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RESEARCH ARTICLE

THE EFFECT OF ESSENTIAL OIL OF *THYMUS VULGARIS* ON GROWTH AND BIOFILM FORMATION OF *CANDIDA TROPICALIS*

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ABSTRACT

Fungi are abundant in different environments and the human body is no exception. They can be the cause of infectious diseases, the most serious being systemic infections. In this context, *C. tropicalis* has been the cause of emerging infections and it has gained importance due to its impact on public health among *Candida* species. In infections caused by species of the genus *Candida*, it is common to find cases of antifungal resistance. However, today it is known that many products of plant origin have antifungal properties such is the case of essential oils.

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INTRODUCTION

As is known, fungi can be found distributed throughout the world. They occupy different environments and can cause different infectious diseases, ranging from superficial mycoses to opportunistic and systemic mycoses (Brown *et al*., 2012). Fungal diseases kill more than 1.5 million people in the world. Serious fungal infections occur as a result of other health problems HIV-AIDS, asthma, cancer, organ transplantation, or corticosteroid therapy (Bongomin *et al*., 2017). On the other hand, in the immunocompetent individuals the species of the genus *Candida* sp. (*C. albicans*, *C. tropicalis*, *C. glabrata, C. dubliniensis, C. parapsilosis,* etc) are part of the microbiota of the skin and oral, gastrointestinal and vaginal mucosa of the human (Munhoz-Alves *et al*., 2021). *C. tropicalis* has become one of the most important *Candida* species. It has been emerged as one of the most important species in terms of epidemiology and virulence. *C. tropicalis* is considered to be the second most virulent *Candida* species, preceded only by *C. albicans* due *C. tropicalis* is a strong biofilm producer with a wide range of other virulence factors (Zuza-Alves *et al*., 2017). *Candida* sp. can also cause various infections in susceptible patients; it is one of the most fungal infections with multiple mechanisms of antifungal resistance (Bhattacharya *et al*., 2020; Fisher *et al*., 2022; Rhodes and Fisher, 2019).

However, it has been reported that extracts of plants and different essential oils have inhibitory effects on growth of bacteria and fungi (Ferreira *et al*., 2011; Flores-Encarnación *et al.*, 2016; Flores-Encarnación *et al.*, 2022). For this reason, in the present work, the effect of essential oil of *T. vulgaris* on growth and biofilm formation of *Candida tropicalis* was studied.

MATERIAL AND METHODS

Source of material*:* In this study, a commercial essential oil of *T. vulgaris* was used. It was obtained from a flavour and fragrance company at Puebla, México.

Biological material: At the beginning of this study, 6 strains of *Candida* sp. from clinical isolates were analyzed and the strain that showed the greatest growth in the tested culture medium was chosen (*C. tropicalis*). The strains of *Candida* sp. were provided by some diagnostic laboratories at Puebla city. The identity of *C. tropicalis* was confirmed by using direct microscopy and streaking on plates of BBLCHROMagar Candida medium, obtaining pure colonies for each species according to manufacturer's specifications.

The strain of *C. tropicalis* was stored at -40°C in nutrient broth added with 5% glucose with 20% glycerol until analysis.

Culture conditions: The nutrient broth added with 5% glucose was used for culture of *C. tropicalis*. Test strain that had been cultured at 37°C for 48 to 96 hours in nutrient broth added with 5% glucose was seeded crosswise in a Petri dish containing nutrient agar added with 5% glucose. The plate was incubated at 37°C for 48 to 96 hours.

Antifungal activity of essential oil: The antifungal activity of *T. vulgaris* essential oil was determined using the technique of disk diffusion in agar. Briefly, nutrient agar (added with 5% glucose) plates containing 20 mL of medium were prepared. Sterile Petri dishes (150 mmin diameter) were used. Plates were inoculated by cross-striation with *C. tropicalis*. It started from a 24-hour preculture with Ab_{560nm}=4. The effect of essential oil of *T. vulgaris* on *C. tropicalis* growth was tested using antimicrobial susceptibility test discs. For this, sterile filter paper disks (5 mm in diameter) were placed on the surface of nutrient agar (added with 5% glucose) plates. Then, different concentrations (1.3 to 13 mg) of the essential oil were used. The agar plates were incubated at 37°C for 24 h. The diameters of the inhibition halos formed were measured using a caliper ruler. The analyses were conducted in duplicate. The fungicide or fungistatic effect was determined by passing the bacteriological handle in the plate area without apparent fungal growth and a fresh nutrient agar (added with 5% glucose) plate was inoculated by cross-streak. The plate was incubated at 37°C for 24 h.

