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RESEARCH ARTICLE

THE EFFECT OF *THYMUS VULGARIS* ESSENTIAL OIL ON THE VIABILITY OF EUKARYOTIC CELLS

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ABSTRACT

Bacterial resistance to antibiotics has led to the search for new substances with bactericidal activity. Among them are some extracts of plant origin, such as some essential oils, which have shown a potent antimicrobial action. At present, the antimicrobial properties of the essential oil of *Thymus vulgaris* have been studied. It has been reported that *T. vulgaris* essential oil has shown high antibacterial activity against *Salmonella enteritidis*, *S. choleraesuis*, *S. typhimurium*, *Vibrio cholerae*, *Proteus mirabilis*, *P. vulgaris*, *Pseudomonas aeruginosa*, *Staphylococcus aureus*, *S. epidermidis*, *Enterococcus faecalis*, *Bacillus cereus, Escherichia coli*. In the present work, the effect of *T. vulgaris* essential oil on the viability of eukaryotic cells was studied.

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INTRODUCTION

At present, different essential oils with antibacterial properties are known. They have even been proposed as an alternative to treat infections caused by bacteria that have developed resistance to antibiotics (Daferera *et al*., 2003; Flores-Encarnación *et al*., 2016; Marston *et al*., 2016). Some oils have been used in food preservation, pharmaceutical, agronomic, aromatherapyand fragrance industries (Prabuseenivasan *et al*., 2006; Saranra and Devi, 2017). In regard to its antibacterial properties, essential oils have demostrated a significant inhibitory effect; so cinnamon, clove, geranium, lemon, lime, orange, rosemary, aniseed, eucaliptus, camphor, thyme oils, have showed antibacterial activity against bacteria both Gram-negative and Gram-positive (Boskovica *et al*., 2015; Prabuseenivasan *et al*., 2006).

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It has been reported that thyme (*T. vulgaris*) essential oil showed high antibacterial activity against *Salmonella enteritidis*, *S. choleraesuis*, *S. typhimurium*, *Vibrio cholerae*, *Proteus mirabilis*, *P. vulgaris*, *Pseudomonas aeruginosa*, *Staphylococcus aureus*, *S. epidermidis*, *Enterococcus faecalis* and *Bacillus cereus* (Al-Shuneigat *et al*., 2014; Hussein *et al*., 2014; Kon and Rai, 2012; Mohsenipour and Hassanshahian, 2015).

In recent study, the growth and biofilm formation of uropathogenic *E. coli* was completely inhibited at low concentrations of *T. vulgaris* essential oil (Flores-Encarnación *et al.*, 2018). On the other hand, most of the works described in the literature refer to the effect of *T. vulgaris* as an antibacterial substance, however little is known about the effect of this essential oil in cells of higher organisms. Therefore, in the present work, the effect of *T. vulgaris* essential oil on the viability of eukaryotic cells was studied.

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* and *Candida albicans* strains were used. *S. cerevisiae* strain from yeast marketed to make bread, while *C. albicans* strain from clinical isolates. The identity of *C. albicans* strain was confirmed using the germ tube test as described by Moya-Salazar and Rojas (2018). Yeasts were stored at -40°C in yeast peptone dextrose (YPD) broth with 20% glycerol until analysis. A freshly prepared erythrocyte suspensión was also used. The strain of uropathogenic *E. coli* CFT073 was used. Bacterial strain was stored in cryovials at -40°C until analysis.

Culture: *S. cerevisiae* and *C. albicans* strains were cultivated on yeast peptone dextrose (YPD) 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. Cultures were grown aerobically at 30°C in a 50 mL-working-volume Erlenmeyer flask stirred at 200 rpm for 24 hours. 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 *S. cerevisiae* and *C. albicans*. The uropathogenic *E. coli* strain was cultured at 37°C for 18 to 24 h in trypticase soy broth (BBL Microbiology Systems, Cockeysville, Md). Then, *E. coli* that had been cultured in trypticase soy broth was seeded crosswise in a trypticase soy agar plate, and was incubated at 37°C for 24 h.

