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RESEARCH ARTICLE

EFFECT OF THYMUS VULGARIS ESSENTIAL OIL ON THE CYTOCHROME ACTIVITY OF UROPATHOGENIC ESCHERICHIA COLI

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ABSTRACT

Essential oils have been used for pharmaceutical purposes and in food due to their aromatic properties and antimicrobial activity. Essential oils have been reported to act by forming pores in cell membranes, however little is known about the mechanism of action of *T. vulgaris* essential oil, which is a potent bactericide. This work presents data suggesting an inhibitory effect on respiration, specifically in decreasing cytochrome reduction in the respiratory chain of uropathogenic *E. coli*.

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INTRODUCTION

Since their emergence, plants have provided many benefits to humans. Currently, many plants with medicinal properties have been reported, and as a result, various substances with pharmacological and even antimicrobial properties have been isolated and studied. In this context, essential oils have shown antibacterial properties. The essential oils are aromatic substances obtained from plant materials as 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). Essential oils have become increasingly popular in a variety of industries in recent years, including aromatherapy, food flavouring, and natural pharmaceutical treatments. As a result, a number of uses, including their, analgesic, anti-inflammatory, antioxidant and antibacterial qualities have been researched (Aziz *et al*., 2018; Burt, 2004; Dhanusha *et al*., 2024; Tongnuanchan and Benjakul, 2014). In regard to its antibacterial properties, essential oils have demostrated a significant antibacterial activity against Gram-negative and Gram-positive bacteria (Boskovica *et al*., 2015). There have been a number of reports validating the in vitro antibacterial and antifungal activities of this essential oil on some human pathogens, including *Staphylococcus aureus, Pseudomonas aeruginosa, E. coli, Candida albicans, Mycobacterium smegmatis, Proteus mirabilis, and Salmonella* sp. (de Lira-Mota *et al*., 2012; Imelouane *et al.*, 2009). On the other hand, some mechanisms from antibacterial activity of essential oils have been reported. One of the reported mechanisms of action is the the ability to alter and to penetrate the lipid membrane of bacteria, making it more permeable and causing leaking ions and cytoplasm (bacterial lysis and death) (Hussein *et al.*, 2018; O'Bryan *et al.*, 2015). In the present work, some evidence is shown about the effect of the essential oil of *T. vulgaris* on uropathogenic *E. coli*, especially on the activity of the cytochromes involved in the respiration of the bacteria.

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

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 (100 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}.

Antimicrobial activity: The antimicrobial activity of *T. vulgaris* essential oil on uropathogenic *E. coli* growth was determined using the technique of disk by diffusion in agar. For this, Petri dishes containing trypticasein soy agar were prepared and seeded as indicated above. Then, sterile filter paper disks (5 mm diameter) were placed on the surface of tripticasein soy agar plates and different amounts of the essential oil were used: 1.2, 2.4, 4.8, 7.2 and 12 mg. The agar plates were incubated at 37°C for 24 - 48 h. The bacterial growth inhibition halos were observed. The analyses were conducted in triplicate. To determine the resistance profile of the isolated bacteria the antibiotic diffusion technique was used. For that, Petri dishes containing trypticasein soy agar were prepared and seeded as indicated above and discs with antibiotics were used: nitrofurantoin (300 µg), furazolidone (100 µg), oxacillin (1 µg), chloramphenicol (30 µg), bacitracin (10 µg), tetracycline (30 µg), amikacin (30 µg), erythromycin (15 µg), ciprofloxacin (5 µg), ertapenem (10 µg) (B BBL, Sensi-Disc). The bacteria was incubated at 37°C during 24 hours. After twenty-four hours proceeded to make the measurement of growth inhibition. Then it proceeded to compare the results with the parameters of sensitivity and resistance following the rules of Clinical and Laboratory Standards Institute. The diameter of zone of inhibition of growth was recorded.

