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The effect of *Thymus vulgaris* on growth and biofilm formation of uropathogenic *Escherichia coli*

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Currently, a problem related to public health is resistance to antibiotics because bacteria have been identified to be resistant. It has been reported that bacteria have developed resistance mechanisms to evade the effect of drugs, especially antibiotics. A resistance mechanism to antibiotics is biofilm formation. The biofilms are microbial communities embedded in an extracellular polymeric matrix and are ubiquitous in the microbial world. In recent years, there has been a special interest in studying new antimicrobial strategies to solve the great problem of resistance to antibiotics; thus, the use of essential oils could be an alternative to fight infections caused by biofilm forming bacteria. It has also been reported that essential oils have antiviral and antibacterial properties. In the present work, the effect of *Thymus vulgaris* on growth and biofilm formation of uropathogenic *Escherichia coli* was studied. This study demonstrated the strong effect of *T. vulgaris* essential oil on the growth and biofilm formation of uropathogenic *E. coli.*

Key words: *Thymus vulgaris*, biofilm, *Escherichia coli*, uropathogenic, growth.

INTRODUCTION

Urinary tract infections represent the most common bacterial infections. Over 150 million cases are estimated worldwide each year (Flores-Mireles et al., 2015). Another major problem has been resistance to antibiotics, since bacteria have been identified to be resistant to antibiotics. Resistance to antibiotics has been attributed to the excessive use and misuse of these drugs (Gould and Bal, 2013; Lushniak, 2014; Read and Woods, 2014; Ventola, 2015). It has been reported that bacteria have developed resistance mechanisms to evade the effect of drugs, especially antibiotics (Lin et al., 2015). So, bacteria that cause urinary tract infections have been found to form biofilm, which are mechanical barrier that protect them from the action of antibiotics (Delcaru et al., 2016). Biofilms are microbial communities embedded in an extracellular polymeric matrix; they are ubiquitous in

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the microbial world (Costerton et al., 1995; Høiby, 2017). Reduced antibiotic susceptibility of biofilms contributes to the persistence of infections (Delcaru et al., 2016). It has been proposed that the poor antibiotic penetration, nutrients limitation, slow growth as an adaptive stress response, the formation of persister cells are essential components for the development of persistent and chronic infections (Delcaru et al., 2016). The above indicates that bacteria forming biofilm are associated with recurrent infections, which represent a major public health problem, and their economic impact is high because they cannot be treated with conventional therapies (Høiby, 2017). Therefore, in recent years, there has been a special interest in studying new antimicrobial strategies to solve the great problem of resistance to antibiotics and bacterial biofilm formation (Sharma et al., 2016). In this wise, the use of essential oils could be an alternative to fight infections caused by biofilm-forming bacteria (Sambyal et al., 2017).

Plant essential oils have been used as natural medicines to combat pathogens such as bacteria, fungi and viruses (Kon and Rai, 2012). The mechanism of action for some of them has been reported, for example: damaging the bacterial cell wall and membrane producing the cell destruction and leakage of the cell content (Sambyal et al., 2017). In the present study, the effect of *T. vulgaris* on growth and biofilm formation of uropathogenic *E. coli* 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.

Bacterial strain

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

Culture conditions

The trypticase soy broth (BBL Microbiology Systems, Cockeysville, Md) was used for bacterial culture. Test strains that had been cultured at 37°C for 18 to 24 h in trypticase soy broth were seeded crosswise in a Petri dish containing trypticase soy agar, and the plate was incubated at 37°C for 24 h.

