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RESEARCH ARTICLE

EFFECT OF ESSENTIAL OIL OF THYMUS VULGARIS ON THE GROWTH AND FORMATION OF BIOFILM OF SACCHAROMYCES CEREVISIAE

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ABSTRACT

As is known, antimicrobial resistance is a global public health problem. More and more bacteria are resistant to antibiotics. Likewise, pathogenic fungal infectious agents have developed resistance to antifungals. For this reason, alternative strategies are currently being studied to combat microbial infections, especially those caused by resistant infectious agents.The use of substances of plant origin (such as essential oils) with antibacterial and antifungal properties is novel. This work presents the effect of *T. vulgaris* essential oil on the growth and biofilm formation of *S.cerevisiae*, a yeast that in recent years has been considered an emerging opportunistic pathogen.

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INTRODUCTION

Essential oils are very complex mixtures of volatile substances obtained from plant material. The primary function of essential oils is to provide scent and flavour to plants. They also play an important communicative role, attracting pollinators and repelling pests. Sometimes, they also act as signals for other plants of the same species (Grażyna and Durczyńska, 2024; Gershenzon *et al*., 2007; Vigan, 2010). Essential oils have been widely applied in folk medicine due to their various properties, such as anti-inflammatory, immunomodulatory, expectorant, sedative effects and antibacterial properties (Grażyna and Durczyńska, 2024; Gironi *et al*., 2008).

In recent years, there has been increasing interest in the use of biologically active organic compounds which are extracted from plant species, that have the ability to eliminate pathogenic microorganisms; this is mainly due to the resistance that microorganisms have developed to antibiotics (Flores-Encarnación *et al*., 2016). One of the mechanisms involved in antibiotic resistance is the production of biofilm. Biofilms are microbial communities embedded in an extracellular polymeric matrix and they reduce susceptibility to antibiotic contributing to the persistence of infections (Costerton *et al*., 1999; Delcaru *et al*., 2016; Flores-Encarnación *et al*., 2018; Høiby, 2017). Most studies using essential oils have been carried out on bacteria, however little is known about their effect on fungi for commercial use. In the present work, the effect of *T. vulgaris*

essential oil on growth and biofilm formation of *S. cerevisiae* 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: The *Saccharomyces cerevisiae* strain was used. The strain of *S. cerevisiae* used was the yeast marketed for making bread. Yeast was stored in cryovials at -40°C in yeast peptone dextrose (YPD) broth with 20% glycerol until analysis.

Culture: *S. cerevisiae*s train were cultivated on yeast peptone dextrose broth containing amoxicillin (16µg/mL) and gentamicin (40 µg/mL) and the following components of medium (g/L): 10 yeast extract, 20 peptone and 20 dextrose. The stationary cultures were grown at 37°C for 24 hours in glass tubes containing 5 mL ofyeast peptone dextrose broth and were used as precultures. The yeast peptone dextrose agar plates containing 20 mL of medium were prepared. Sterile Petri dishes (150 mm) were used. Plates were inoculated by crossstriation with a stationary 24-hour preculture of *S. cerevisiae* in yeast peptone dextrose broth (Ab_{560nm}= 5.3).

Assay of antifungal activity: The antifungal activity of *T. vulgaris* essential oil was determined using the technique of diffusion in agar using paper discs. For it, yeast peptone dextrose agar plates (containing 20 mL of medium) were prepared. Sterile Petri dishes (150 mm) were used. Plates were inoculated by crossstriation with a stationary 24-hour preculture of *S. cerevisiae* in yeast peptone dextrose broth (Ab_{560nm}= 5.3). Then, sterile filter paper disks (5 mm diameter) were placed on the surface of yeast peptone dextrose agar plates. Different concentrations (1.3, 2.6, 5.2, 7.8 and 13.2 mg) of essential oil were used. The agar plates were incubated at 37°C for 24 h. The diameters of the inhibition halos formed were measured. The analyses were conducted in triplicate. As reference, yeast peptone dextrose agar plates were inoculated by crossstriation with *S. cerevisiae* and sterile filter paper disks were placed on them, adding different concentrations (0.25, 0.50, 1, 1.5, 2.5µg) of amphotericin B. The antifungal activity of amphotericin B was also determined using the technique of diffusion in well. For it, yeast peptone dextrose agar plates were used. Plates were inoculated by crossstriation with a stationary 24-hour preculture of *S. cerevisiae* in yeast peptone dextrose broth (Ab_{560nm}= 5.3). Subsequently, 3 wells were made on yeast peptone dextrose agar plate with the aid of the mouth piece of a sterile glass Pasteur pipette. Then, 10 µg of amphotericin B were placed in two wells. Only 20 µL of sterile distilled water was placed in the other well. The plates were incubated at 37°C for 24 h. The diameters of the inhibition halos formed were measured. The analyses were conducted in triplicate.