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 uropathogenic *E. coli* were used. Cells of uropathogenic *E. coli* were obtained from a culture in an Erlenmeyer flask containing 50 mL of trypticase soy broth, incubated at 37 °C with shaking at 150 r.p.m. for 24 hours. Cell suspension reached an optical density (OD_{560nm})=3-4. Then, 1 mL of the cell suspension was removed and cells of uropathogenic *E. coli* were recovered by centrifuging at 8,000 rpm for 10 min at 4 °C; cells were washed twice with 50 mM Tris-HCl buffer (pH 7.0). The reaction mixture (final volumen= 6 mL) contained: 50 mM Tris-HCl buffer (pH 7.0), 40 mM glucose or 40 mM succinate. The reactions were initiated adding washed uropathogenic *E. coli* cells (resuspended in 200 uL of 50 mM Tris-HCl buffer pH 7.0 buffer). The oxygen consumption kinetics were recorded for 30 min. The temperature was kept constant at 37°C. In all tests, the respiratory activities of uropathogenic *E. coli* was reported as consumed nmol O_2 min^{-1}. The analyses were conducted in triplicate.

Effect of *T. vulgaris* essential oil on respiratory activity: The effect of *T. vulgaris* on respiratory activity of uropathogenic *E. coli* was determinated adding the different concentrations of essential oil: 0.65 and 1.3 mg. For that, washed cells of uropathogenic *E. coli* were obtained as indicated above (cell suspension at OD_{560nm}= 3-4) and resuspended in 200 uL of 50 mM Tris-HCl buffer pH 7.0 buffer. Then cells were incubated with *T. vulgaris* essential oil for 20 min at 37 °C (facilitating contact between complete cells and essential oils). At the end of incubation, the reaction mixture (final volumen= 6 mL) contained: 50 mM Tris-HCl buffer (pH 7.0), 40 mM glucose or 40 mM succinate. The reactions were initiated adding the uropathogenic *E. coli* cells treated with the essential oil. The oxygen consumption kinetics were recorded for 30 min. The temperature was kept constant at 37°C. In all tests, the respiratory activities of uropathogenic *E. coli* was reported as consumed nmol O_2 min^{-1}. The analyses were conducted in triplicate.

Effect of *T. vulgaris* essential oil on cytochromic activity of uropathogenic *E. coli:* To determine cytochrome activity, cells of uropathogenic *E. coli* were obtained from a culture in an Erlenmeyer flask containing 50 mL of trypticase soy broth, incubated at 37 °C with shaking at 150 r.p.m. for 24 hours. Once culture reached an optical density (OD_{560nm})=3-4, cells of uropathogenic *E. coli* were recovered by centrifuging at 8,000 rpm for 10 min at 4 °C. Cells were washed twice with 50 mM Tris-HCl buffer (pH 7.0). The recovered cells were resuspended in 4 mL of 20 mM phosphate buffer (pH 7) containing 30% (vol/vol) glicerol. Samples (1 mL, 250 mg wet weight) were oxidized with a few grains of ammonium persulfate and reduced with 80 mM succinate or 80 mM glucose, at room temperature for 30 min. Then, samples were then frozen at -13 °C for 30 min and analyzed in a DU-Beckman spectrophotometer (scanning visible region from 400 to 700 nm). Difference spectra were recorded in cuvettes with a 1-cm light path. To determine the effect of *T. vulgaris* essential oil on cytochromic activity of uropathogenic *E. coli,* washed cells of uropathogenic *E. coli* were obtained as indicated above. So, samples (1 mL, 250 mg wet weight) were incubated at room temperature for 20 min adding different concentrations of essential oil: 6.5 and 13 mg, independently. At the end of incubation, cells were oxidized with a few grains of ammonium persulfate and reduced with 80 mM succinate or 80 mM glucose, at room temperature for 30 min and then were frozen at -13 °C for 30 min. Cytochrome analysis was performed usinga DU-Beckman spectrophotometer (scanning visible region from 400 to 700 nm). Difference spectra were recorded in cuvettes with a 1-cm light path.

