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# RESEARCH ARTICLE

## A SIMPLE METHOD FOR OBTAINING AN ESSENTIAL OIL WITH ANTIMICROBIAL PROPERTIES

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**ABSTRACT**

Essential oils are volatile hydrophobic substances with pleasant scents that have demonstrated antimicrobial properties. Their natural origin makes essential oils a potential alternative for treating infectious diseases. They possess antibacterial, antifungal, and other properties. Therefore, this paper shows a simple method for extracting essential oil from *T. vulgaris* and some of its antimicrobial properties.



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# INTRODUCTION

In recent years, various plant-based substances have been the subject of study due to their antimicrobial properties because plants are rich sources of secondary metabolites (Flores-Encarnación *et al*., 2016; Flores-Encarnación *et al*., 2022*c*; Gaikwad *et al.*, 2025). Among these substances are essential oils which are substances obtained from flowers, leaves, fruits, branches, seeds, bark by different methods (Burt, 2004; Citarasu, 2010; Cowan, 1999; Flores-Encarnación *et al*., 2016). The essential oils are secondary metabolites produced by plants in order to provide a defense function or attraction (Butkiené *et al*., 2015). Due to the antimicrobial properties of essential oils, they have been proposed as possible alternative substances to combat pathogenic microorganisms especially when there are cases of resistance to antibiotics (Aljeldah, 2022; Galgano *et al.*, 2022). There have been a number of reports validating the in vitro antibacterial and antifungal activities of this essential oil on some human pathogens, including *Pseudomonas aeruginosa*, *Staphylococcus aureus*, *Escherichia coli*, *Proteus mirabilis*, *Salmonella* sp. *Mycobacterium smegmatis* (Flores-Encarnación *et al*., 2020). Therefore, this work shows a simple method for obtaining an essential oil and the antimicrobial properties.

# MATERIAL AND METHODS

**Source of material:** In this study, the essential oil of *T. vulgaris* was distilled from partially crushed fresh thyme leaves. Thyme was obtained of a typical market at Puebla city, México. The essentail oil was prepared by distillation dragging water vapor from thyme leaves.

**Biological material:** The strain of uropathogenic *E. coli* CFT073 was used. Bacterial strain was stored in cryovials at -40°C until analysis. 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.

**Distillation dragging water vapor:** To obtain the essential oil of *T. vulgaris*, 20 grams of partially crushed fresh thyme leaves were used. The grinding was carried out using a sterile mortar

and pestle. The thyme leaves were placed in a 500 mL round glass flask containing 200 mL of distilled water, which was heated to boiling using a heat source. Using 5 mm glass tubing, the water vapor generated in the first round flask was passed to a second 500 mL flask, containing 20 grams of the ground thyme leaves. The second round-bottom flask was connected to a condenser to recover the *T. vulgaris* essential oil entrained by the water vapor. The condensate was recovered in a 250 mL Erlenmeyer flask. After 3 hours, about 100 mL of distillate was recovered and protected from light using aluminum foil. Then, the distillate obtained was then placed in a 500 mL glass separation funnel and 15 mL of chloroform was added, stirring vigorously for 30 min (releasing excess gas periodically). This process was carried out at room temperature in a gas extraction system. Phase separation between chloroform (below) and water (above) was immediately observed. To recover a larger amount of *T. vulgaris* essential oil, the separating funnel was left to stand for 24-48 hours at room temperature in low light. The chloroform phase (below, containing the essential oil) was recovered in an amber glass bottle. Chloroform was removed from the *T. vulgaris* essential oil using a continuous low flow of air passed over the surface of the chloroform phase for 3 hours at room temperature within a gas extraction system. The obtained *T. vulgaris* essential oil was stored in a sterile 1.5 mL centrifuge tube and protected from light.

**Culture:** The uropathogenic *E. coli* strain was cultured at 37°C for 18 to 24 h in trypticase soy broth (BBL Microbiology Systems, Cockeysville, Md). For it, sterile Petri dishes (150 mm) were used and tripticasein soy agar plates (containing 20 mL of medium) were prepared. Plates were inoculated by crossstriation with uropathogenic *E. coli*. Each inoculum contained approximately $10^6$ CFU $mL^{-1}$. *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).