Antimicrobial activity

The antimicrobial activity of essential oil was determined using the technique of disk diffusion in agar with some modifications and the antimicrobial susceptibility test discs. Briefly, tripticasein soy agar plates containing 20 mL of medium were prepared. Sterile Petri dishes (150 mm) were used. Plates were inoculated by cross-striation with uropathogenic *E. coli*. Each inoculum contained approximately 10^6 CFU mL^{-1}. Subsequently, 5 wells were made on the tripticasein soy agar plate with the aid of the mouthpiece of a sterile glass Pasteur pipette. Then, different concentrations (13.3 to 59.4 mg mL^{-1}) of the essential oil were placed in each well. The agar plates were allowed to stand for about 20 min at room temperature. Then, the plates were incubated at 37°C for 24 h. The effect of essential oil of *T. vulgaris* on uropathogenic *E. coli* growth was also tested using antimicrobial susceptibility test discs. For this, the plates were inoculated by cross-striation 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 using a caliper ruler. The analyses were conducted in triplicate. The bactericidal or bacteriostatic effect was determined by passing the bacteriological handle in the plate area without apparent bacterial growth and a fresh tripticasein soy agar plate was inoculated by cross-streak. The plate was incubated at 37°C for 24 h.

Determination of minimum inhibitory concentration

The minimal inhibitory concentration was determined spectrophotometrically using trypticase soy broth and different concentrations (2.64 to 79.2 µg mL^{-1}) of the essential oil. For this, cultures containing approximately 10^6 CFU mL^{-1} were prepared and the working volume was adjusted to 1 mL. The tubes containing trypticase soy broth were incubated at 37°C for 24 h; then, the absorbance at 560 nm was determined. The minimal inhibitory concentration was defined as the lowest concentration of compound at which no growth was evident.

Detection and measurement of biofilm

For the detection of biofilm, uropathogenic *E. coli* was pre cultured during 24 h in tripticasein soy broth. Then, *E. coli* was cultivated in tripticasein soy agar plates containing 0.2% calcofluor and incubated at 37°C for 24 h. Subsequently, the agar plates were irradiated with UV light. The bacterial biofilm was also measured using crystal violet staining. The bacteria were grown in four-fold on the 96-well sterile polystyrene plates and 250 µl of trypticase soy broth were used. A medium without bacteria incubated under the same conditions was treated as a negative control. The plate was incubated at 37°C for 48 to 72 h. After that time, the culture broth was removed and each well was washed with sterile phosphate buffer at pH 7.0; the biofilm was detected by staining with 0.1% violet crystal for 20 min at room temperature. The violet crystal was then solubilized using ethanol and measured spectrophotometrically at 595 nm.

The effect of essential oil on the formation of biofilm

In order to show the effect of *T. vulgaris* on biofilm formation, uropathogenic *E. coli* was inoculated on tripticasein soy agar plates containing 0.2% calcofluor dye. Different concentrations of *T. vulgaris* (1.32 to 13.2 mg mL^{-1}) were placed in wells made in the agar as described above. Plates were incubated at 37°C for 24 h. Later, the plates were irradiated with UV light. The effect of *T. vulgaris* on biofilm formation was also observed in 96 well plates using 0.1% crystal violet dye and different concentrations (0.66 to 52.8 mg mL^{-1}) of esterified essential oil. Thus, the plates were incubated at 37°C for 48 to 72 h and then the biofilm was quantified as indicated in the previous section.