Ubiquinone inactivation assay: For this essay, cells of uropathogenic *E. coli* were obtained from a culture in an Erlenmeyer flask containing 50 mL of trypticase soy broth, incubated at 37 °C with shaking at 150 r.p.m. for 24 hours. Once culture reached an optical density (OD_{560nm})=3-4, cells of uropathogenic *E. coli* were recovered by centrifuging at 8,000 rpm for 10 min at 4 °C. Cells were washed twice with 50 mM Tris-HCl buffer (pH 7.0). The recovered cells (1 gram) were resuspended in 5 mL of 50 mM Tris-HCl buffer (pH 7.0) and placed in a sterile 60 mm diameter Petri dish. The Petri dish was surrounded with finely powdered ice and the cell suspension was continuously stirred using a magnetic stirrer. Then, a short-wave ultraviolet light lamp was placed above the Petri dish (5 cm distance) and ultraviolet light was applied in darkness to the cell suspension for 90 min. At the end of irradiation, the uropathogenic *E. coli* cells were recovered by centrifuging at 8,000 rpm for 10 min at 4 °C. The recovered cells were resuspended in 3 mL of 20 mM phosphate buffer (pH 7) containing 30% (vol/vol) glicerol. Samples (1 mL) were oxidized with a few grains of ammonium persulfate and reduced with 80 mM succinate or 80 mM glucose, at room temperature for 30 min. Then, samples were then frozen at -13 °C for 30 min and analyzed in a DU-Beckman spectrophotometer (scanning visible region from 400 to 700

nm). Difference spectra were recorded in cuvettes with a 1-cm light path.

RESULTS

In this study, effect of *T. vulgaris* essential oil on the cytochrome activity of uropathogenic *E. coli* was determined. For this, antimicrobial activity of *T. vulgaris* essential oil on uropathogenic *E. coli* growth was determined using the technique of disk by diffusion in agar. Briefly, sterile filter paper disks (5 mm diameter) were placed on the surface of tripticasein soy agar plates and different amounts of the essential oil were used: 1.2- 12 mg, as was indicated in Materials and Methods. The results are shown in Fig. 1. As shown in Fig. 1A, *T. vulgaris* essential oil completely inhibited the growth of uropathogenic *E. coli*. This result was observed at all additions of essential oil tested. As can be seen in the image, the essential oil showed a strong inhibitory effect since the surface of the trypticasein soy agar lacked bacterial growth. To compare previous results, sensitivity tests to different antibiotics were determinated. For that, Petri dishes containing trypticasein soy agar were seeded and discs with antibiotics were used as described in Materials and Methods. The bacteria was incubated at 37°C during 24 hours. After twenty-four hours proceeded to make the measurement of growth inhibition.

The results are shown in Fig. 1C and Fig. 1D. As shown in Fig. 1C, uropathogenic *E. coli* was found to be sensitive to nitrofurantoin, furazolidone, chloramphenicol, and tetracycline antibiotics, while the bacteria showed resistance to oxacillin and bacitracin antibiotics. Fig. 1D shows that uropathogenic *E. coli* was moderately sensitive to amikacin and erythromycin antibiotics. The greatest sensitivity was recorded with ciprofloxacin and ertapenem antibiotics. Based on the above results and given the effectiveness essential oil in preventing the growth of uropathogenic *E. coli*, it was proposed that *T. vulgaris* could have several mechanisms of antibacterial action. One of these could be related to the alteration of bacterial respiration. Therefore, respiratory activity was determinated polarographically using a Clark oxygen electrode according to the methodology described in Materials and Methods. So, reaction mixture contained 50 mM Tris-HCl buffer (pH 7.0), 40 mM glucose or 40 mM succinate and reactions were initiated adding washed uropathogenic *E. coli* cells treated and untreated with the *T. vulgaris* essential oil. Table 1 shows respiratory activity of uropathogenic *E. coli* cells using succinate and glucose as substrates.

Table 1. Effect of essential oil of *T. vulgaris* on the respiratory activity of uropathogenic *E. coli*

Substrates	Essential oil (mg)	Respiratory activity[a] (nmol O_2 min^{-1})	Decreased oxidase activity (%)
Succinate	0	22.78	0
	0.65	0	100
	1.3	0	100
Glucose	0	21.75	0
	0.65	0	100
	1.3	0	100