**Antimicrobial activity of essential oil:** The antibacterial activity of *T. vulgaris* essential oil was determined using the technique of disk by diffusion in agar on uropathogenic *E. coli* and *S. cerevisiae* growth. For this, Petri dishes containing trypticasein soy agar and yeast peptone dextrose agar were seeded as follows: half of the agar plate was seeded with the bacteria, then 2 additions of 14.4 mg of the *T. vulgaris* essential oil were made in two different areas of the trypticasein soy agar plate. Other half of the agar plate was seeded with uropathogenic *E. coli* (control). The agar plates were incubated at 37°C for 24 h. The inhibition zones formed were observed. The analyses were conducted in triplicate. Each inoculum contained approximately $10^6$ CFU $mL^{-1}$. 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: 0.72, 1.44, 2.88, 4.32 and 7.2 mg. The agar plates were incubated at 30°C for 24 h. The inhibition zones formed were observed. The analyses were conducted in triplicate.

**Cell viability assay:** The cell viability assay was performed using *S. cerevisiae* cells and the trypan blue dye according to modified 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 viability of *S. cerevisiae* cells was determined as follows. The cell suspension was prepared and mixed with the trypan blue dye as described before. Then, 0.72 mg of *T. vulgaris* essential oil was added; this mixture was incubated at room temperature at 15 min. The preparations were observed at ***4*0X** power. All determinations were made in triplicate.

# RESULTS

In this study, a simple method for obtaining an essential oil and the antimicrobial properties were determinated. So, *T. vulgaris* essential oil of was prepared by distillation dragging water vapor from 20 grams of partially crushed fresh thyme leaves (Fig. 1A). As described in Materials and Methods, thyme leaves were placed in a 500 mL round glass flask containing 200 mL of distilled water, which was heated to boiling using a heat source. The water vapor generated in the first round flask was passed to a second 500 mL flask, containing the ground thyme leaves. The second round-bottom flask was connected to a condenser to recover the *T. vulgaris* essential oil entrained by the water vapor (Fig. 1B). The condensate was recovered in a 250 mL Erlenmeyer flask. After 3 hours, about 100 mL of distillate was recovered and protected from light using aluminum foil (Fig. 1C). Then, the distillate obtained was then placed in a 500 mL glass separation funnel and 15 mL of chloroform was added, stirring vigorously for 30 min (releasing excess gas periodically). This process was carried out at room temperature in a gas extraction system. Phase separation between chloroform (down) and water (up) was immediately observed (Fig. 1D). To recover a larger amount of *T. vulgaris* essential oil, the separating funnel was left to stand for 24-48 hours at room temperature in low light. The chloroform phase (below, containing the essential oil) was recovered in an amber glass bottle. Chloroform was removed from the *T. vulgaris* essential oil using a continuous low flow of air passed over the surface of the chloroform phase for 3 hours at room temperature within a gas extraction system. The obtained *T. vulgaris* essential oil was stored in a sterile 1.5 mL centrifuge tube and protected from light (Fig. 1E). On the other hand, the antimicrobial activity of *T. vulgaris* essential oil was determined. For this, the technique of disk by diffusion in agar