Formation of biofilm by C. tropicalis: For the formation of biofilm, *C. tropicalis* was placed in Petri dish containing 5 mL of nutrient broth added with 5% glucose. Sterile Petri dishes (60 mmin diameter) were used. The broth was inoculated with 125 µL of a 24-hour preculture of *C. tropicalis* in the same medium incubated at 37 ºC (Ab_{560nm}=4). Then with the help of sterile forceps, a glass coverslip was submerged in the inoculated nutrient broth, ensuring that the coverslip remained at the bottom of the Petri dish. The inoculated plates were incubated at 37°C for 72 to 96 hours keeping in humidity chamber. After time, the glass coverslips were removed from Petri dish. The formation of biofilm was observed using direct microscopy and it was stained with 0.1% violet crystal for 5 min at room temperature. Similarly, the formation of *C. tropicalis* biofilm was observed at the bottom of the petri dish (after the culture medium was removed) and it was dyed with 0.1% calcofluor white for 20 min at room temperature. The Petri dishes were irradiated with UV light to evidence biofilm formation of *C. tropicalis*. The analyses were conducted in duplicate.

The effect of T. vulgaris on the formation of biofilm: To determine the effect of *T. vulgaris* on the formation of *C. tropicalis* biofilm, the procedure described above was followed. Thus, once the coverslip was removed from the Petri dish 5 mg of *T. vulgaris* essential oil was added on the surface of the biofilm of *C. tropicalis* and it was observed at different times using direct microscopy. The analyses were conducted in duplicate.

RESULTS

In this study, the effect of essential oil of *T. vulgaris* on growth and biofilm formation of *C. tropicalis* was determined. As mentioned earlier, the strain of *Candid*a sp. that showed the greatest growth in nutrient broth added with 5% glucose was chosen (data not shown). The identity of fungal strain was confirmed by using direct microscopy and and observing the growth on plates of BBL CHROMagar Candida medium (Fig. 1). Fig.1A shows the blastoconidia and pseudohyphae of *C. tropicalis*. It is important to mention that *C. tropicalis* strain was able to grow in nutrient broth added with 5% glucose showing the characteristic morphology. Fig.1B shows the confirmatory test for the identity of *C. tropicalis*, turning blue when the fungus was grown on a chromoagar plate for *Candida* sp. (according to manufacturer's specifications). Fig. 1C shows *C. tropicalis* growing on nutrient agar plates (added with 5% glucose).

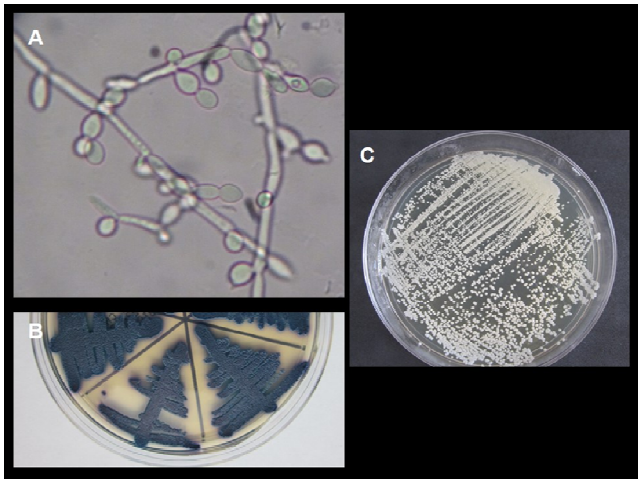

Fig. 1 The growth of *C. tropicalis.* A. Characteristic structures in nutrient broth added with 5% glucose. B. Growth of *C. tropicalis* on a chromoagar Candida plate. C. Growth of *C. tropicalis* on nutrient added with 5% glucose agar plate

As seen in Fig. 1C, a large number of monomorphic, cream-colored, smooth, glabrous colonies were observed on the tested culture medium, which consistent with the colony morphology of *C. tropicalis*. To determine the effect of essential oil of *T. vulgaris* on growth *C. tropicalis* the technique of diffusion in agar using sterile filter paper disks as mentioned in Materials and Methods. Different concentrations (1.3, 2.6, 5.2, 6.5 and 13 mg) of the essential oil were used. The agar plates were incubated at 37°C for 24 h. The results obtained are shown in Fig. 2.