[a]Oxidase activity

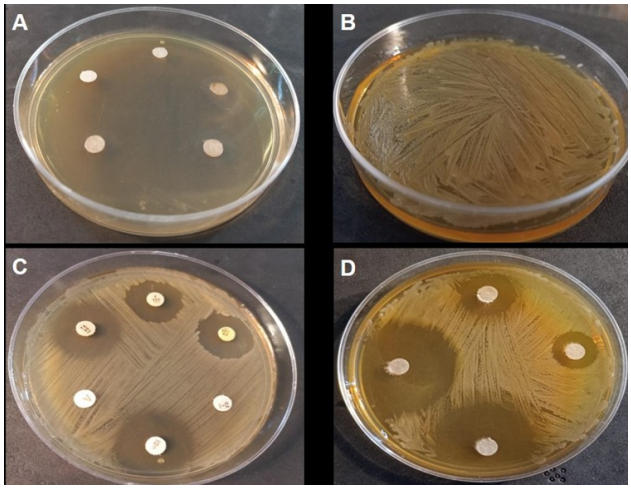

Fig. 1 Effect of *T. vulgaris* essential oil and antibiotics on uropathogenic *E. coli* growth. A. Antibacterial activity of *T. vulgaris* essential oil (1.2, 2.4, 4.8, 7.2 and 12 mg). Essential oil was placed in increasing amounts in the counterclockwise direction, starting with the top. B. Control condition. C. Sensitivity test to different antibiotics: nitrofurantoin, furazolidone, oxacillin, chloramphenicol, bacitracin and tetracycline. D. Sensitivity test to antibiotics: amikacin, erythromycin, ciprofloxacin and ertapenem. The discs with antibiotics were placed clockwise.

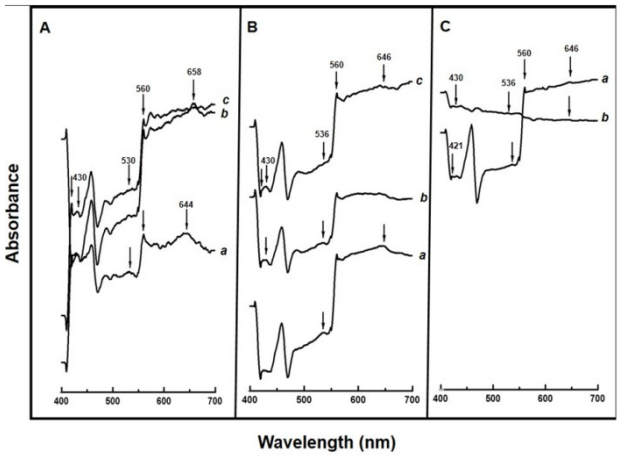

Fig. 2. Effect of *T. vulgaris* essential oil on the reduction of cytochromes in uropathogenic *E. coli*. A. Reduced minus oxidized difference spectra of *E. coli* cells generated by adding succinate (without *T. vulgaris*, spectrum *a*; 0.65 mg *T. vulgaris*, spectrum *b;* 1.3 mg *T. vulgaris*, spectrum *c*). B. Reduced minus oxidized difference spectra of *E. coli* cells generated by adding glucose (without *T. vulgaris*, spectrum *a*; 0.65 mg *T. vulgaris*, spectrum *b;* 1.3 mg *T. vulgaris*, spectrum *c*). C. Reduced minus oxidized difference spectra of *E. coli* cells irradiated with UV light, generated by adding succinate (spectrum *a*) and glucose (spectrum *b*).

In both cases, succinate oxidase and glucose oxidase activities were measured in whole *E. coli* cells. The oxidase activity for glucose and succinate showed a value around 22 nmol O_2 min^{-1} in the absence of *T. vulgaris* essential oil. As indicated in Materials and Methods uropathogenic *E. coli* cells were incubated for 20 min with 0.65 and 1.3 mg of *T. vulgaris* essential oil. Thus, as indicated in Table 1, the activities of succinate oxidase and glucose oxidase were completely lost. These tests provided evidence of the inhibitory action of *T. vulgaris* essential oil on respiratory activity on uropathogenic *E. coli* (measured as succinate and glucose oxidases).