Cell viability assay: The cell viability assay was performed using the trypan blue dye according to methodology described by Castillo *et al*., (2009). For that, 1 mL of an active culture of *S. cerevisiae* (5 hours of culture, Ab_{560nm}=1.62) was centrifuged at 2,500 r.p.m. for 3 min. The supernatant was removed and 100 µL of fresh yeast peptone dextrose broth were added (cell suspension). The cell viability assay was determined by mixing 10 µL of cell suspension and 10 µL of 0.1% trypan blue dye, and then placing 10 µLof the mix on a slide observing at 40X power. Dead cells were observed in a deep blue color. All determinations were made in triplicate. For negative control, non-viable cells of *S. cerevisiae* were used. This cells were obtained by heating at 60°C for 20 minutes. The effect of *T. vulgaris* essential oil on cell viability was determined as follows. The cell suspension was prepared and mixed with the trypan blue dye as described before. Then, 0.065and 0.13 mg of *T. vulgaris* essential oil was added to the separately mixture. The preparation was observed at at 40X power. All determinations were made in triplicate.

Formation of biofilm of *S. cerevisiae:* For the formation of biofilm, *S. cerevisiae* was placed in Petri dish containing 5 mL of fresh yeast peptone dextrose broth. Sterile polystyrene Petri dishes (60 mm in diameter) were used. The broth was inoculated with 200 µL of a 24-hour stationary preculture of *S. cerevisiae* in the same medium incubated at 37°C (Ab_{560nm}= 5.3). Then with the help of sterile forceps, a glass coverslip was submerged in the inoculated yeast peptone dextrose 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 at 10X and 40X power. Similarly, the formation of *S. cerevisiae* biofilm was observed at the bottom of the petri dish (after the culture medium was removed). The formation of biofilm was observed using direct microscopyat 10X and 40X power. The analyses were conducted in triplicate.

Effect of *T. vulgaris* essential oil on the formation of biofilm: To determine the effect of *T. vulgaris* essential oil on the formation of *S. cerevisiae* biofilm, the procedure described above was followed. Thus, once *S. cerevisiae* biofilm was observed at the bottom of the petri dish, 0.13 mg of *T. vulgaris* essential oil was added on the surface of the biofilm, a coverslip was placed an immediately it was observedat 40X power. The analyses were conducted in triplicate. In all cases, 0.1% trypan blue staining was used to determine cell viability. So, 10 µL of 0.1% trypan blue dye were placed directly on the surface of the biofilm and it was observedat 40X power. To determine the effect of *T. vulgaris* essential oil 10 µL of 0.1% trypan blue was mixed with 0.13 mg of *T. vulgaris* essential oil. Then, mixture was placed directly on the surface of the biofilm and it was observedat 40X power.

Effect of *T. vulgaris* essential oil on actively growing cells of *S. cerevisiae:* To determine the effect of *T. vulgaris* essential oil on actively growing cells, *S. cerevisiae* was grown in a 125 mL Erlenmeyer flask containing 50 mL of yeast peptone dextrose broth and incubating at 37 °C with shaking at 150 r.p.m. for 5 hours. Then, 1 mL of culture (Ab_{560nm}=1.62) was centrifuged at 2,500 r.p.m. for 3 min. The supernatant was removed and 100 µL of fresh yeast peptone dextrose broth were added (cell suspension). Then, 10 uL of cell suspensión, 10 uL of 0.1% trypan blue dye and 1.3 or 0.65µg (sublethal dose) of the *T. vulgaris* essential oil (diluted in dimethyl sulfoxide, DMSO) were added. The mixture was prepared in a 1.5 mL centrifuge tube. Immediately, 10 µL of this mixture was placed on a slide and the preparation was observed at 40X power.

All determinations were made in triplicate. As a control, *S. cerevisiae* cells were directly exposed to dimethyl sulfoxide.