Antimicrobial Activity of *T. vulgaris:* The antimicrobial activity of *T. vulgaris*was determined using the technique of diffusion in agar using paper discs. For it, tripticasein soy agar plates (containing 20 mL of medium) were prepared. Sterile Petri dishes (150 mm) were used. Plates were inoculated by crossstriation with uropathogenic *E. coli*. Each inoculum contained approximately 10^6 CFU mL^{-1}. Then, sterile filter paper disks (5 mm diameter) were placed on the surface of tripticasein soy agar plates. Different concentrations (0.66 to 13.2 mg mL^{-1}) of the essential oil were used. The agar plates were incubated at 37°C for 24 h. The diameters of the inhibition halos formed were measured. The analyses were conducted in triplicate. Similarly, yeast peptone dextrose agar plates (containing 20 mL of medium) were prepared. Plates were inoculated by crossstriation with *S. cerevisiae* and *C. albicans*. Sterile filter paper disks were placed on the surface of yeast peptone dextrose agar plates and different concentrations (1.3, 3.9, 6.5, 9.1 and 13.2 mg mL^{-1}) of essential oil were used. The agar plates were incubated at 30°C for 24 h. The diameters of the inhibition halos formed were measured. As reference, yeast peptone dextrose agar plates were inoculated by crossstriation with *C. albicans* and sterile filter paper disks were placed on them, adding different concentrations (0.25, 0.75, 1.25, 1.75 and 2.5 µg) of amphotericin B. The agar plates were incubated at 30°C for 24 h.

Cell Viability Assay: The cell viability assay was performed using *S. cerevisiae* and *C. albicans* cells and a freshly prepared erythrocyte suspensión, according to methodology described by Castillo *et al*., (2009). For this, 10 µL of *S. cerevisiae* and *C. albicans* 24-hour precultures were used (growing in yeast peptone dextrose broth at 30°C and stirring at 200 rpm). The cell viability assay was determined by mixing 10 µL of culture with 10 µL of 0.4% trypan blue, incubating 5 minutes at room temperature. Then, 10 µL of cell suspension were placed in a Neubauer chamber for observation at 40x. Dead cells were observed in a deep blue color. All determinations were made in duplicate. For negative control, non-viable cells of *S. cerevisiae* were used. This cells were obtained by heating at 50°C for 20 minutes. To determine the cell viability in erythrocytes, it was used a freshly prepared erythrocyte suspension. For this, 1 µL of whole blood was used and 99 µL of isotonic saline was added. The cell viability was determined by mixing 10 µL of erythrocyte suspension with 10 µL of 0.4% trypan blue, incubating 5 minutes at room temperature. Then, 10 µL of erythrocyte suspension were placed in a Neubauer chamber for observation at 40x. All determinations were made in duplicate.

The Effect of *T. vulgaris* on Cell Viability: To determine the effect of *T. vulgaris* on cell viability, 50 µL of *S. cerevisiae* and *C. albicans* were mixed with different concentrations of *T. vulgaris*: 1.3, 3.9, 6.5 y 9.1 mg, incubating 10 min at room temperature. The cell viability assay was determined by mixing 10 µL of the previous mix with 10 µL of 0.4% trypan blue. Then, 10 µL of cell suspension were placed in a Neubauer chamber for observation at 40x. Similarly, 10 µL of erythrocyte suspension (described above) were mixed with different concentration of *T. vulgaris* in the order: 1.3, 2.6 and 3.9 mg, and incubated 10 min at room temperature. The cell viability was determined by mixing 10 µL of erythrocyte suspension with 10 µL of 0.4% trypan blue; 10 µL were placed in a Neubauer chamber for observation at 40x. All determinations were made in duplicate.

