ISSN: 0975-833X

Available online at http://www.journalcra.com

International Journal of Current Research

Vol. 16, Issue, 07, pp.29109-29113, July, 2024
DOI: https://doi.org/10.24941/ijcr.47462.07.2024

INTERNATIONAL JOURNAL OF CURRENT RESEARCH

RESEARCH ARTICLE

THE INSTANT ANTIFUNGAL EFFECT OF THYMUS VULGARIS ESSENTIAL OIL

Flores-Encarnación M.[1*], Ocaña-Lozano D.[1], López-Vázquez I.[1], Xicohtencatl-Cortes J.[2]

[1]Laboratorio de Microbiología Molecular y Celular. Laboratorio 421 Edif. EMA1. Biomedicina, Facultad de Medicina, Benemérita Universidad Autónoma de Puebla. Puebla. Puebla. México; [2]Laboratorio de Bacteriología Intestinal. Hospital Infantil de México Federico Gómez. Ciudad de México. México

ARTICLE INFO

Article History:
Received 19th April, 2024
Received in revised form
15th May, 2024
Accepted 20th June, 2024
Published online 29th July, 2024

Key words:
Essential oil,yeast, *Saccharomyces cerevisiae*, antifungal, *Thymus vulgaris*.

ABSTRACT

Antibiotic resistance is a public health problem in the world. Therefore, efforts to find new substances with antimicrobial activity are increasing. Thus, it has been reported that essential oils represent an option because they have shown antimicrobial properties. Thyme (*T. vulgaris*) essential oil has antibacterial and antifungal properties. *T. vulgaris* essential oil is a powerful inhibitor of the growth of bacteria and also fungi. In this work, some data are presented regarding the antifungal action of *T. vulgaris* essential oil and the immediate effect as a fungicidal agent, using *S. cerevisiae* as a biological model.

***Corresponding author:**
M. Flores-Encarnación

Citation: Flores-Encarnación M., Ocaña-Lozano D., López-Vázquez I., Xicohtencatl-Cortes J. 2024. "The instant antifungal effect of *thymus vulgaris* essential oil.*". International Journal of Current Research*, 16, (07), 29109-29113.

INTRODUCTION

Currently, efforts have increased to reduce the use of chemicals as antimicrobial agents in the field of nutrition (Nazzaro *et al.*, 2017). However, the number of cases of microorganisms (for example, bacteria) that are resistant to antimicrobials is increasing, so increasing number of infections caused by antibiotic-resistant bacterial pathogens over the last few decades has become a critical global health problem, the scale of which has led to it being named a "silent pandemic". For common bacterial infections, high rates of resistance against the antibiotics frequently used to treat them have been observed worldwide, indicating that we are running out of effective antibiotics (Soto, 2023). Fungal infections are no exception. It has been reported that there are increased reports of antifungal drug resistance amongst emerged human fungal pathogens and is often found in emerging fungal pathogens. For example, *Cryptococcus gattii* has experienced a shift in species distribution to the Pacifc Northwest, and more recently *Candida auris* has emerged as an important nosocomial infection on all continents except Antarctica (Fraser *et al*., 2005; Hui *et al*., 2024; Rhodes, 2019; Satoh *et al.*, 2009). In this context, various studies are being carried out looking for alternative substances to combat infectious diseases caused by bacteria or fungi.

Thus, different substances of plant origin seem to be a good alternative since they have shown important antimicrobial activity. This is the case of some essential oils (Bertella *et al*. 2018; Flores-Encarnación *et al*., 2016; Flores-Encarnación *et al*., 2018). Over 2,500 essential oils have been identified, with many having commercial value in the pharmaceutical, agricultural, food, medical, cosmetic, and perfume industries. Many studies are currently being conducted to test the antibacterial effects of essential oils and their active compounds against bacteria in planktonic and sessile forms (Bouguenoun *et al*., 2023; Iseppi *et al*. 2020; Nuță*et al.*, 2021). Therefore, this work shows the antifungal action of *T. vulgaris* essential oil and the immediate effect as a fungicidal agent in *S. cerevisiae*.

MATERIALS 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* strain 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 30°C for 24 hours in glass tubes containing 5 mL of yeast 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).