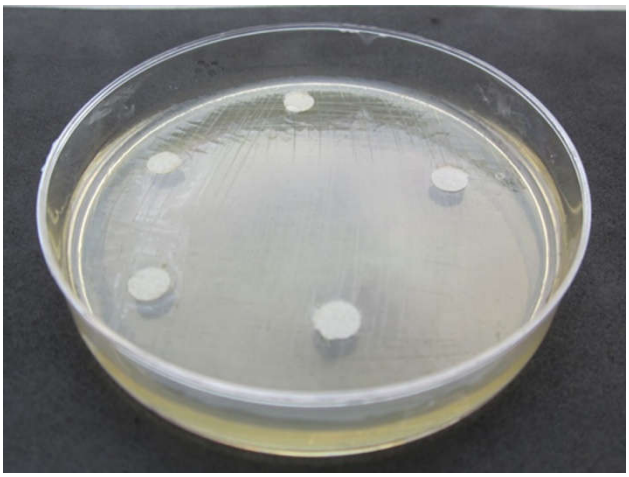

Fig. 2. The inhibition of growth from *C. tropicalis* using essential oil of *T. vulgaris*

The Fig. 2 shows the surface of a nutrient agar (added with 5% glucose) plate where *C. tropicalis* was cultivated, observing that the growth was completely inhibited by action of the essential oil of *T. vulgaris* at concentrations tested. As can be seen in the image, the surface of the nutrient agar completely lacks fungal growth and appears shiny, reflecting the surrounding light. The fungicide or fungistatic effect was determined by passing the bacteriological handle in the plate area without apparent no fungal growth (by action of the essential oil of *T. vulgaris*). Then a fresh nutrient agar (added with 5% glucose) plate was inoculated by cross-streak. The plate was incubated at 37°C for 24 h and there was no growth of *C. tropicalis* (data not shown). To determine the effect of essential oil of *T. vulgaris* on the formation of biofilm, *C. tropicalis* was placed in Petri dish containing nutrient broth added with 5% glucose as mentioned in Materials and Methods. Once the coverslip was removed from the

culture broth, the biofilm formed by *C. tropicalis* was observed by direct microscopy and with 0.1% crystal violet dye (Fig. 3).

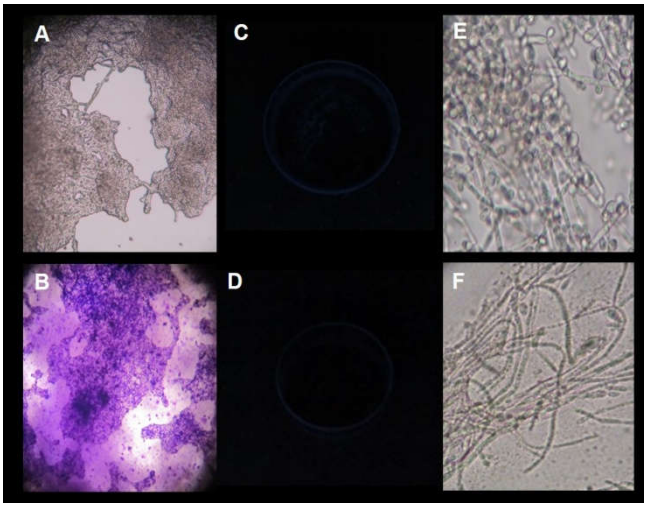

Fig. 3. Biofilm formation by *C. tropicalis* and the effect of essential oil of *T. vulgaris*. A. Biofilm observed by direct microscopy (at 10X power). B. Biofilm observed staining with crystal violet dye (at 10X power). C. Fluorescence emission by biofilm of *C. tropicalis*. D. Petri dish without biofilm (control condition). E. The blastoconidia and pseudohyphae in biofilm of *C. tropicalis* (at 40X power). F. Effect of essential oil of *T. vulgaris* on the biofilm of *C. tropicalis* (at 20 min of incubation)

