed that concentrations below 0.06% did not present antifungal activity against *C. albicans* strains analyzed.

Antifungal activity of artisanal oils was compared to that of industrialized by Sousa et al. (2013), using as challenger the fungal genus of mycelial growth *Pestalotiopsis* spp, and its resistance to *Copaifera langsdorffii* Desf. (Copaíba) and *Ricinus communis* L. (Mamona). Both plants showed inhibition in the mycelial growth of the fungus with its extracts, and in the industrial oil there was an increase of 3.7% in the average of inhibition, proving to be more efficient under the conditions tested.

Silva et al. (2008), after studies of antimicrobial activity using dehydrated leaves commercially acquired in the city of São José dos Campos - SP under *Candida* strains, verified that the essential oil of the plant did not inhibit the growth of *C. albicans* (ATCC 36802), from the 50% dilution of the infusion into tubes containing Saboraud Dextrose in broth. The detailed infusion method for this author resembles that necessary to obtain the artisanal form of the essential oil of oregano.

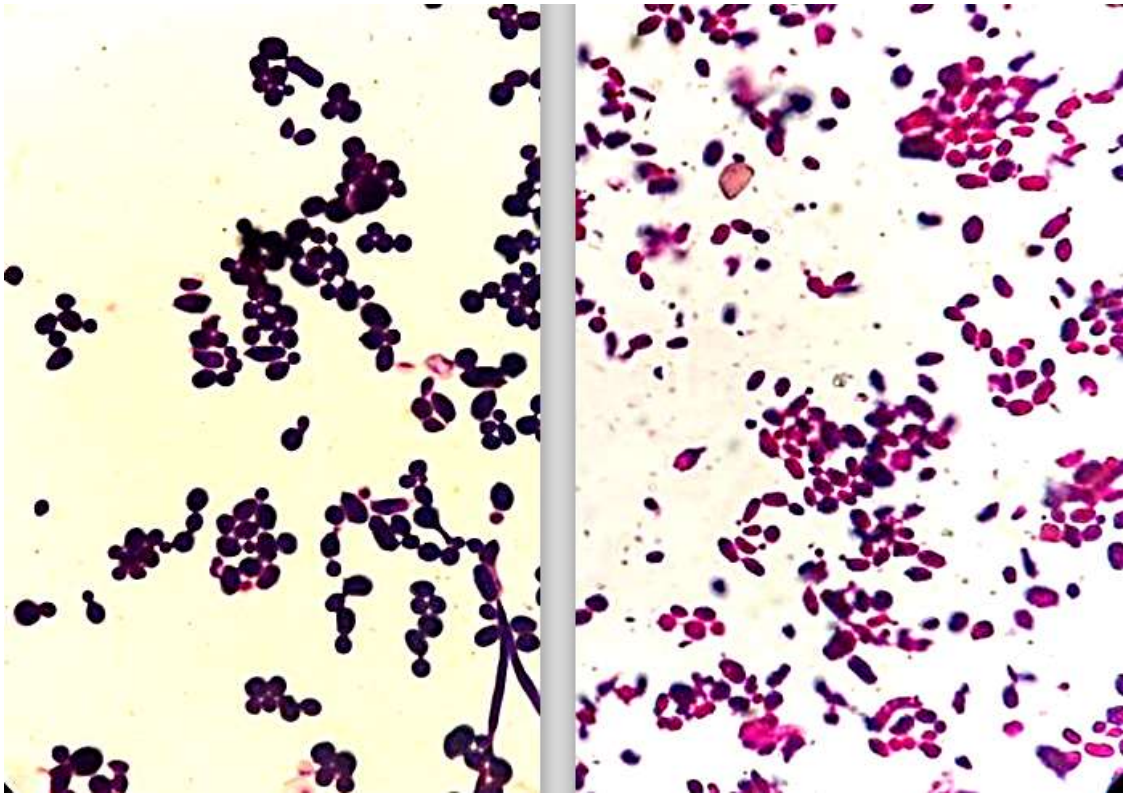
Variations observed in the test results may be related to the various factors that influence the fungal activity of plant essential oils, crop and crop conditions, as well as climatic and geographic factors. Problems of dry product quality, adulteration, falsification, misidentification of packaging may interfere with the compounds present at the end of oil extraction [Simões & Spitzer, 2004). In addition, to heterogeneity of oils, differences between the methodologies and culture medium of works may cause certain variations in spectrum of susceptibility and fungal resistance concentrations. Besides knowing the activity of the oil to be used, it is necessary to understand the mechanisms that lead to the effect or not.