Antifungal activity assay: 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). Then, sterile filter paper disks (5 mm diameter) were placed on the surface of yeast peptone dextrose agar plates. Different amounts of essential oil were used: 1.3, 2.6, 5.2, 7.8 and 13.2 mg. The agar plates were incubated at 30°C for 24 h. The diameters of the inhibition zones formed were measured. The analyses were conducted in triplicate. As reference, yeast peptone dextrose agar plates were inoculated by cross striation with *S. cerevisiae* and sterile filter paper disks were placed on them, adding different antifungal compounds: fluconazole (30 µg), ketoconazole (50 µg), amphotericin B (2 µg). The paper discs were also impregnated with 96% alcohol (10 µL) and 70% alcohol (10 µL). The plates were incubated at 30°C for 24 h. The diameters of the inhibition zones 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* (18-24 hours of culture, Ab_{560nm}=5) was centrifuged at 3,000 r.p.m. for 10 min. The supernatant was removed and 200 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 µL of 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 100°C for 10 minutes.

Effect of *T. vulgaris* essential oil on cell viability: 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, 1.3 mg of *T. vulgaris* essential oil was added; this mixture was incubated at room temperature at 1 min and 5 min. The preparations were observed at *40X* power. All determinations were made in triplicate. To determine the effect of *T. vulgaris* essential oil on cell viability at short time, the cell suspension was prepared and mixed with the trypan blue dye as described before. *T. vulgaris* essential oil (1.3 mg) was directly added into a cell of the Neubauer chamber, then 10 µL of cell suspension and trypan blue were added. At 30 seconds, the observation was carried out at *40X* power. In order to know if *T. vulgaris* completely inactivated to *S. cerevisiae*, the cells that were incubated for 5 min with the essential oil were washed twice using 50 mM Tris HCl buffer pH 7. Then, the pellet was resuspended with 200 µL of the same buffer and was plated by cross-striation on a yeast peptone dextrose agar plate. The agar plate were incubated at 30°C for 24 h.

RESULTS

The instant effect of *T. vulgaris* essential oil on *S. cerevisiae* cells was determinated. So, yeast peptone dextrose agar plates were prepared as described in Materials and Methods. Plates were inoculated by crossstriation, and sterile filter paper disks were placed on the surface of yeast peptone dextrose agar plates. On filter paper disks different amounts of essential oil were applied (1.3 to 13.2 mg). The agar plates were incubated at 30°C for 24 h. The results were shown in Fig. 1. As shown in Fig. 1A, *T. vulgaris* essential oil had a strong inhibitory effect on the growth of *S. cerevisiae*. In this image, the yeast peptone dextrose agar surface lacked growth by yeast and the surface of the agar acquired a bright appearance. There was no fungal growth at any of the amounts of *T. vulgaris* essential oil tested. As reference, different antifungal compounds were tested. So, yeast peptone dextrose agar plates were inoculated by crossstriation with *S. cerevisiae* and sterile filter paper disks were impregnated the antifungal compounds. The results are shown in Fig. 1B. As shown in Fig. 1B, *S. cerevisiae* was sensitive to ketoconazole showing the formation of a very noticeable zone of growth inhibition. As seen in the image, fluconazole and amphotericin B did not inhibited the growth of *S. cerevisiae*. As described in Materials and Methods, the effect of sanitizing compounds such as 70% and 96% ethanol was also tested. The results indicated that *S. cerevisiae* is capable of growing in the presence of ethanol at the concentrations tested (Fig. 1B). As could be seen, in none of the cases (using antifungal and sanitizing compounds) was obtained a similar result inhibiting the growth of *S. cerevisiae*, as when the *T. vulgaris* essential oil was used. Fig. 2C shows the growth inhibition zones of *S. cerevisiae* when low amounts of essential oil were used. As seen in the image, *T. vulgaris* essential oil (using 1 mg) produced a growth inhibition zone similar to that obtained using ketoconazole, while by doubling the amount tested the zone of inhibition of the growth of *S. cerevisiae* was much larger. In Fig. 1A, the different growth inhibition zones of *S. cerevisiae* could not be seen due to the synergistic effect of the essential oil (overlapping of growth inhibition zones).