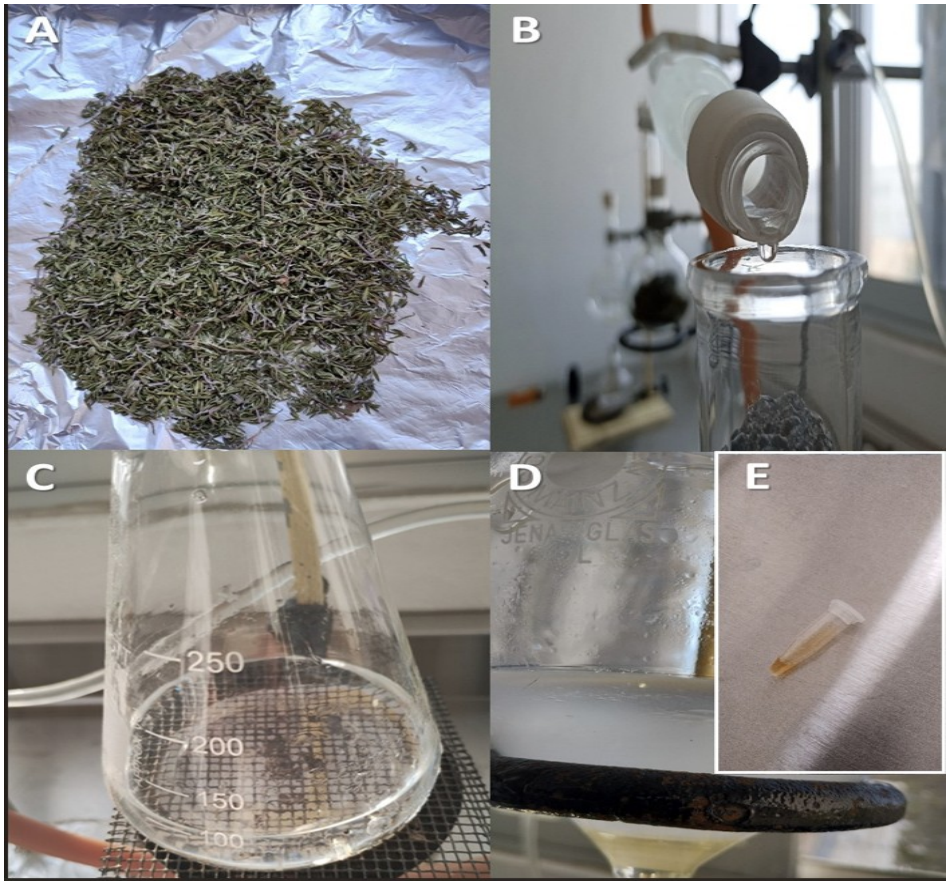


**Fig. 1 A simple method for obtaining an essential oil. A. Partially crushed fresh thyme leaves. B. Distillation dragging water vapor. C. Recovered distillate. D. Chloroform phase containing the essential oil (down). E. *T. vulgaris* essential oil obtained.**