Therefore, the effect of the essential oil on the reduction of cytochromes (cytochrome activity) of uropathogenic *E. coli* was determined. As described in Materials and Methods samples were oxidized with a few grains of ammonium persulfate and reduced with 80 mM succinate or 80 mM glucose for 30 min. Cells of uropathogenic *E. coli* were incubated with different concentrations of essential oil (6.5 and 13 mg). Difference spectra were recorded. The results are shown in Fig. 2. So, the reduced minus oxidized difference spectra shown in Fig. 2A*a* and Fig. 2B*a* show the reduction of cytochromes from uropathogenic *E. coli* that were reduced with succinate and glucose. In both cases, the uropathogenic *E. coli* cells were not incubated with the *T. vulgaris* essential oil. In the difference spectra, the presence of *b*-type and *d*-type cytochromes was observed by the signals obtained at 430, 530, 536, 560, 644 and 646 nm, highlighting a better reduction of the cytochromes using succinate as a substrate (Fig. 2A*a*). Using glucose as a substrate, the reduction of cytochromes was lower (Fig. 2B*a*). The difference spectra corresponding to uropathogenic *E. coli* cells treated with *T. vulgaris* essential oil were shown in *b* and *c* of Fig. 2A and Fig. 2B. In both cases, a decrease in the reduction of cytochromes was observed, especially in the spectral signals recorded at 560 and 644 nm, corresponding to *b*-type and *d*-type cytochromes, respectively (Fig. 2A*b* and Fig. 2A*c*). Similar results were obtained in Fig. 2B*b* and Fig. 2B*c* showing a decrease in the reduction of *b*-type and *d*-type cytochromes in the presence of the essential oil. These results indicated that *T. vulgaris* essential oil inhibits the reduction of cytochromes present in the respiratory chain of uropathogenic *E. coli* and this could explain why the respiratory activity of uropathogenic *E. coli* decreased considerably when the activities of succinate and glucose oxidases were measured in the present study. Thus, *T. vulgaris* essential oil affected the cytochrome activity of uropathogenic *E. coli*. Based on the above results, it was suggested that *T. vulgaris* essential oil might have some effect on the lipophilic ubiquinone transporter of *E. coli*. Therefore, an assay was conducted that involved inactivating ubiquinone by irradiating *E. coli* cells with ultraviolet light. As described in Materials and Methods, cells of uropathogenic *E. coli* were irradiated using a short-wave ultraviolet light lamp in darkness for 90 min. Then, difference spectra were recorded using succinate and glucose as substrates.The results are shown in Fig. 2C. As can be seen in the figure, irradiation of cells with ultraviolet light produced a decrease in the reduction of *b*-type and *d*-type cytochromes, losing the spectral signals that had been previously observed in cells not treated with the essential oil (430, 560 and 646 nm). These results are consistent with those obtained by treating uropathogenic *E. coli* cells with *T. vulgaris* essential oil, to say significantly reducing the reduction of cytochrome components associated with *E. coli* respiration.