RESULTS

To determine the antibacterial activity of *T. vulgaris* essential oil, the technique of diffusion in agar was used. For this, the uropathogenic *E. coli* strain was seeded crosswise in a trypticase soy agar plate and sterile filter paper disks were placed on the surface of agar plates. Different concentrations of essential oil were placed on paper disks: 0.66, 1.3, 3.9, 6.5 and 13.2 mg mL^{-1}. The agar plates were incubated at 37°C for 24 h. Similarly, the yeast peptone dextrose agar plates were inoculated by crossstriation with *S. cerevisiae* and *C. albicans* and different concentrations of essential oil were used: 1.3, 3.9, 6.5, 9.1 and 13.2 mg mL^{-1}. The agar plates were incubated at 30°C for 24 h. The results obtained are shown in the Fig. 1. As shown in Fig. 1A, the *T. vulgaris* essential oil completely inhibited the growth of uropathogenic *E. coli*. This inhibitory effect was observed at all concentrations tested. As seen in Fig. 1A, the surface of trypticase soy agar plate was bright and free of bacterial growth. In Fig. 1B, the antifungal activity of *T. vulgaris* essential oil is shown. As seen in this figure, *T. vulgaris* had a different effectin growth of *S. cerevisiae*: the growth on the surface of yeast peptone dextrose agar plates was observed at the concentrations tested. Regarding the growth of *C. albicans*, the *T. vulgaris* essential oil produced inhibition of yeast growth, however the inhibition was not as strong as observed with the growth of *E. coli* (Fig. 1C). The growth inhibitory effect with *T. vulgaris* was greater in *C. albicans* than in effect observed with *S. cerevisiae*. *T. vulgaris* showed greater antifungal activity in *C. albicans*. To compare the antimicrobial effect of *T. vulgaris*, different concentrations of amphotericin B were used: 0.25, 0.75, 1.25, 1.75 and 2.5 µg.

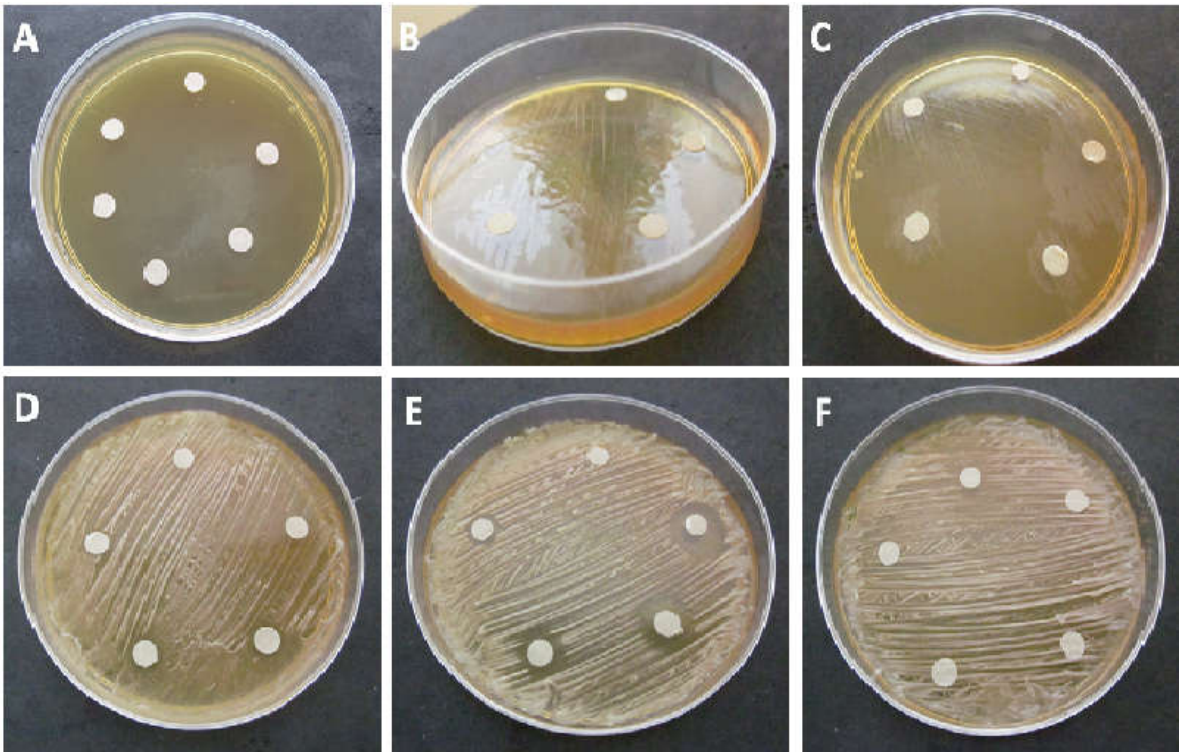

Fig. 1. The effect of *T. vulgaris* essential oil in microbial growth.A. Uropathogenic *E. coli*; B. *S. cerevisiae;* C. *C. albicans;* D. Growth of *S. cerevisiae* in the presence of amphotericin B; E. Growth of *C. albicans* in the presence of amphotericin B; F. Control condition (*C. albicans*). In all cases, the test substance was placed on the paper disk counterclockwise starting with the paper disk located at the top

Table 1. Effect of *T. vulgaris* essential oil in cell viability

	T. vulgaris					
Eukariotic cell	0 mg	1.3 mg	3.4 mg	6.5 mg	9.1 mg	13 mg
S. cerevisiae	100%[a]	82%	55%	30%	0%	0%
C. albicans	100%	76%	44%	0%	0%	0%
Erythrocytes	100%	59%	38%	0%	0%	0%

[a]The cell viability was expressed as percentage rate with respect to living cells in absence of *T. vulgaris*.