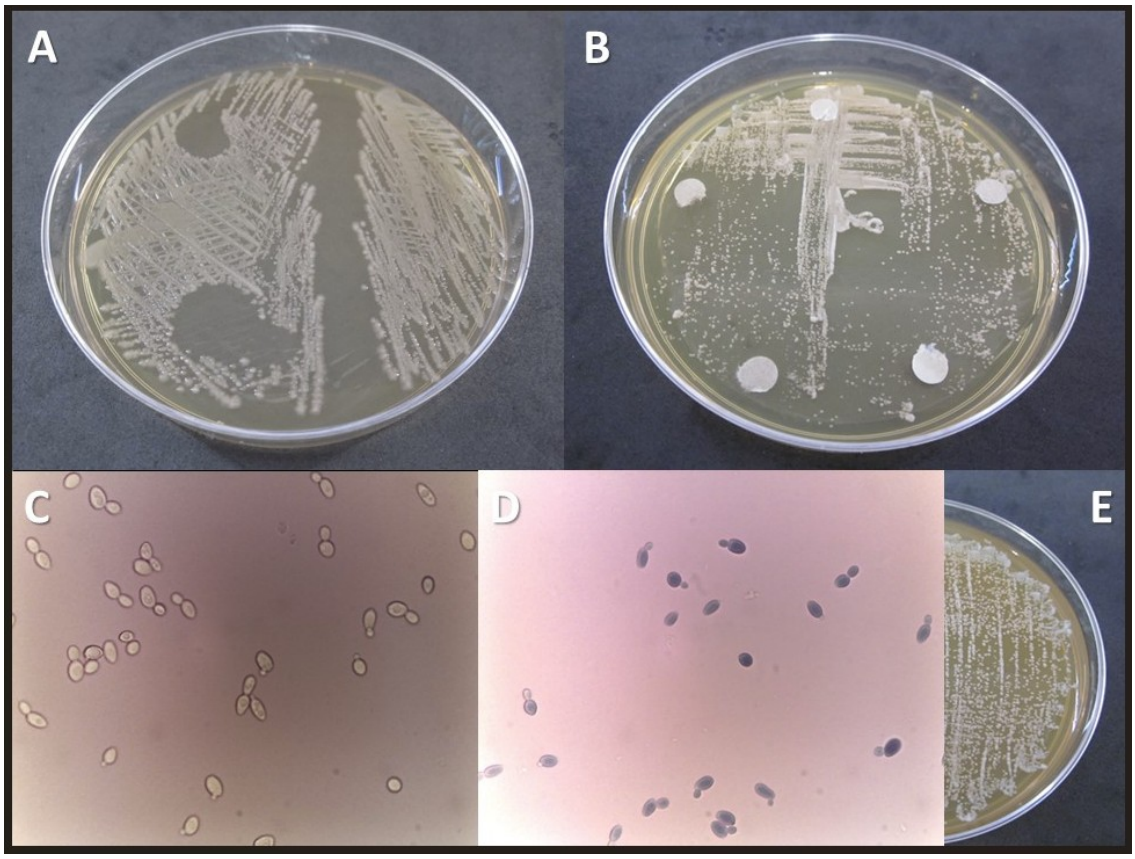


**Fig. 2 The antimicrobial activity of *T. vulgaris* essential oil. A. *T. vulgaris* essential oil on uropathogenic *E. coli* growth. B. *T. vulgaris* essential oil on *S. cerevisiae* growth. Essential oil increasing amounts (0.72, 1.44, 2.88, 4.32 and 7.2 mg) were placed in the clockwise direction, starting with the top. C. Cells of *S. cerevisiae* mixed with 0.1% trypan blue dye. D. Cells of *S. cerevisiae* treated with *T. vulgaris* essential oil for 15 min and stained with 0.1% trypan blue dye. E. Yeast peptone dextrose agar plate inoculated with *S. cerevisiae***

on uropathogenic *E. coli* and *S. cerevisiae* growth was used. So, Petri dishes containing trypticasein soy and yeast peptone dextrose agar were seeded as described in Materials and Methods. The results are shown in Fig. 2. The antibacterial activity of obtained *T. vulgaris* essential oil was shown in Fig. 2A. A half of trypticasein soy agar plate was seeded with uropathogenic *E. coli* and 2 additions of 14.4 mg of the *T. vulgaris* essential oil were made. The agar plates were incubated at 37°C for 24 h. The zones of growth inhibition of uropathogenic *E. coli* were obtained (Fig. 2A). The essential oil obtained showed antibacterial activity. The antifungal activity of *T. vulgaris* essential oil was determined using *S. cerevisiae* and technique of diffusion in yeast peptone dextrose agar. Plates were inoculated by crossstriation and then sterile filter paper discs were placed on the surface of yeast peptone dextrose agar plates adding different amounts of essential oil: 0.72 - 7.2 mg. The agar plates were incubated at 30°C for 24 h. The results are shown in Fig. 2B. As shown in the figure, low amounts of *T. vulgaris* essential oil did not inhibit the growth of *S. cerevisiae*. However, at the highest quantities of *T. vulgaris* oil tested, it was possible to observe zones of growth inhibition of *S. cerevisiae*. With this, the antifungal effect of the essential oil of *T. vulgaris* was verified.

To determine the direct effect of the essential oil, *S. cerevisiae* cells were treated with *T. vulgaris* during 15 min and cell viability was determinated using trypan blue dye as described in Materials and Methods. Dead cells were observed in a deep blue color. The results are shown in Fig. 2C and Fig. 2D. Fig. 2C shows *S. cerevisiae* cells stained with trypan blue dye and not treated with *T. vulgaris* essential oil. As seen in this image, the cells of *S. cerevisiae* were not stained by trypan blue which indicated that the cells were intact. Fig. 2D shows the results obtained when the *S. cerevisiae* cells were incubated for 15 min with *T. vulgaris* essential oil. *S. cerevisiae* cells were stained due to the action of *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. The cells maintained their characteristic morphology but not their viability.