As shown in Fig. 3A and Fig. 3B, *C. tropicalis* formed biofilm on the glass surface of coverslip, covering it completely and showing a homogeneous spread. As a mature biofilm (produced after incubation for up to 48 hours), consisted of a dense network of blastoconidia and pseudohyphae of *C. tropicalis*, yeasts were not observed (Fig. 3E). The formation of *C. tropicalis* biofilm was also observed using calcofluor White and and staining the bottom of the petri dish as mentioned in Materials and Methods. Thus, the presence of the extracellular matrix of the *C. tropicalis* biofilm was revealed observing fluorescence emission when Petri dish was irradiated with UV light (Fig. 3C). The Petri dish without biofilm did not show fluorescence when irradiated with UV light (Fig. 3D). Finally, the effect of essential oil of *T. vulgaris* on formation of biofilm of *C. tropicalis* was determined. Thus, once the coverslip (containing biofilm formed of *C. tropicalis*) was removed from the Petri dish, 5 mg of *T. vulgaris* essential oil was added on the surface of biofilm. The results obtained are shown in Fig. 3F. As shown in Fig. 3F, the essential oil of *T. vulgaris* altered the structure of biofilm of *C. tropicalis*, observing a disintegration, destruction and important changes in the characteristic morphology of the blastoconidia and pseudohyphae *C. tropicalis*. In Fig. 3F, a large amount of cell debris is also observed, so it is proposed that the essential oil of *T. vulgaris* causes cell destruction with the release of cytoplasmic content.

DISCUSSION

Fungi cause various diseases in humans, ranging from allergic syndromes to life-threatening, invasive fungal diseases, affecting more than a billion people worldwide (Bongomin *et al*., 2017; Brown *et al*., 2012; Fisher *et al*., 2022). Currently, many cases of resistance to antifungals are known (Fisher *et al*., 2022). Species of the genus *Candida* are commensal fungi, which are part of the human microbial flora. They are located in the skin and the gastrointestinal genital tract. However, *Candida* sp. it can also cause disease in susceptible patients. It is one of the most frequent infections worldwide, presenting various resistance mechanisms (Bhattacharya *et al.*, 2020; Pfaller, 2007). So, *C. albicans* is the most common causative agent of mucosal infections and systemic infection, and it is responsible for about 70% of fungal infections around the world (Morad *et al.*, 2018; Talapko *et al.*, 2021). Similarly, *C. tropicalis* has emerged as one of the most important *Candida* species and it has been considered the second most virulent Candida species, only preceded by *C. albicans*.

Besides, this species has been recognized as a very strong biofilm producer, it produces a wide range of other virulence factors, such as adhesion to buccal epithelial and endothelial cells, the secretion of lytic enzymes (proteinases, phospholipases, and hemolysins) and others (Zuza-Alves *et al.*, 2017). In this context, the study of effect of *T. vulgaris* essential oil on growth and biofilm formation of *C. tropicalis* was determinated. It's known that the essential oils are plant metabolites that protect them from various agents (Butkiené *et al*., 2015; Flores-Encarnación *et al.*, 2016). They have been used as flavorings, preservatives or for cosmetic purposes (Ali *et al*., 2015). In more recent years, essential oils have been shown to possess antioxidant and antimicrobial properties, especially antifungal properties (Flores-Encarnación *et al*., 2022; Sacchetti *et al*., 2005). In this study, the strain of *C. tropicalis* from a clinical isolate was grown in nutrient broth added with 5% glucose. As mentioned above, the tested culture medium favored the growth of the fungus, showing the the typical morphological characteristics: a large number of monomorphic colonies, cream-colored, with smooth and glabrous colonies. Using the direct microscopy, *C. tropicalis* strain showed the blastoconidia and pseudohyphae, characteristic of its morphology. In addition, a chromogenic agar to differentiate *Candida* species was used. Thus, the strain of *C. tropicalis* showed a turning blue. According to other authors, it has been reported that the use of chromogenic media, with different substrates that react with specific enzymes of the *Candida* species induce the formation of colonies with different colors and has been used for the identification of *C. tropicalis* (Zuza-Alves *et al.*, 2017). On the other hand, the effect of essential oil of *T. vulgaris* on growth *C. tropicalis* was determinated using the technique of diffusion in agar. As shown in the Results section, the essential oil of *T. vulgaris* showed a strong inhibitory effect on the growth of *C. tropicalis*.