So, the yeast peptone dextrose agar plates were inoculated with *C. albicans* and sterile filter paper disks were added with amphotericin B and were incubated at 30°C for 24 h. The results obtained are shown in the Fig. 1D and E. As shown in Fig. 1D, amphotericin B had no effect on the growth of *S. cerevisiae* at the concentrations tested. The inhibition halos formation of growth was not observed. The Fig. 1E shows the growth of *C. albicans* in the presence of amphotericin B. As can be seen, the growth inhibition at the highest concentrations of amphotericin B was recorded. *C. albicans* strains were more sensitive to the presence of the antifungal tested compared to *S. cerevisiae*. However, in both cases at these concentrations of amphotericin B it was not possible to observe the effect recorded with *T. vulgaris*. In order to determine the effectiveness of the essential oil of *T. vulgaris* in a short time (10 min), the cell viability assay was performed using *S. cerevisiae, C. albicans* and anerythrocyte suspensión. So, 50 µL of *S. cerevisiae* and *C. albicans* were mixed (independently) with different concentrations of *T. vulgaris*: 0, 1.3, 3.9, 6.5, 9.1 and 13 mg and incubating 10 min at room temperature. For cell viability assay, 10 µL of the previous mix with 10 µL of 0.4% trypan blue were mixed. Then, 10 µL of cell suspension were placed in a Neubauer chamber for observation at 40x. The results obtained are shown in the Table 1. As shown in Table 1, the percentage of viable *S. cerevisiae* cells decreased by 28% when they were exposed at 1.3 mg of *T. vulgaris* for 10 min. At a *T. vulgaris* concentration of 6.5 mg, the viability of *S. cerevisiae* cells was 30%.

At concentrations of 9.1 and 13 mg of T. *vulgaris*, the *S. cerevisiae* cells were completely lysed. In the case of *C. albicans* cells, a greater decrease in viability was observed, when the yeast was subjected to the action of *T. vulgaris*. As shown in Table 1, the percentage of viable *C. albicans* cells decreased by 24% when they were exposed at 1.3 mg of *T. vulgaris*. At a *T. vulgaris* concentration of 3.4 mg, the viability of *C. albicans* cells was 44%. At concentrations of 6.5, 9.1 and 13 mg of *T. vulgaris*, the *C. albicans* cells were completely lysed. To determine the effect of *T. vulgaris* in erythrocytes, the cell viability test was performed using trypan blue, considering as intact erythrocyte cells those that do not stain with the dye. For that, 10 µL of an erythrocyte suspension were mixed with different concentration of *T. vulgaris*: 1.3, 2.6 and 3.9 mg, and incubated 10 min at room temperature.

Then, 10 µL of erythrocyte suspension with 10 µL of 0.4% trypan blue were mixed; 10 µL were placed in a Neubauer chamber for observation at 40x. As shown in Table 1, the percentage of intact erythrocyte cells decreased by 41% when they were exposed at 1.3 mg of *T. vulgaris*. At a *T. vulgaris* concentration of 3.4 mg, the percentage of intact erythrocyte cells decreased by 62%. At concentrations of 6.5, 9.1 and 13 mg of *T. vulgaris*, the erythrocyte cells were completely lysed, as it happened with *C. albicans* cells. It is important to note that in these tests high concentrations of *T. vulgaris* were used, compared to the number of cells tested for yeasts and erythrocytes, that is, the cells were subjected to high doses of the essential oil in a short period of time. Apparently, the eukaryotic cells tested in these assays showed greater resistance to the essential oil of *T. vulgaris* compared to uropathogenic *E. coli*, which was highly sensitive.

