ISSN: 0975-833X

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

*International Journal of Current Research*

**Vol. 16, Issue, 11, pp.30522-30527, November, 2024**
**DOI: https://doi.org/10.24941/ijcr.48047.11.2024**

**INTERNATIONAL JOURNAL OF CURRENT RESEARCH**

# RESEARCH ARTICLE

## THYMUS VULGARIS ESSENTIAL OIL AND ITS EFFECT ON *SACCHAROMYCES CEREVISIAE*

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**ARTICLE INFO**

***Article History:***
Received 24th August, 2024
Received in revised form
17th September, 2024
Accepted 29th October, 2024
Published online 30th November, 2024

***Key Words:***
Essential oil, *Thymus vulgaris*, Saccharomyces cerevisiae, antimicrobial, respiratory activity.

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**ABSTRACT**

For a long time, some properties of essential oils have been known. One of these is their antimicrobial effect. Due to a significant increase in resistance to antimicrobials (especially antibiotics), essential oils has been proposed as an alternative to combat antibiotic-resistant bacterial infections. Essential oils are also good antifungals but little about their effects is known. Therefore, this work shows some data related to the effect of *T. vulgaris* essential oil on *S. cerevisiae*.



**Citation: Flores-Encarnación M. and Hernández-Hernández F.C. 2024.** "Thymus vulgaris essential oil and its effect on *saccharomyces cerevisiae".* *International Journal of Current Research*, 16, (11), 30522-30527.

# INTRODUCTION

The need for new therapeutic approaches to control bacterial infections is crucial. Treatments of bacterial infections are complicated because antibiotic resistance. So, discovering new antibiotics from medicinal plants, able to kill drug-resistant bacteria is essential to saving modern medicine (Lahlou *et al.,* 2023). For a long time now, the antimicrobial and other properties of essential oils are known (Flores-Encarnación *et al*., 2016). The essential oils have emerged as alternative antimicrobial products due to their strong and wide-spectrum activity against microorganisms, in addition to their eco-friendly and human safety status (Amassmoud *et al*., 2023; Bhattacharya *et al*., 2021; de Souza Pedrosa *et al*., 2019). Essential oils are secondary metabolites extracted from aromatic plants, primarily colorless, lipophilic, and volatile in nature and are found in various plant organs, including fruits, bark, rhizomes, roots, flowers, resins, seeds leaves, and wood. Essential oils consist of a mixture of compounds including terpenes and aroma compounds like phenols, hydrocarbons, aldehydes, alcohols, methoxy derivatives, and methylenedioxy compounds (Abdi-Moghadam *et al.*, 2023; Bahmani *et al.*, 2022; Bora *et al*., 2020; Falahi *et al.*, 2019; Mazaheri *et al.*, 2019; Shamloo *et al.*, 2023).

Studies are known that have shown that essential oils inhibit or slow the growth of yeasts and molds (Flores-Encarnación *et al*., 2022). Maness and Zubov (2019) reported that essential oils of *Rosmarinus officinalis*, *Cinnamomum verum* and *Citrus paradise* inhibited the growth of *Trichophyton mentagrophytes*, *Microsporum gypseum* and *Rhizopus stolonifer*. There is evidence about antimicrobial activities attributed to specific compounds related to monoterpenes such as thyme, carvacrol, α- pinene, linalool, methyl salicylate, eugenol and geraniol (Monzote-Fidalgo *et al*., 2004; Prasanth *et al*., 2014; Scalas *et al*., 2018; Wińska *et al*., 2019). Most studies using essential oils have been carried out on bacteria, however little is known about of effect on fungi and the mechanisms involved in antifungal activity of essential oils. In the present work, the effect of *T. vulgaris* essential oil on *S. cerevisiae* was studied.

# MATERIAL AND METHODS

**Source of material:** In this study, the commercial essential oil of thyme 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 pH 6.5. 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 (100 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 (100 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*.

**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 at 4 °C. 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 30 seconds. The preparations were observed at 40X power. All determinations were made in triplicate.

**Respiratory activity:** The respiratory activity was measured polarographically with a Clark oxygen electrode according to the methodology established by Flores-Encarnación *et al*., (2020). For it, cells of *S. cerevisiae* were used. The cells of *S. cerevisiae* were obtained from a culture in a 125 mL Erlenmeyer flask containing 50 mL of fresh yeast peptone dextrose broth pH 6.5. Erlenmeyer flask was inoculated with 625 µL from stationary 24-hour preculture of *S. cerevisiae* in yeast peptone dextrose broth ($Ab_{560nm}$=6) and was incubated at 30°C with shaking at 150 r.p.m. for 18-24 hours. In the end, the shaken culture showed an $Ab_{560nm}$= 9.4. To determine the respiratory activity of *S. cerevisiae*, 3 mL of the cell suspension were used. The cells of *S. cerevisiae* were washed twice with buffer 50 mM Tris-HCl pH 7.0, centrifuging at 3,000 r.p.m. for 10 min at 4 °C. The pellet was resuspended using 200 µL of buffer 20 mM phosphate pH 6.5 (cell suspension). The reaction mixture (final volumen= 6 mL) contained: buffer 20 mM phosphate pH 6.5 and 40 mM glucose. The reactions were initiated adding the cell suspension. The oxygen consumption kinetics were recorded for 30 min. The temperature was kept constant at 30°C. In all tests, the respiratory activities of *S. cerevisiae* were reported as consumed nmol $O_2$ $min^{-1}$. The analyses were conducted in triplicate.