The action of the essential oil was fungicidal as could be demonstrated through the subsequent tests carried out. Little is known about the action of *T. vulgaris* on the growth of *C. tropicalis*, however it has been reported that the activity of some essential oils such as *T. vulgaris*, *Citrus limonum*, *Pelargonium graveolens*, *Cinnamomum cassia*, *Ocimum basilicum*, and *Eugenia caryophyllus* in clinical isolates of *C. albicans* and *C. glabrata*, reporting that those essential oils exhibited both fungistatic and fungicidal activity against the *C. albicans* and *C. glabrata* isolates (Gucwa *et al.*, 2018). In relation to the mechanism of action of essential oils, it has been reported that *T. vulgaris* and *C. limonum* affected the cell membranes. Furthermore, it has been shown that *T. vulgaris* produced a potassium ion efflux and it inhibited the transition of yeast to mycelium form (Feyaerts *et al.*, 2018; Flores-Encarnación *et al*., 2022; Ramage *et al.*, 2006). As is known, candidiasis (frequently caused by *C. albicans, C. glabrata, C. tropicalis, C. krusei*, or *C. parapsilosis*) is associated with the formation of biofilms on the surface of implanted medical devices and tissues or on host epithelial cell surfaces. Cells forming biofilm increase the resistance to antimicrobial agents and to host defenses (Bizerra *et al*., 2008; Feyaerts *et al*., 2018). According to the above, in this study the effect of essential oil of *T. vulgaris* on the formation of biofilm of *C. tropicalis* was determinated. As shown in the Results section, a mature biofilm of *C. tropicalis* was used. Blastoconidia and pseudohyphae were observed in the biofilm of *C. tropicalis*. The formation of *C. tropicalis* biofilm was confirmed using crystal violet and calcofluor White (this last dye indicated the presence of extracellular matrix of the *C. tropicalis* biofilm). The results obtained in this study shown that the essential oil of *T. vulgaris* altered the structure of biofilm of *C. tropicalis*, observing important changes in the characteristic morphology of the blastoconidia and pseudohyphae of *C. tropicalis*. The disintegration, disorganization, cell lysis and the escape of cytoplasmic content, were some of the effects observed by the action of the essential oil of *T. vulgaris* on the cells of *C. tropicalis*. It has been reported that the clove and thyme essential oils can be efficiently used preventing the formation of biofilm in abiotic surfaces (glass, polyethylene terephthalate, polypropylene) by *Candida* sp.; clove and thyme essential oils showed antibiofilm

activity (Flores-Encarnación *et al.*, 2022; Rajkowska *et al*., (2019). As has been reported by several authors, in bacteria the essential oils destabilize the cellular architecture, leading to the breakdown of membrane integrity and increased permeability, which disrupts many cellular activities, including energy production (membrane-coupled), membrane transport, and other metabolic regulatory functions, including cellular respiration (Flores-Encarnación *et al.*, 2020; Oussalah *et al*., 2006; Raut and Karuppayil, 2014; Saad *et al*., 2013; Swamy *et al.*, 2016). Something similar must happen in fungi. Little is known about the mechanisms of action of essential oils in fungi, however some authors have reported that the essential oils contain different monoterpene components, which are responsible for the observed effects such as the alteration of cell permeability, for example: *Thymus kotschyanus* essential oil contained thymol (60.48%), carvacrol (3.08%), p-cymene (5.56%), and γ-terpinene (6.67%) (Ghasemi *et al.*, 2020).

CONCLUSION

The genus *Candida* is composed of pleomorphic fungi that colonize the human gastrointestinal and genitourinary tracts. The most invasive forms of candidiasis manifest themselves as candidemia infecting the abdomen, lungs, bones, kidneys, central nervous system, and others. Given the reported cases of resistance to antifungals, it is necessary to search for new compounds with antifungal activity. The essential oils, including *T. vulgaris*, have shown antimicrobial properties. This work showed some data confirming the action of *T. vulgaris* inhibiting the growth and biofilm formation of *C. tropicalis*.

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