DISCUSSION

Essential oils are rich in beneficial chemical elements and have a wide range of applications in medicine, food, cosmetics, agricultura, and are derived from a variety of sources, including flowers, fruits, spices, herbs, and contain a wide range of constituents, with hydrocarbon monoterpenes. Essential oils have antimicrobial properties that are dependent on their chemical composition and the number of single components (Angane *et al.*, 2022; Hintz *et al*., 2015; Mehidi *et al*., 2024; Nazzaro *et al.*, 2013). For instance, cinnamon is widely employed in a variety of applications, including pharmaceuticals, seasonings, cosmetics, foods, beverages, commodity essences, and chemical industries. Cinnamon essential oil is composed terpenes, limonene, cinnamaldehyde, and aromatic compounds and shows a high antibacterial action against foodborne pathogens such as *Escherichia coli* and *Listeria monocytogenes* (Liu *et al*., 2021; Mahazir and Othman, 2021; Yang *et al.*, 2021). On the other hand, it has been reported chemical composition of *T. vulgaris* essential oil which mainly contains geraniol, linalool, gamma-terpineol, trans-thujan-4-ol/terpinen-4-ol, carvacrol and thymol. These latter components have been reported to be responsible for the antimicrobial activity as well as strong antioxidant properties (Borugă *et al*., 2014; Grigore *et al.*, 2010; Rota *et al.*, 2008; Sacchetti *et al*., 2005; Thompson *et al*., 2003). In the present study, antimicrobial activity of *T. vulgaris* essential oil on uropathogenic *E. coli* growth was determined using the technique of disk by diffusion in agar. As shown in the Results section, *T. vulgaris* essential oil completely inhibited the growth of uropathogenic *E. coli*. The above is in agreement with what was reported by other authors. In this work, sensitivity tests to different antibiotics for uropathogenic *E. coli* were also made. The results showed that uropathogenic *E. coli* was sensitive to nitrofurantoin, furazolidone, chloramphenicol, tetracycline, amikacin and, erythromycin antibiotics and it showed resistance to oxacillin and bacitracin antibiotics. It has been reported that ability to effectively treat a wide range of ailments caused by bacteria, parasites, viruses, and fungi is gradually endangered by antimicrobial resistance. So, resistant pathogens and antimicrobial agents are widespread, affect humans, animals, plants, and the environment and have the potential for species-to-species and border-to-border transmission (Wirtu *et al*., 2024). Recent surge in infections caused by multidrug-resistant bacteria in recent years has heightened interest in the antibacterial activities of medicinal plants and their metabolites. So, a study of the antibacterial capabilities of essential oils and their constituents could reveal new applications in health, aiding in minimizing the risks associated with antibiotic resistance, a growing problem due to the excessive use of antibiotics in medicine (Helmy *et al.*, 2023; Mehidi *et al.*, 2024). Based on the results obtained in this study, the *T. vulgaris* essential oil was very effective in inhibiting the growth of uropathogenic *E. coli*. For this, it was proposed that *T. vulgaris* could have several mechanisms of antibacterial action. It has been reported that essential oils or their constituents can attack multiple sites inside a bacterial cell to inactivate the bacteria. Authors have reported a robust binding affinity between the components of oils and enzymes, including isoleucyl-tRNA synthetase, DNA gyrase, and the penicillin-binding protein. For instance, thymol and viridoflorol showed significant binding affinity, suggesting potent antibacterial potential against multidrugresistant strains (Aouf *et al*., 2022; Mehidi *et al.*, 2024). In the present study, a new mechanism of action of *T. vulgaris* essential oil was proposed, which should directly affect the respiration of bacteria, specifically affecting the reduction of cytochromes of the respiratory chain of uropathogenic *E. coli*. Therefore, respiratory activity was determinated polarographically. As shown in the Results section, the activities of succinate and glucose oxidases were completely lost, after treating the cells with the essential oil. These tests provided evidence of inhibitory action of *T. vulgaris* essential oil on respiratory activity on uropathogenic *E. coli* (measured as succinate and glucose oxidases). Therefore, the direct effect on cytochrome activity was

determined (observing reduction of cytochromes). The results showed that uropathogenic *E. coli* cells previously incubated with the *T. vulgaris* essential oil substantially decreased the reduction in cytochromes *b*-type and *d*-type (by difference spectra), that is, the essential oil produced a blockage in the transport of electrons to cytochromes *b* and *d*. As indicated above, uropathogenic *E. coli* cells were reduced using succinate and glucose as substrates. These results suggested that *T. vulgaris* essential oil inhibits the reduction of cytochromes present in the respiratory chain of uropathogenic *E. coli* and this could explain why the respiratory activity of uropathogenic *E. coli* decreased also considerably. Thus, *T. vulgaris* essential oil affected the cytochrome activity of uropathogenic *E. coli*, this blocked bacterial respiration and inhibited growth. Finally, ubiquinone of *E. coli* was destroyed by irradiation with ultraviolet light. It has been reported that the use of ultraviolet light destroys bound quinones and it has been well established as a means of studying the role of these compounds in microbial metabolism (Adair, 1968). As described in Materials and Methods, cells of uropathogenic *E. coli* were irradiated using a short-wave ultraviolet light lamp in darkness for 90 min. Then, difference spectra were recorded using succinate and glucose as substrates. The results showed that irradiating *E. coli* cells with ultraviolet light produced a decrease in the reduction of *b*-type and *d*-type cytochromes, similar to what occurs in cells that were treated with the *T. vulgaris* essential oil. The above suggested that *T. vulgaris* essential oil might have some effect on the lipophilic ubiquinone transporter of *E. coli*.

CONCLUSION

Essential oils and their antimicrobial properties have been reported in various studies. Little is known about the mechanism of action of *T. vulgaris* essential oil and its antibacterial activity. This study showed evidence of the inhibitory effect of *T. vulgaris* essential oil on the respiratory activity of uropathogenic *E. coli*. It was also found that *T. vulgaris* significantly decreased the reduction of cytochromes *b*-type and *d*-type in uropathogenic *E. coli*. These data led to the proposal of a new mechanism for inhibiting bacterial growth by *T. vulgaris* essential oil.

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