DISCUSSION

The plant extracts contain substances with antimicrobial activity, such is the case of essential oils widely used in the world since ancient times for different purposes (Sendra *et al.*, 2016). At present, the essential oils has been used greatly increased in the food, cosmetic and pharmaceutical industry (Elshafie and Camele, 2017). It has been reported that essential oil of *T. vulgaris* is a potent bactericide against *Staphylococcus aureus*, *Salmonella typhimurium*, *Escherichia coli*, including some fungi like *Cryptococcusneoformans*, *Aspergillus* sp. (Inayatullah *et al*., 2017; Johny *et al*., 2010). In the present work, the antibacterial activity of *T. vulgaris* essential oil was tested using uropathogenic *E. coli* strain as described in materials and methods. The results obtained showed that *T. vulgaris* essential oil completely inhibited the growth of uropathogenic *E. coli* at all concentrations tested (Fig. 1).

In previous works, it was reported that *T. vulgaris* essential oil inhibited the growth of uropathogenic *E. coli* and also the biofilm formation (Al-Shuneigat *et al*., 2014; Flores-Encarnación *et al.*, 2018; Hussein *et al*., 2014). As shown in Fig. 1, the surface of trypticase soy agar plate was bright and free of bacterial growth. The observed effect of *T. vulgaris* in uropathogenic *E. coli* was bactericide, not bacteriostatic (data not shown). As reported by some authors, the bacterial growth inhibitory effect observed with *T. vulgaris* is attributed to thymol and carvacrol, two important chemical components in the essential oil of *T. vulgaris* (Khan*et al.*, 2017). In addition to *T. vulgaris*, carvacrol is present in essential oils of *Origanum vulgare*, *Trachyspermum ammi*, *Lepidium africanum*, *Citrus bergamia* (Dandlen *et al*., 2011). Thymol and carvacrol are hydrophobic monoterpenes that penetrate the cell membranes of bacteria, leading to disruption of cell membrane integrity; it results in dissolution of the proton motive force and subsequent reduction in ATP synthesis (reduction in other energy-dependent cell processes including synthesis of enzymes and toxins). As well as a release of bacterial cell content (Nostro and Papalia, 2012). It has been reported that the antibacterial action of carvacrol is stronger against Gram-positive as *Bacillus cereus*, *Enterococcus faecalis*, *Listeria monocytogenes*, *Staphylococcus aureus*, than Gram-negative bacteria as *E. coli* O157:H7, *Pseudomonas fluorescens*, *Salmonella typhimurium*, *Vibrio cholerae*, *V. vulnificus* (Langeveld *et al*., 2014; Magi *et al*., 2015). In this work, once the effect of *T. vulgaris* in uropathogenic *E. coli* was known, other tests were performed to determine the effect of essential oil on eukaryotic cells using yeasts (*S. cerevisiae* and *C. albicans*) and erythrocytes as biological models. The results obtained showed that *T. vulgaris* produced inhibition of growth in both yeasts, however the inhibition was not as strong as observed with the growth of *E. coli* (Fig. 1). The *T. vulgaris* essential oil had a different effect in growth of *S. cerevisiae* and *C. albicans*. *T. vulgaris* showed greater antifungal activity in *C. albicans*. It has been reported that the essential oils of plants show also activity against a wide range of microorganisms including both yeast and filamentous fungi (Helal *et al*., 2006). For example: the essential oil of *Cymbopogon citratus* inhibited the growth of *Neurospora sitophila*, *Penicillium digitatum*, *Aspergillus parasiticus* and *A. flavus* (Mishra and Dubey, 1994). It has been reported that both thymol and the essential oil of *T. vulgaris* exhibited strong fungistatic and fungicidal activities against *Aspergillus*, *Penicillium*, *Cladosporium*, *Trichoderma*, *Mucor* and *Rhizopus* and that the inhibition effect of thymol was three times greater than the essential oil of *T. vulgaris* (Klaric *et al*., 2007). Bachir raho *et al*., (2018) reported that 100 ppm of *T. vulgaris* oil did not show any antifungal activity against *S. cerevisiae*.