This results showed the inhibition of the growth of *S. cerevisiae* after 20 hours, so in order to know the effect of the *T. vulgaris* essential oil at short times, it was determined the effect of essential oil on cell viability. For that, a cell suspension was prepared mixing with trypan blue dye as described in Materials and Methods. Then, 1.3 mg of *T. vulgaris* essential oil was added; this mixture was incubated at room temperature at different times: 1 and 5 min. The preparations were observed at *40X* power. For a time of 30 seconds, the test was carried out directly in a Neubauer chamber as indicated in Materials and Methods. At 30 seconds, the observation was carried out at *40X* power. The results are shown in Fig. 2. Fig. 2A shows cells of *S. cerevisiae* mixed with trypan blue.

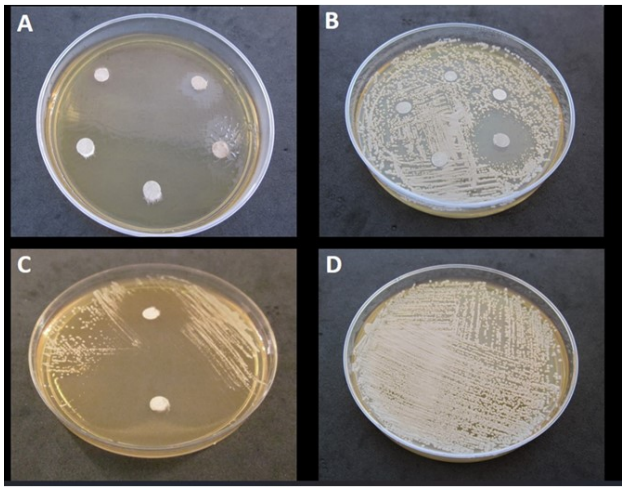

Fig. 1 Effect of *T. vulgaris* essential oil and antifungal compounds on the growth of *S. cerevisiae*. A. Strong antifungal activity of *T. vulgaris* essential oil. Essential oil increasing amounts (1.3 to 13.2 mg) were placed in the counterclockwise direction, starting with the top. B. Antifungigram for *S. cerevisiae*: 96% alcohol, fluconazole (30 µg), ketoconazole (50 µg), 70% alcohol and amphotericin B (2 µg). Antifungal compounds were placed in clockwise direction, starting with the top. C. Inhibition of the growth of *S. cerevisiae* by *T. vulgaris* essential oil at low amounts: 1.3 mg (top) and 2.6 mg (bottom). D. Control condition

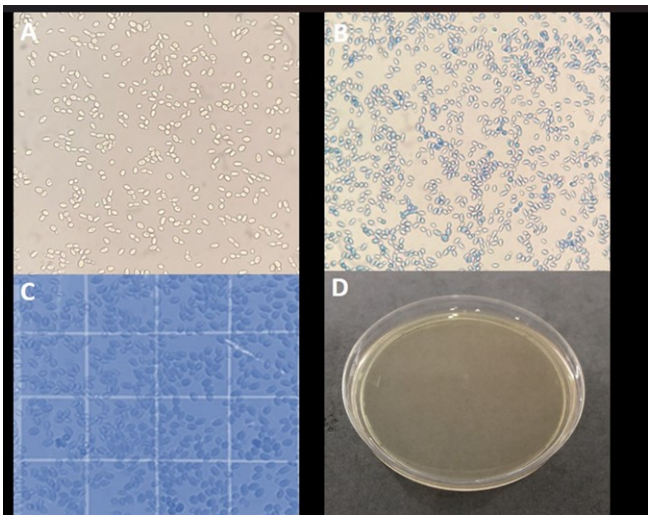

Fig. 2. Effect of *T. vulgaris* essential oil on cell viability. A. Cells of *S. cerevisiae* mixed with 0.1% trypan blue dye. B. Cells of *S. cerevisiae* treated with *T. vulgaris* essential oil for 1 min and stained with 0.1% trypan blue dye. C. Cells of *S. cerevisiae* treated with *T. vulgaris* essential oil for 30 seconds and stained with 0.1% trypan blue dye. D. Yeast peptone dextrose agar plate inoculated by cross-striation using with *S. cerevisiae* cells treated with *T. vulgaris* essential oil

As seen in this image, the cells of *S. cerevisiae* were not stained blue which indicated that the cells were intact. Fig. 2B shows the results obtained when the *S. cerevisiae* cells were incubated for 1 min with *T. vulgaris* essential oil. Similar results were obtained when incubating *S. cerevisiae* cells with *T. vulgaris* essential oil for 5 min (data not shown). The cells of *S. cerevisiae* stain blue due to the action of the *T. vulgaris* essential oil. Dead cells were observed in a deep blue color. In this image it also can be seen that almost all *S. cerevisiae* cells are intracellularly permeated by the dye, meaning that they are most likely dead cells due to the action of the *T. vulgaris* essential oil. As you can see, the cells retain their characteristic morphology but not their viability.