RESULTS

In this study, the effect of essential oil of *T. vulgaris* on growth and biofilm formation of *S. cerevisiae* was determined. As mentioned earlier, antifungal activity of *T. vulgaris* essential oil was determined using the technique of diffusion in agar using paper discs. Plates were inoculated with *S. cerevisiae* and different concentrations of essential oil were used, as described in Materials and Methods. The results are shown in Fig. 1.

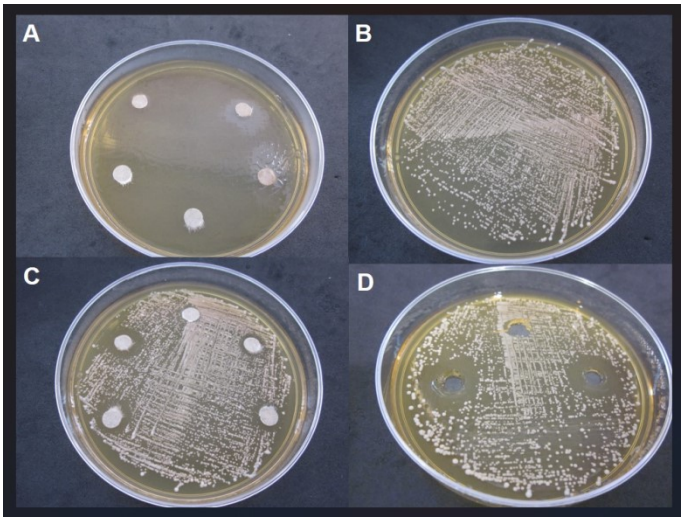

Fig. 1. The inhibition of growth from *S. cerevisiae* using *T. vulgaris* essential oil. A. Antifungal activity of *T. vulgaris* essential oil (1.3 to 13 mg). B. Control. C. Antifungal activity of amphotericin B (0.25 to 2.5µg). D. Antifungal activity of amphotericin B (10µg). Bottom wells containing amphotericin B; upper well without amphotericin B. In A and C, essential oil and amphotericin B were placed in increasing amounts in the counterclockwise direction, starting with the top.

As shown in Fig. 1A, the essential oil of *T. vulgaris* completely inhibited the growth of the yeast, with a gloss being observed on the surface of yeast peptone dextrose agar plates, at all tested amounts of the essential oil. The effect was fungicidal since viable cells of *S. cerevisiae* no could be recovered after scraping the surface of the agar without growth and plating on the surface of fresh yeast peptone dextrose agar plates (data not shown). As a reference, yeast peptone dextrose agar plates were inoculated by crossstriation with *S. cerevisiae* and sterile filter paper disks were placed on them, adding different concentrations of amphotericin B: 0.25 - 2.5µg. The antifungal activity of amphotericin B was also determined using the technique of diffusion in well to increase the concentration of amphotericin B up to 10 µg. The results are shown in Fig. 1C and Fig. 1D. As can be seen in both figures, amphotericin B produced halos of inhibition in the growth of *S. cerevisiae*, registering halos of around 8 to 15 mm at amounts of 0.25 to 2.5 µg of amphotericin B, while the halos recorded in well test (containing 10 µg of amphotericin B) measured around 20 mm. According to the results obtained, it was observed that the essential oil of *T. vulgaris* completely inhibited the growth of *S. cerevisiae*.

On the other hand, the effect of *T. vulgaris* essential oil on biofilm formation by *S. cerevisiae* was determined. For it, *S. cerevisiae* was placed in Petri dish containing yeast peptone dextrose broth and incubated at 37°C, as described in Materials and Methods. After 72 to 96 hours of incubation at 37 °C, the glass coverslips were removed from Petri dish to observe biofilm formation using direct microscopy at 10X and 40X power. In this case, biofilm formation by *S. cerevisiae* no could be observed on the used glass coverslips (data not shown). In this study, *S. cerevisiae* did not adhere to the glass to form the biofilm. So, the formation of *S. cerevisiae* biofilm was observed at the bottom of the petri dish (after the culture medium was removed) using direct microscopy at 10X and 40X power. The results obtained are shown in Fig. 2.