Other studies have shown the antifungal activity of essential oils of various chemotypes of *T. vulgaris* against *C. albicans* (Giordani *et al.*, 2004). This data are related to the results obtained in the present study, where it was observed that *T. vulgaris* mostly inhibited the *C. albicans* growth. It was suggested that the *C. albicans* cell membrane may be more susceptible to the action of *T. vulgaris* by cell wall/membrane damage, disrupt the permeability of cell membranes and possibly inhibit respiration (Giordani *et al*., 2004; Thakre *et al*., 2017). Cox *et al*., (2000) suggested that the variations in the rate of monoterpene penetration through the fungal cell wall and cell membrane may explain the differences in the efficacy of the monoterpenes on the yeasts. The above could explain the differences observed in the growth of *S. cerevisiae* and *C. albicans* when by *T. vulgaris* were treated. As is known, candidiasis is one of the most common fungal infections in humans caused by the *Candida* species, most notably *C. albicans* **(**Deorukhkar and Roushani, 2018). In this case, the essential oil of *T. vulgaris* could be proposed for the treatment of infections by *C. albicans* (at least in the treatment of superficial mycoses). In the present study, the amphotericin B it had no effect on the growth of *S. cerevisiae* at the concentrations tested. The inhibition halos formation of growth was not observed, which indicated a resistance to the antifungal used (Fig. 1). The *C. albicans* strain was more sensitive to the presence of the antifungal tested compared to *S. cerevisiae*.

However, in both cases at these concentrations of amphotericin B (up to 2.5 µg) it was not possible to observe the effect recorded with *T. vulgaris*. In recent years, iIt has been observed that the incidence of invasive infections and resistance to antifungal therapy continue increasing (Deorukhkar and Roushani, 2018). Resistance to antifungal drugs has become a very serious problem, especially in immunocompromised individuals, because it is associated with increased incidences of opportunistic infections and systemic fungal infections (Deorukhkar *et al*., 2014). Therefore, it is necessary to search for new substances or drugs with antifungal activity and the use of essential oils could be an option. To determine the effectiveness of the essential oil of *T. vulgaris* in a short time, the cell viability assay was performed using *S. cerevisiae, C. albicans* and an erythrocyte suspension. As shown in Table 1, the percentage of viable *S. cerevisiae* cells decreased by 28% when they were exposed at 1.3 mg of *T. vulgaris* for 10 min. At a *T. vulgaris* concentration of 6.5 mg, the viability of *S. cerevisiae* cells was 30%. At concentrations of 9.1 and 13 mg of *T. vulgaris*, the *S. cerevisiae* cells were completely lysed.

In the case of *C. albicans* cells, a greater decrease in viability was observed, when the yeast was subjected to the action of *T. vulgaris*. As shown in Table 1, the percentage of *S. cerevisiae*, *C. albicans* and erythrocyte viable cells decreased at 82, 76 and 59%, respectively, when they were exposed at 1.3 mg of *T. vulgaris*. At a *T. vulgaris* concentration of 3.4 mg, the viability of *S. cerevisiae*, *C. albicans* and erythrocyte cells decreased at 55, 44 and 38%, respectively. The most affected cells were erythrocytes followed by *C. albicans*. Rojas-Armas *et al.*, (2019) evaluated the acute and repeated 28-day oral dose toxicity of *T. vulgaris* essential oil in rats, and they reported that the essential oil has moderate oral toxicity. The hematological and biochemical parameters were not altered. In the present study, the erythrocytes were challenged directly to the essential oil and it was observed that 38% of them remained intact up to 3.4 mg of *T. vulgaris*, which suggested that the essential oil have moderate toxicity to erythrocyte cells. Using higher concentrations of *T. vulgaris* essential oil, all cells were completely lysed. In all previous tests, the cells were subjected to high doses of the essential oil in a short period of time, which would indicate that the eukaryotic cells tested showed greater resistance to the essential oil of *T. vulgaris* compared to uropathogenic *E. coli* (highly sensitive).

Conclusion

The essential oil of *T. vulgaris* has been used for many years for different purposes. As a potent bactericide, *T. vulgaris* acts on both Gram positive and negative bacteria. Apparently, bacteria do not develop resistance to essential oils due to their rapid action. In this study, it was shown that essential oil of *T. vulgaris* inhibited the growth of yeasts such as *S. cereviceae* and *C. albicans*. However, higher concentrations of the essential oil were required to cause damage to yeast cells in a short time. Something similar happened with erythrocyte cells. The essential oil of *T. vulgaris* had a moderate toxicity to yeast and erythrocyte cells.

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