This was corroborated when the cells of *S. cerevisiae* (that were incubated for 5 min with the essential oil) were washed twice using 50 mM Tris HCl buffer pH 7, resuspended in same buffer and plated by cross-striation on a yeast peptone dextrose agar plate. The agar plate were incubated at 30°C for 24 h. The results obtained indicated that the cells of *S. cerevisiae* were not viable because they did not grow on yeast peptone dextrose agar plate (Fig. 2D). Finally, Fig. 2C shows the results obtained by incubating cells of *S. cerevisiae* for 30 seconds in the presence of *T. vulgaris*. The results indicated that at that time, the cells of *S. cerevisiae* stained blue. The relevant thing about this is that apparently the *T. vulgaris* essential oil performs its antifungal action instantly.

DISCUSSION

The emergence of microbial infections has motivated the search for new drugs to combat pathogenic microorganisms, especially when there are cases of resistance to drugs (such as the bacteria to antibiotics). An alternative has been the use of natural products, through their extracts or essential oils obtained from plants and vegetables (Aljeldah, 2022; Flores-Encarnación *et al*., 2022; Galgano *et al*., 2022). Essential oils are aromatic compounds found in great quantities in oil sacs or oil glands present at different depths in the fruit peel, mainly flavedo part and cuticles. Essential oils are aromatic oil liquids extracted from different parts of plants for instance, leaves, barks, seeds, flowers and peels (Herman *et al.*, 2019; Mahato *et al*., 2017; Tongnuanchan and Benjakul, 2014). The essential oils are secondary metabolites produced by plants in order to provide a defense function or attraction (Butkiené *et al*., 2015; Flores-Encarnación *et al*., 2016). Essential oils are characterized by a complex mixture of several compounds belonging to different classes of organic chemistry, for example: hydrocarbons, phenols, terpenes, alcohols, aldehydes, ketones, esters, ethers and others (Butkiené *et al*., 2015; Castañeda *et al*., 2007; Flores-Encarnación *et al*., 2016; Hassan *et al*., 2016; Kordali *et al*., 2005). It has been reported that thyme (*T. vulgaris*) essential oil produce total inhibition of growth of uropathogenic *E. coli*; it is a potent inhibitor of the growth of uropathogenic *E. coli* (Flores-Encarnación *et al*., 2018). In general, different essential oils have been tested on both Gram-negative and Gram-positive bacteria, as well as fungi. In these cases, it has been observed that essential oils have antimicrobial properties. However, in this study we were interested in determining the time required for the *T. vulgaris* essential oil produce growth inhibition, using *S. cerevisiae* as a model. As shown in Results, the *T. vulgaris* essential oil had a strong inhibitory effect on the growth of *S. cerevisiae.* There was no fungal growth at any of the amounts of *T. vulgaris* essential oil tested. The above is in accordance with Konuk and Ergüden, (2017) and Kovacik *et al*., (2022) which reported strong antimicrobial activity of thyme and oregano essential oils on *S. cerevisiae*. They determined minimal inhibition concentration at the level of 0.2 - 0.3 µL/mL for both essential oils. Bennis *et al*., (2004) used pure thymol, one of the main components of *T. vulgaris* essential oil, for inhibition of *S. cerevisiae* growth. For complete inhibition of *S. cerevisiae* growth, 3 mM of thymol dispersed in 0.2% agar solution was sufficient. Using concentration higher than minimal inhibition concentration caused total mortality (Kovacik *et al*., 2022). In this study, it was observed that *S. cerevisiae* cells treated with *T. vulgaris* essential oil (washed several times) die; the results obtained indicated that the cells of *S. cerevisiae* were not