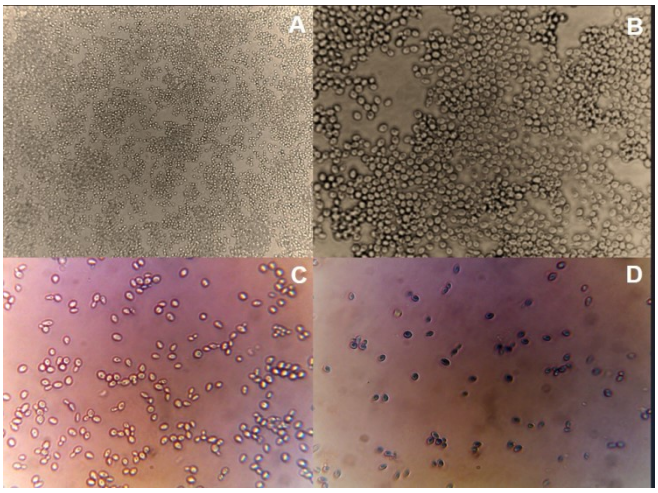

Fig. 2 Biofilm formation by *S. cerevisiae*. A. Direct microscopy at 10X power. B. Direct microscopy at 40X power. C. Viability of *S. cerevisiae* cells using 0.1% trypan blue dye. D. Effect of *T. vulgaris* essential oil on cells in biofilm of *S. cerevisiae.*

Fig. 2A and Fig. 2B show the biofilm formed by *S. cerevisiae* on the bottom of the used Petri dish. Polystyrene is the manufacturing material of Petri dishes, so this material favored the formation of the biofilm. The cell viability was determined using 0.1% trypan blue dye, so 10 µL of 0.1% trypan blue dye were placed directly on the surface of the biofilm and it was observed at 40X power (Fig. 2C). Fig. 2C shows that the *S. cerevisiae* cells were intact, that is, with their complete membranes since the trypan blue dye could not penetrate them. Commonly, dead cells are observed in a deep blue color. To determine the effect of *T. vulgaris* essential oil on the formation of *S. cerevisiae* biofilm, 10 µL of 0.1% trypan blue was mixed with 0.13 mg of *T. vulgaris* essential oil, a coverslip was placed an immediately it was observed at 40X power. The results obtained are shown in Fig. 2D. As can be seen in this figure, the number of complete cells of *S. cerevisiae* decreased considerably and all *S. cerevisiae* cells were observed in a deep blue color, which indicated that the integrity of the membrane was lost due to the addition of *T. vulgaris* essential oil. Therefore, *T. vulgaris* essential oil disrupted the biofilm formed by *S. cerevisiae* and affected the integrity of the cell membrane. On the other hand, it is known that cells growing in biofilm present a different phenotype than planktonic cells. Thus, actively growing cells of *S. cerevisiae* were obtained with shaking at 150 r.p.m, 37°C for 5 hours, as described in Materials and Methods. So, 10 µL of cell suspension, 10 µL of 0.1% trypan blue dye and 0.65 µg

(sublethal dose) of the *T. vulgaris* essential oil (diluted in dimethyl sulfoxide, DMSO) were added. Immediately, 10 µL of this mixture was placed on a slide and the preparation was observed at 40X power at different times. The results obtained are shown in Fig. 3. As can be seen in Fig. 3A, around 50% of the *S. cerevisiae* cells were not penetrated by the trypan blue dye (t=0 min). However, after 4 min all *S. cerevisiae* cells were stained deep blue color and a decrease in the number of complete cells was recorded, which indicated that the integrity of all cells was affected by the essential oil of *T. vulgaris* (Fig. 3B).

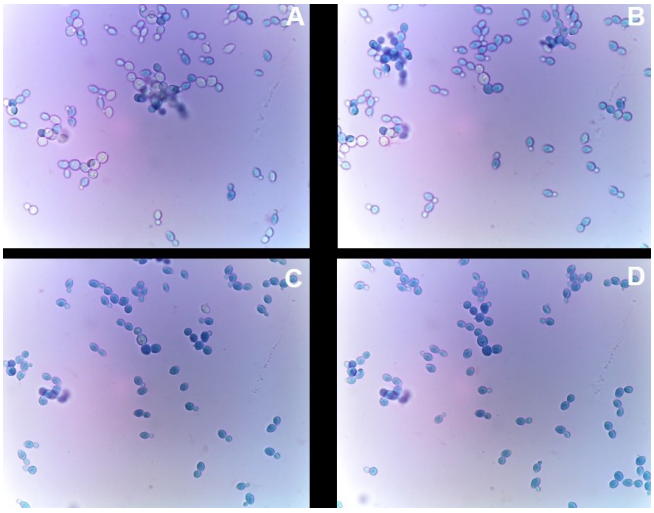

Fig. 3. Effect of *T. vulgaris* essential oil on actively growing cells of *S. cerevisiae*. A. Viable *S. cerevisiae* cells in the presence of *T. vulgaris* essential oil (0 minutes). B, C and D. *S. cerevisiae* cells stained deep blue color (at 1, 3 and 4 minutes)