**Effect of *T. vulgaris* on respiratory activity:** The effect of *T. vulgaris* essential oil on respiratory activity of *S. cerevisiae* was determinated. For that, 3 mL of washed cells of *S. cerevisiae* were resuspended in 200 uL of buffer 20 mM phosphate pH 6.5 and then incubated with *T. vulgaris* essential oil (1.3 mg and 2.6 mg, separately) for 10 min at room temperature. At the end of incubation, the reaction mixture contained (final volumen= 6 mL): buffer 20 mM phosphate pH 6.5 and 40 mM glucose. The reactions were initiated adding the cell suspension incubated with *T. vulgaris*. The oxygen consumption kinetics were recorded for 30 min. The temperature was kept constant at 30°C. In all tests, the respiratory activities of *S. cerevisiae* were reported as consumed nmol $O_2$ $min^{-1}$. The analyses were conducted in triplicate.

**Detection of cytoplasmic protein released by *T. vulgaris* essential oil:** To detect cytoplasmic protein released by *T. vulgaris* essential oil, 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 at 4 °C. The pellet was washed 3 times using buffer 50 mM Tris-HCl pH 7.0. The supernatant obtained after the third wash of *S. cerevisiae* pellet was collected in a 1.5 mL sterile centrifuge tube and stored at -40 °C until use. The pellet was resuspended using 200 µL of buffer 50 mM Tris-HCl pH 7.0 (cell suspension); then 50 µL of cell suspension was placed in a 1.5 mL centrifuge tube and 13 mg of *T. vulgaris* essential oil were added. The mixture was incubated 5 min at room temperature. At the end of incubation, the mixture was centrifuged at 3,000 r.p.m. for 10 min at 4 °C and the supernatant was collected and stored at -40 °C until use. The protein concentration of supernatant was determined by a modification of the Lowry method (Dulley and Grieve, 1975). For reference, 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 at 4 °C. The pellet was washed 3 times using buffer 50 mM Tris-HCl pH 7.0. The pellet was then disrupted using a laboratory-implemented alkaline SDS lysis technique (data not shown). The analyses were conducted in triplicate.

# RESULTS

In this study, the effect of *T. vulgaris* essential oil on cells of *S. cerevisiae* was determined. So, the antifungal activity of essential oil was determined using the technique of diffusion using yeast peptone dextrose agar plates.

Sterile filter paper disks were placed on the surface of yeast peptone dextrose agar plates as described in Materials and Methods. Different amounts of essential oil were used: 1.3, 2.6, 5.2, 7.8 and 13.2 mg. The results obtained are 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 agar acquired a bright appearance. On the other hand, the effect of *T. vulgaris* essential oil on viability of *S. cerevisiae* cells directly was determined as described in Materials and Methods. So, 10 µL of cell suspension were mixed with 10 µL of 0.1% trypan blue dye and 1.3 mg of *T. vulgaris* essential oil was added. This mixture was incubated at room temperature at 1 min and 30 seconds observing at 40X power. The results obtained are shown in Fig. 1C. As shown in Fig. 1C, *S. cerevisiae* cells were stained by trypan blue dye after being incubated with *T. vulgaris* essential oil (both at 30 seconds and at 1 min). Cells of *S. cerevisiae* were intracellularly permeated by the dye, meaning that they are most likely dead cells 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 the cells retain their characteristic morphology but not their viability. Cells that were not incubated with *T. vulgaris* essential oil did not show the blue color when stained with trypan blue (Fig. 1D). Based on the above results, it was proposed that the *T. vulgaris* essential oil produced pores in the envelope of *S. cerevisiae* cells and thus the release of cytoplasmic content. Therefore, detection of released protein was carried out, as described in Materials and Methods. The protein concentration of supernatant was determined by a modification of the Lowry protein assay.