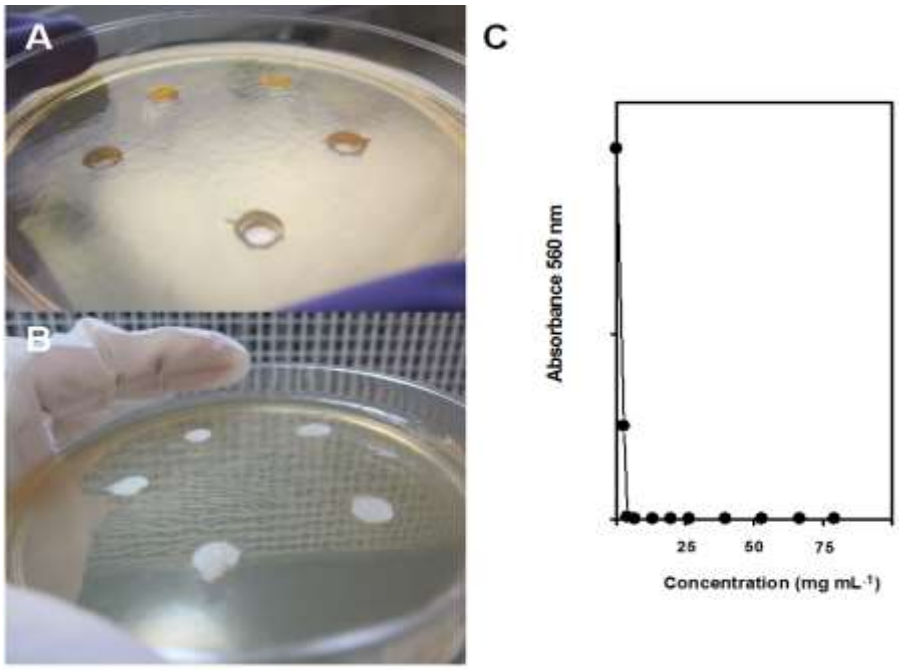

Figure 1. The inhibition of growth from uropathogenic *E. coli* using essential oil of *T. vulgaris*. **A.** The technique of disk diffusion in tripticasein soy agar plates; **B.** The antimicrobial susceptibility test discs; **C.** The minimum inhibitory concentration of *T. vulgaris* on growth of uropathogenic *E. coli*.

Obtaining the esterified essential oil

The esterification of essential oil was made in accordance with the official Mexican standard NMX-F-174-S-1981. 2 mL of *T. vulgaris* was placed in an Erlenmeyer flask containing 20 mL of potassium hydroxide in an alcoholic solution. A reflux condenser was added to the flask, which was placed in a boiling water bath for 15 min with constant stirring. When the saponification was completed, 400 µL of the 1.0% phenolphthalein indicator solution was added and it was titrated in the cold with 0.5 N HCl.

RESULTS

As described above, the antimicrobial activity was determined using different concentrations of the essential oil: 13.2, 19.8, 26.4, 39.6 and 59.4 mg mL^{-1}. The plates were incubated at 37°C for 24 h. The Figure 1A shows the surface of a tripticasein soy agar plate where uropathogenic *E. coli* was cultivated, observing that the growth was completely inhibited. On the other hand, the effect of essential oil of *T. vulgaris* on uropathogenic *E. coli* growth was also tested using antimicrobial susceptibility test discs. So the plates were inoculated by cross-striation with uropathogenic *E. coli* and sterile filter paper disks (containing different concentrations of the essential oil: 0.66, 1.32, 2.64, 6.6 and 13.2 mg mL^{-1}) were placed on the surface of tripticasein soy agar. The agar plates were incubated at 37°C for 24 h. The results obtained are shown in the Figure 1B. Although, lower concentrations of essential oil were used, the uropathogenic *E. coli* growth was completely inhibited as shown in Figure 1B. This test showed greater effectiveness because the essential oil of *T. vulgaris* diffused more easily in the agar and it was not diluted as it occurred in the test of wells on the agar. As mentioned earlier, the bactericidal or bacteriostatic effect was determined by passing the bacteriological handle in the plate area without apparent bacterial growth and a fresh tripticasein soy agar plate was inoculated by cross-streak and incubated at 37°C for 24 h. The results obtained indicated a bactericidal effect because the growth was not recorded (data not shown).