As can be seen in Fig. 3A, around 50% of the *S.cerevisiae* cells were not penetrated by the trypan blue dye (t=0 min). However, after 4 min all *S. cerevisiae*cells were stained deep blue color and a decrease in the number of complete cells was recorded, which indicated that the integrity of all cells was affected by the essential oil of *T. vulgaris* (Fig. 3B).

DISCUSSION

It has been reported that certain fungal genera, including *Fusarium* sp., *Penicillium* sp., *Aspergillus* sp., and *Alternaria* sp., are capable of producing secondary metabolites known as mycotoxins, which can be lethal or toxic in both humans and animals. To combat food contamination by fungi different synthetic fungicides has been used, however they produce adverse effects on consumer health. So, to mitigate the potential harm caused by synthetic compounds, numerous studies have sought to develop natural alternatives capable of inhibiting fungal growth. In this context, essential oils are gaining increasing popularity due to their alignment with the contemporary trends of "green," "safe," and "healthy" food additives (Abdi-Moghadam *et al*., 2023; Achimon *et al*., 2021; Farhadi *et al*., 2022; Flores-Encarnación*et al.*, 2023*b*; Mutlu-Ingok *et al*., 2020; Taghizadeh *et al*., 2023; Wang *et al*., 2023; Wu *et al*., 2022; Yousefi *et al*., 2022). Aromatic plants and extracts prepared from them are known for their medicinal properties (antiseptic, bactericidal, antiviral, and fungicidal) for centuries. For this reason, they have been used in embalming, religious ceremonies, food preservation, insecticides and parasiticides in agriculture, antispasmodic, sedative, topical anaesthetic and anticancer agents (Bassolé *et al.*, 2012; Flores-Encarnación *et al*., 2023*a*; Kowalczyk *et al.*, 2023; Mostafa *et al*., 2023; Swamy *et al*., 2016; Żukowska G. and Durczyńska, 2024). The severity of the fungal infection depends on the inoculum charge, the host's immunological state and resistance. However, essential oils can represent one of the most promising natural products for fungal inhibition. In fact, many kinds of essential oils obtained from different plants exhibited intense antifungal properties (Bakkali *et al*., 2008; Ghalem, 2016; Hu*et al*., 2007; Kalemba and Kunicka, 2003; Lang and Buchbauer, 2012; Nazzaro *et al*., 2017; Prakash *et al*., 2012). On the other hand, similar to what happens with bacteria, many cases of resistance to antifungal been reported (Fisher *et al*., 2022). Of the best known cases, infections caused by strains of *Candida* sp. have been reported. *Candida* species are commensals, they are part of the normal human flora and are localized on skin and gastrointestinal and genital tracts (Bhattacharya *et al*., 2020; Flores-Encarnación *et al.* 2022*a*; Pfaller and Diekema, 2007). However, *Candida* sp. causes also various infections in susceptible patients; it is one of the most common fungal infections globally with multiple mechanisms of antifungal resistance (Bhattacharya *et al.*, 2020). *S. cerevisiae* is widespread used in the biotechnology sector. *S. cerevisiae* has always had "generally regarded as safe" status and can, therefore, legally be used in the food and pharmacological sector. Classically, *S. cerevisiae* has been regarded as nonpathogenic, however numerous cases of clinical infection caused by *S. cerevisiae* have been reported. Some isolates are virulent and have been implicated in the induction of disease, particularly in immunocompromised individuals. *S. cerevisiae* is one of the emerging fungal pathogens with a unique characteristic: its presence in many food products. *S. cerevisiae* has an impeccably good food safety record compared to other microorganisms like virus, bacteria and some filamentous fungi. However, humans unknowingly and inadvertently ingest large viable populations of *S. cerevisiae* (home-brewed beer or dietary supplements that contain yeast) (Murphy and Kavanagh, 1999; Pérez-Torrado and Querol, 2016; Schreuder *et al*., 1996). As mentioned before, in this study the effect of *T. vulgaris* essential oil was determined on growth and biofilm formation of *S. cerevisiae*. Antifungal activity of *T. vulgaris* essential oil was determined using the technique of diffusion in agar. The results indicated a complete inhibition of the growth of *S. cerevisiae* at the amounts of the essential oil tested; the effect observed was fungicidal. The above is novel because there are few studies that have reported the antifungal activity of *T. vulgaris* essential oil against *S. cerevisiae*, as an emerging agent in public health. In recent years, it was reported that people with severe coronavirus disease-2019 (COVID-19) infection often exhibit an immunosuppression condition, resulting in greater chances of developing co-infections with bacteria and fungi, including opportunistic yeasts belonging to the *Saccharomyces* and *Candida* genera (Ramos *et al.,* 2023). In this study, another important aspect is that the essential oil of *T. vulgaris* had a more potent effect on the growth of *S. cerevisiae* than the antifungal drug used as reference. Using amphotericin B, some resistance to the drug was observed, however with the essential oil of *T. vulgaris* the growth of *S. cerevisiae* was completely inhibited. Since biofilm is considered a multifunctional structure with both virulence and resistance properties, capacity to form biofilm and the effect of *T. vulgaris* essential oil on biofilm formation were determined. As shown before, biofilm formation of *S. cerevisiae* was observed on the