The protein concentration quantified from the obtained supernatants was around 3.8 µg · µL cell suspension$^{-1}$. The protein concentration of *S. cerevisiae* cells not treated with *T. vulgaris* essential oil was also quantified. The results were similar to the protein concentration quantified in the supernatants (data not shown), which suggested that *T. vulgaris* essential oil released a large amount of cytoplasmic proteins to the exterior of cells. This could explain the strong inhibitory effect observed on the growth of *S. cerevisiae*. Finally, the respiratory activity of *S. cerevisiae* cells was determinated. The respiratory activity was measured polarographically with a Clark oxygen electrode according to the methodology described in Materials and Methods. The respiratory activities of *S. cerevisiae* were reported as consumed nmol $O_2$ min$^{-1}$. To determine the effect of *T. vulgaris* essential oil on *S. cerevisiae*, cells were incubated in the presence of essential oil (1.3 and 2.6 mg) for 10 min at room temperature. The results were shown in Table 1. As shown in Table 1, the respiratory activity rate (measured as oxidase activity) of *S. cerevisiae* cells was approximately 16 nmol $O_2$ min$^{-1}$, using glucose as a substrate. The addition of *T. vulgaris* essential oil inhibited the respiratory activity. The addition of 1.3 mg of *T. vulgaris* inhibited respiratory activity by approximately 50%, while the addition of 2.6 mg of the essential oil produced an 80% inhibition of respiratory activity. As can be seen, the presence of the essential oil produced a significant decrease in the respiratory rates of *S. cerevisiae* cells. This could be attributed to a significant loss in the protein components present in the cytoplasm of *S. cerevisiae* cells, as could be detected in the assays performed directly on *S. cerevisiae* cells in this study.

It is also proposed that *T. vulgaris* essential oil could affect some other components involved in the respiration of *S. cerevisiae*, probably related with mitochondrial activity.

# DISCUSSION

Essential oils are very complex mixtures of volatile substances obtained from plants. 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 (Gershenzon *et al*., 2007; Vigan, 2010; Żukowska and Durczyńska 2024). Essential oils are widely used in medical and health industries due to their antioxidant, anti-inflammatory, antitumor, antibacterial and other health benefits. They have been used in various fields like food, biomedicine, textile, and agriculture for their preservatives, pharmacological and antimicrobial effects (Calo *et al*., 2015; Jugreet *et al*., 2020; Prakash *et al*., 2015; Zhao *et al*., 2023). Essential oils are a rich source of broad-spectrum antifungal plant-derived metabolites that inhibit both fungal growth and their production of toxic metabolites. Some of these essential oils that displayed effectiveness in reducing fungal decay are tea seed, camellia, oregano, cinnamon, lemongrass, sunflower seed, clove, and fennel. As a result, the production and consumption of essential oils have expanded over the world in recent years (Abdel-Khalek *et al.,* 2022; Allaguie*t al*., 2024; Basaglia *et al.,* 2021; Lee *et al*., 2020; Liu *et al*., 2020; Moumni *et al*., 2021; Phuong *et al*., 2023; Sharma *et al*., 2023; Sun *et al*., 2021; Wani *et al.*, 2021; Xiong *et al*., 2020; Zhou *et al*., 2023).

In the present study, the effect of *T. vulgaris* essential oil on *S. cerevisiae* was determined. The results obtained shown that *T. vulgaris* essential oil had a strong inhibitory effect on the growth of *S. cerevisiae*. This was interesting because essential oils represent compounds that could be used in antifungal treatments. The results of this study were obtained using *S. cerevisiae* as a biological model, however essential oils have been tested against other pathogenic fungi (molds and yeasts) that cause diseases and are important in public health (Flores-Encarnación *et al.*, 2022). 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 (Flores-Encarnación *et al.*, 2022; Gucwa *et al.*, 2018). Maness and Zubov (2019) reported that essential oils of *Rosmarinus officinalis, Cinnamomum verum* and *Citrus paradis i*inhibited the growth of *Trichophyton mentagrophytes, Microsporum gypseum* and *Rhizopus stolonifer*. The yeast *S. cerevisiae*, a close relative of the pathogenic *Candida* species, has traditionally been considered a non-pathogenic fungus. However, there are reports about its role as an emerging opportunistic pathogen fungus. 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 temperatures (Byron *et al*. 1995; Corrêa-Moreira *et al*., 2024; Clemons *et al.* 1994; Ellouze*et al.*, 2016; Flores-Encarnación *et al.*, 2024; Goldstein *et al*., 2001; Gupta *et al*., 2019; Hazen, 1995; McCusker *et al*. 1994; Murphy and Kavanagh, 1999).