It was apparent the presence of turbidity in the negative control tubes, with 0.25 on the McFarland scale. In the same ones, no growth of yeast colonies in the plates was verified, excluding the possibility of external contamination, being therefore the own artesian oil of turbid appearance. According to Xavier et al. (2012), some plant compounds produce an extract and turbid oils from certain proteins extracted during the process. This turbidity does not make the tests invalid, however, difficult to read the results, as highlighted by Casaroto et al. (2012), and it is therefore advisable to check it by optical microscopy.

The number of colonies was homogeneous, with growth on the entire plaque surface of the study concentrations, and it was not possible to count actual colonies and the possible determination of CFU. In this case, the reading was considered as> 300 colonies per plaque in the ten concentrations at 24 and 48h (Table 1). In later observations, on plates of Muller Hinton Agar, with readings at 24 and 48h, it was presented that, there was no total inhibition in the growth of colonies with regard to number. The present colonies have a typical character in macroscopic fungal structure, creamy to whitish, smooth and shiny, with soft consistency, according to Miotto et al. (2004) and Lacaz et al. (1998).

A significant change was observed in the micromorphology of *Candida albicans* cells treated with AOE, indicating interference in the virulence mechanism. According to Oliveira et al. (2016), cell morphology is one of several factors that influence and contribute to pathogenicity and host adaptations. Some authors have shown that the alteration in wall structure and cell membrane permeability is the most likely mechanism of action against essential oil pathogens (Oliveira et al., 2016; Burt, 2004) (Figure 2).

Figure 2. *Candida albicans* cells on detail, in optical microscopy. On the right, the positive control cells. On the left, cells under the *Origanum vulgare* artisanal oil interference.



Nikon Eclipse E100: 100X. Source: Silva Rocha (2021).

The integrity of the cell wall was altered, making the cells more elongated, evidencing that the artisanal essential oil exerts an effect under the yeast cells, which can lead to a decrease in virulence. Besides the shape, the arrangement was impaired, having a great influence on the biofilm formation and the power of infection. According to Braga et al. (2017), the ability to form biofilm is one of the virulence factors of this fungus, which makes it more resistant to conventional drugs and antifungals.

The AOE promoted a decrease in the uptake of violet crystal from the Gram staining process, indicating the occurrence of changes in cell wall permeability, since this dye complex is easily removed when the wall is damaged, giving the cells a appearance. Vieira & Santos (2017) highlight the integrity of the cell wall and membranes as one of the most important

in the maintenance and growth of *C. albicans*, once these damaged structures, both the antifungal action can be benefited, development itself is controlled.

The development of hyphae and pseudohyphas make the phagocytosis process difficult and allow the pathogen to be adhered to the tissues, thus, it is associated with virulence factors presented in *Candida*. The morphological changes of the cells may influence the pathogenicity of yeast (Romani et al., 2003). The results show that the AEO at the concentrations used is able to decrease the formation of hyphae, pseudohyphas. Oliveira et al. (2016) verified the interference of oils and substances of plant origin on the pathogenic fungi micromorphology and found concordant results.

In this study, it is possible to highlight that *Origanum vulgare* artisanal essential oil obtains the concentration of compounds capable of reducing the pathogenicity of species of the genus *Candida*, similar to the oils commercially acquired, once the morphology structure was affected. The AEO is incorporated as a promising candidate for the development of antifungal drugs for clinical use. Although its mechanism of action has not yet been clarified in its entirety, it is assumed that this oil is capable of causing damage to the shells, thus interfering in morphological structures associated with the pathogenicity of these micro-fungi.

4. Conclusion

In the tested conditions, *Candida albicans* presented resistance to essential oil of *Origaum vulgare* (orégano), extracted from dry leaves marketed at concentrations, 2.5 μL, 5.0 μL, 10 μL, 25 μL, 50 μL 100 μL, 250 μL, 500 μL, 750 μL, 1000 μL. However, the colonies have an atypical size, revealing the deforming effect of the oil on the morphology of the yeast, considering these aspects as possible interference in its pathogenicity mechanisms. Although the total number of cells in the cell count did not change, there was a significant reduction in the concentration of whole cells and growth of non-intact and non-viable cells. Phenolic compounds and flavonoids have high levels in artisanal oil, which may be related to the observed effects. In the future, studies are needed to develop methodologies capable of discussing the occurrence of this action, and which structures are directly affected, as well as *in vivo* studies that demonstrate the same effects and reveal low toxicity in human cells.

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