polystyrene surfaces of the Petri dishes, but not on the glass surfaces of the used coverslips. When determining the effect of *T. vulgaris* essential oil on the formation of *S. cerevisiae* biofilm it was observed that the number of complete cells of *S. cerevisiae* decreased considerably and all *S. cerevisiae* cells were observed in a deep blue color, which indicated that the integrity of the membrane was lost due to the addition of *T. vulgaris* essential oil. *T. vulgaris* essential oil disrupted the biofilm formed by *S. cerevisiae* and affected the integrity of the cell membrane. Little is known about the mechanisms of action of the antifungal activity of essential oils. Some authors have reported that hydrophobicity of essential oils allows them to insert themselves between the lipids of the cell membrane of fungi and also in the membranes of mitochondria, thus increasing permeability and causing the release of intracellular constituents and interfering in different biological processes (Cristani *et al*., 2007; da Silva Bomfim *et al.*, 2015; Flores-Encarnación*et al*., 2022*a*; Paul *et al*., 2011; Wang *et al*., 2019). Other proposals related to the mechanisms of action of essential oils and their antifungal activity are known. So, it has been reported that essential oils could produce filamentation inhibition, disappearance of extracellular matrix, biofilm destruction, ergosterol inhibition, cytoplasm leak, and generation of reactive oxygen species (Daniellia *et al*., 2018; Jeong-Eun *et al.*, 2019; Rajkowska *et al*., 2019). As could be seen in this study, the action of *T. vulgaris* essential oil altering the permeability of the *S. cerevisiae* membrane is almost instantaneous. So, actively growing cells of *S. cerevisiae* were confronted with essential oil of *T. vulgaris*. The results obtained showed that immediately the cells were permeated by the trypan blue dye through the action of the essential oil, which indicated that the integrity of all cells of *S. cerevisiae* were affected by the essential oil of *T. vulgaris*. It has been reported that the essential oil of *T. vulgaris* contains several chemical components. Thymol, carvacrol and eugenol are the most active constituents with a wide antimicrobial spectrum. Both constituents destabilizes the cytoplasmic membrane (Bassolé *et al.* 2010; Al-Shuneigat *et al.*, 2014; Flores-Encarnación *et al.*, 2017; Flores-Encarnación *et al.*, 2022*a*; Flores-Encarnación *et al.*, 2022*b*; Soković *et al.*, 2010; Ultee *et al.*, 2002).

CONCLUSION

Microbial infections caused by bacteria, viruses and fungi are a common problem throughout the world. Antimicrobial resistance has exacerbated the problem. Therefore, the search for new compounds with antimicrobial activity is a common objective in many research groups. In this context, essential oils are novel due to their multiple properties. One of them is to be powerful antifungals, as is the case with *T. vulgaris* essential oil. In this work, some results were presented showing the antifungal potential of *T. vulgaris* in the growth and biofilm formation of *S. cerevisiae*, an emerging pathogenic yeast.

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