# DISCUSSION

Essential oils have been used for thousands of years in various cultures for medicinal and health purposes. They are concentrated hydrophobic liquid containing volatile chemical compounds from 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 (Herman *et al*., 2019; Mahato *et al.*, 2019). In addition, essential oils are aromatic oily liquids extracted from different parts of plants for instance, leaves, barks, seeds, flowers and peles (Herman *et al*., 2019; Tongnuanchan and Benjakul, 2014). On the other hand, as is well known, antibiotic resistance is a global problem that is increasing. New strains of resistant bacteria are emerging every day. With their ever-increasing use and misuse, microorganisms have developed antimicrobial resistance. The phenomenon of antimicrobial resistance refers to the potential of microorganisms including bacteria, viruses, fungi, and parasites to thrive and continue to grow in the midst of drugs designed to kill them. Infections caused by antimicrobial-resistant organisms are not only difficult to treat, there is also always an increased chance of severe illness and even death due to these infections (Salam *et al*., 2023). Faced with this problem, several authors have reported the search for new substances with antimicrobial properties. One of these alternatives are essential oils extracted from plants and containing terpenoids (Flores-Encarnación *et al.*, 2016). Erkana *et al*., (2012) demonstrated the antibacterial activity *versus* Gram-negative bacteria of a group of terpenoids extracted from leaves of *Murraya koenigii* (curry). Prabuseenivasan *et al*., (2006) reported that 19 essential oils showed antibacterial activity. They demostrated a significant inhibitory effect by cinnamon, clove, geranium, lemon, lime, orange and rosemary oils. Therefore, in this study, the *T. vulgaris* essential oil was extracted using a simple methodology and the antimicrobial effect of the essential oil obtained was determined. *T. vulgaris* essential oil was prepared by distillation dragging water vapor from 20 grams of partially crushed fresh thyme leaves. Although the yield of the extracted essential oil was not very high (only about 200 uL were recovered, data not shown), the antimicrobial activity was good: at the concentrations tested the growth of uropathogenic *E. coli* and *S. cerevisiae* was inhibited. The above is in agreement with what was reported by other authors who the antimicrobial activity of different concentrations evaluated after 1 min, 3 min and 5 min of contact at real time with suspensions of different bacterial strains; they also evaluated bacterial growth after 24 h and 48 h of incubation (Galgano *et al.*, 2022; Galgano *et al*. 2023). We previously reported the antimicrobial activity of commercial *T. vulgaris* essential oil against uropathogenic *E. coli* and *S. cerevisiae*. The results were similar to those reported in this study, especially antifungal activity of *T. vulgaris* essential oil evaluated after 15 min of contact (at real time) with suspensions of *S. cerevisiae* (Flores-Encarnación *et al.*, 2018; Flores-Encarnación *et al.*, 2020; Flores-Encarnación *et al.*, 2024). The *T. vulgaris* essential oil in this study showed antibacterial activity producing zones of growth inhibition of uropathogenic *E. coli* at the highest quantities tested. However, this results were not the same as those obtained in previous studies using the commercial essential oil of *T. vulgaris*; in previous studies the total growth inhibition of uropathogenic *E. coli* was observed, even at the lowest quantities tested (data not shown). This could be attributed to the fact that commercial essential oil is more concentrated than the essential oil extracted in this study. Therefore, the chemical components responsible for antimicrobial activity are more diluted. It has been reported that essential oil of *T. vulgaris* (thyme) 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%). Additionally, monoterpene derivatives (as thymol and carvacrol) are the major ingredients in thyme essential oil with antimicrobial properties such as anti-oxidative activity (Ben *et al*., 2019; Flores-Encarnación *et al*., 2022*b*; Kianersi *et al*., 2021; Lopez,2004; Powers *et al.*, 2018; Sakkas and Papadopoulou, 2007). The antimicrobial activity of *T. vulgaris* has been reported against Gram-negative bacteria such as *Salmonella enteritidis, Salmonella choleraesuis, S. typhimurium, Vibrio cholerae, Proteus mirabilis, P. vulgaris, Pseudomonas aeruginosa,* uropathogenic *E. coli,* and Gram-positive bacteria as *S. aureus, S. epidermidis, Enterococcus faecalis and Bacillus cereus*. Gram-negative bacteria are more resistant to essential oils than Gram-positive bacteria. This feature is related to structure of the cell walls of Gram-positive and Gram-negative bacteria due 90%–95% of the cell wall of

Gram-positive bacteria consists of peptidoglycan and other molecules such as teicoic acid and proteins (Al-Shuneigat *et al*., 2014; Flores-Encarnación *et al*., 2018; Hussein *et al.*, 2014; Kon and Rai, 2012; Mohsenipour and Hassanshahian, 2015; Nazzaro *et al*., 2013; Trombetta *et al.*, 2005). In relation to fungi, it has been reported that essential oils inhibit or slow the growth of yeasts and molds. Maness and Zubov (2019) reported that essential oils of *Rosmarinus officinalis*, *Cinnamomum verum* and *Citrus paradisi* inhibited the growth of *Trichophyton mentagrophytes*, *Microsporum gypseum* and *Rhizopus stolonifer*. In previous studies, it was reported that *T. vulgaris* essential oil inhibited the growth and biofilm formation of *Candida tropicalis* and inhibited the growth of *S. cerevisiae* (Flores-Encarnación *et al.*, 2022*a*; Flores-Encarnación *et al*., 2024). In the present study, another important aspect to consider is that the process for extracting the essential oil was low-cost, which is important because this methodology can be used to produce novel substances with antimicrobial properties that are highly useful for combating antimicrobial-resistant microorganisms.

# CONCLUSION

Antimicrobial resistance is a global public health problem. The search for new substances with antimicrobial properties to treat infectious diseases is a topic of interest that warrants research. The essential oils have demonstrated antibacterial and antifungal activity and other properties, making them potential substances that could be used as drugs to treat infectious diseases. Therefore, in this work, a simple method for extracting the *T. vulgaris* essential oil and some antimicrobial properties was presented.

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