**Table 1. The effect of *T. vulgaris* essential oil on respiratory activity of *S. cerevisiae***

| *T. vulgaris* essential oil (mg) | Glucose-oxidase activity (nmol $O_2$ $min^{-1}$) | Relative respiratory activity (%) |
|---|---|---|
| 0 | 15.645 | 100 |
| 1.3 | 7.995 | 51.1 |
| 2.6 | 3.109 | 19.9 |

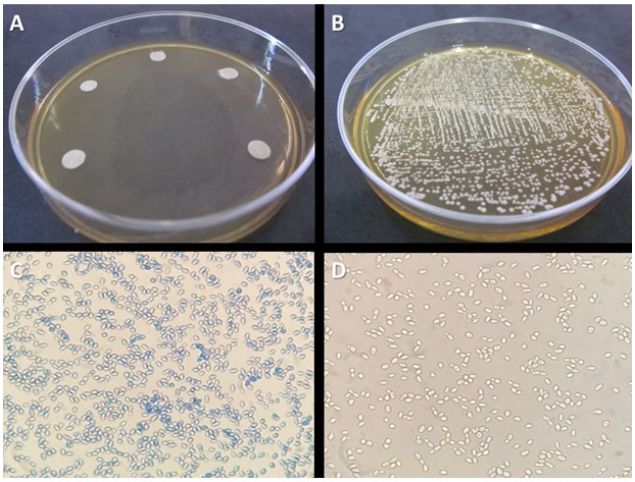


**Fig. 1. The antifungal activity of *T. vulgaris* essential oil.A. Strong antifungal activity of *T. vulgaris* essential oil (yeast peptone dextrose agar plate assay). Essential oil increasing amounts (1.3 to 13.2 mg) were placed in the counterclockwise direction, starting with the top. B. Growth of *S. cerevisiae* on yeast peptone dextrose agar (control condition). C. The viability of *S. cerevisiae* cells was affected by *T. vulgaris* essential oil (direct assay in cells). Dead cells were stained by trypan blue dye. D. Intact *S. cerevisiae* cells stained with trypan blue (control condition)**

On the other hand, it has been reported that *T. vulgaris* essential oil contains monoterpenes as: thymol (49%), ρ-cimene (18%), carvacrol (6%), γ- terpinene (9%), linalool (3%), car-3-eno (2%), β- mirceno (2%), α- pinene (1%), limonene (1%) and camphane (0.5%), and that thymol and carvacrol are the major ingredients in *T. vulgaris* essential oil with antimicrobial and pharmacological properties (such as anti-metastatic, anti-oxidative, anti-inflammatory effects) (Ben *et al*., 2019; Flores-Encarnación *et al.*, 2022; Sakkas and Papadopoulou, 2007). In the present study, the effect of *T. vulgaris* essential oil on viability of *S. cerevisiae* cells was also determined. The results showed that *S. cerevisiae* cells were stained by trypan blue (cells were observed in a deep blue color) after being incubated with *T. vulgaris* essential oil, which indicated that the cells were dead. However, the cells retained their characteristic morphology; they were not lysed, only their viability was affected. Thus, it was proposed that the *T. vulgaris* essential oil produced pores in the envelope of *S. cerevisiae* cells and loss of cytoplasmic content. In this regard, some authors have reported that hydrophobicity of essential oils allows them to insert themselves between the lipids of the cell membranes of fungi, as well as in the membranes of mitochondria 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; Paul *et al*., 2011; Wang *et al*., 2019). Wang *et al*., (2019) found that high concentration of essential oil led to the membrane permeability increased and nucleic acid released from *C. gloeosporioides*, as well as a marked decrease in the protein content of fungal cells by increasing the concentration of essential oil, affecting the permeability of cell membrane and wall. To verify the loss of cytoplasmic content, in the present study the protein concentration in the supernatants obtained after treating *S. cerevisiae* cells with *T. vulgaris* essential oil was quantified. The results indicated that *T. vulgaris* essential oil released a large amount of cytoplasmic proteins to the exterior of cells and this could explain the strong inhibitory effect observed on the growth of *S. cerevisiae*. Finally, in order to know if *T. vulgaris* essential oil had any effect on the mitochondrial activity of *S. cerevisiae*, the respiratory activity in complete cells was determinated. The results indicated that the two quantities of essential oil tested the respiratory activity decreased considerably (up to 80%). So, the presence of *T. vulgaris* essential oil caused the loss of respiratory activity in *S. cerevisiae* cells, which is directly related to the loss of cytoplasmic content. As noted above, the hydrophobicity of essential oils should facilitate them to insert between the lipids of cell membranes, including mitochondrial membranes.

# CONCLUSION

As is known, most of the studies carried out with essential oils for their antimicrobial properties have been done on bacteria. In this study, it was observed that *T. vulgaris* essential oil had a strong inhibitory effect on the growth of *S. cerevisiae*, showing effects at the level of membrane permeability and thus, producing the release of cytoplasmic content and also affecting cellular respiration.

# ACKNOWLEDGEMENTS

We appreciate the enthusiastic collaboration and technical support of Ocaña-Lozano D. and López- Vázquez I. from Biomedicina-BUAP. Thanks to Facultad de Medicina-BUAP and Grupo de Académicos de Puebla SC for the facilities provided for the development of this work.

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