viable because they did not grow on yeast peptone dextrose agar plate. Bennis *et al*., (2004) reported that scanning electron microscopy clearly showed surface of many cells is deformed and cells in the background showed important damage consisting in apparent cracks. In the present study, the latter explains the entry of the trypan blue dye into *S. cerevisiae* cells, when they are treated with the *T. vulgaris* essential oil. As could be seen in the images, the *S. cerevisiae* cells elongated slightly when exposed to the *T. vulgaris* essential oil and yeast lysis was not observed. Despite its non-pathogenic association, there is a growing prevalence of *S. cerevisiae* isolation from various human anatomical sites, including the gastrointestinal, respiratory and genital tracts, driven by emerging reports of infections caused by this once-considered benign species. While the frequency of the most severe infections, such as fungemia caused by *S. cerevisiae* yeast, remains unknown, it is estimated that they may account for 0.1-3.6% of all cases of bloodstream fungal infections (Górzyńska *et al*., 2024; Roeske *et al*., 2020; Seng *et al*., 2016). In recent years it has been reported that *S. cerevisiae*, a close relative of the pathogenic *Candida* species, is an emerging opportunistic pathogen. Therefore, it is common to find *S. cerevisiae* in different parts of the body and clinically, in different types of patients as it is an emerging opportunistic pathogen. Unlike laboratory *S. cerevisiae* strains and other non-clinical strains, *S. cerevisiae* strains from clinical isolates have characteristics that resemble those found in pathogenic fungi, such as profuse pseudohyphal formation and growth at high temperaturas (Flores-Encarnación *et al*., 2024). According to the above, *S. cerevisiae* was chosen as a model to study the effects of *T. vulgaris* essential oil. It has been reported that infections associated with *S. cerevisiae* from organ-specific to generalized mycoses, including fungemia, endocarditis, liver abscesses, and pneumonia and vulnerable populations involve mostly premature infants, individuals over 60, and those under immunosuppression or intensive care, and instances of infection have also been documented in immunocompetent patients. The infection routes involve translocation from the intestine to the bloodstream, dissemination to other organs, and catheter-related infections, particularly central venipuncture. A notable risk factor is the intake of probiotics containing *S. cerevisiae* var. *boulardii*, surpassing the cumulative incidence of infections caused by probiotic bacteria (Gómez-López *et al*., 2008; Górzyńska *et al*., 2024; Roeske *et al*., 2020). On the other hand, in this study *S. cerevisiae* was sensitive to ketoconazole, However, the *T. vulgaris* essential oil produced the greater growth inhibition than ketoconazole. Fluconazole and amphotericin B did not inhibited the growth of *S. cerevisiae* at the concentrations tested. It has been reported that there is little knowledge about the drug susceptibility of *S. cerevisiae*.

Górzyńska *et al.*, (2024) reported susceptibility of 55 clinical isolates identified as *S. cerevisiae* finding that most strains were sensitive to amphotericin B, flucytosine and echinocandins. They also reported isolates that expressed high minimum inhibitory concentrations values for azoles, indicating cross-resistance. In this study, *S. cerevisiae* was able to grow in the presence of ethanol at different concentrations. Wolf *et al*., (2023) reported in *S. cerevisiae* that longevity, peroxisomal, energy, lipid, and RNA/protein metabolisms are the core processes that drive etanol tolerance. Finally, the most relevant about this study was that *T. vulgaris* essential oil produced the death of *S. cerevisiae* cells in very short times, almost instantaneously.

At 30 seconds, the cells of *S. cerevisiae* were dyed blue due to the action of *T. vulgaris* essential oil, which reaffirms that the essential oil of *T. vulgaris* is a powerful antifungal, with fungicidal function. *T. vulgaris* essential oil performs its antifungal action instantly.

CONCLUSION

As is known, resistance to antimicrobials is increasing, resistance to antifungal compounds being no exception. Therefore, new compounds are tested every day. Essential oils have shown important antimicrobial properties. Their effect has been studied on Gram positive and negative bacteria, as well as on different fungi. However, the studies have been carried out for long periods of time (18 to 24 hours). In this work, the antifungal action of *T. vulgaris* essential oil was confirmed, demonstrating that it has a powerful growth inhibitory effect on *S. cerevisiae* and that its action is immediate.

ACKNOWLEDGEMENTS

Thanks to Facultad de Medicina-BUAP and Grupo de Académicos de Puebla SC for the facilities provided for the development of this work. We also thank G. Sayago, Biomedicine student, for his valuable technical assistance.

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