Once the inhibitory effect of the essential oil was determined, the minimum inhibitory concentration was calculated using spectrophotometer at absorbance of 560 nm with trypticase soy broth and different concentrations (0, 2.64, 3.96, 6.66, 13.2, 19.8, 26.4, 39.6, 52.8, 66.6 and 79.2 µg mL^{-1}) of the essential oil. The results obtained are shown in the Figure 1C. Figure 1C shows that low concentrations of essential oil of *T. vulgaris* produced growth inhibition of uropathogenic *E. coli*. From the results obtained, the minimum inhibitory concentration of *T. vulgaris* was calculated at approximately 4 µg mL^{-1}. This low concentration of the essential oil explains the strong inhibitory effect of the growth observed in the tripticasein soy agar shown earlier.

To determine the effect of *T. vulgaris* on the formation of biofilm, the essential oil was chemically esterified because previous biofilm measurements were not

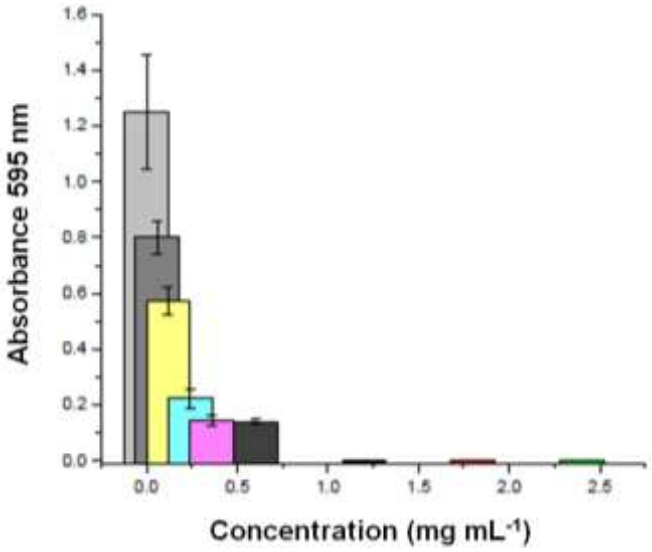

Figure 2. The inhibition of the biofilm formation of uropathogenic *E. coli* by esterified essential oil of *T. vulgaris*.

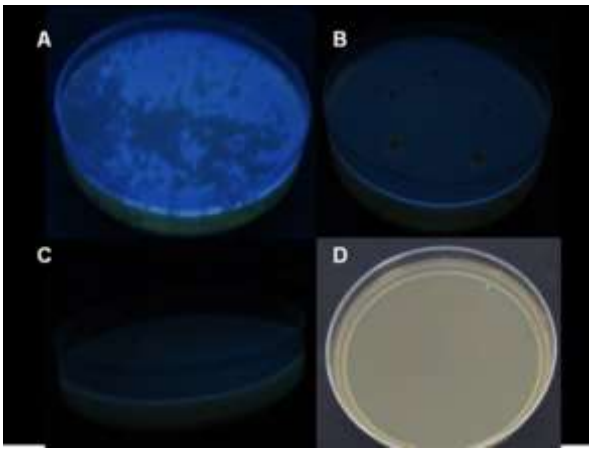

Figure 3. The production of exopolysaccharides by uropathogenic *E. coli* and the effect of essential oil of *T. vulgaris*. **A.** Presence of exopolysaccharides; **B.** Inhibition of the production of exopolysaccharides; **C.** The tripticasein soy agar plate containing 0.2% calcofluor dye and exposed to UV light; **D.** The tripticasein soy agar plate containing 0.2% calcofluor dye.

appropriate which was attributed to the insolubility of *T. vulgaris* in culture broths. The esterified form of the essential oil showed bactericidal activity similar to the non-esterified oil (data not shown). For the measurement of biofilm, *E. coli* was grown on the 96-well sterile polystyrene plates as indicated earlier. The biofilm was determined spectrophotometrically using 0.1% violet crystal. The concentrations of the essential oil used were: 0, 0.06, 0.12, 0.24, 0.36, 0.6, 1.2, 1.8 and 2.4 mg mL^{-1} (Figure 2). As shown in the figure, the formation of biofilm decreases with increasing concentration of esterified essential oil. In addition, at concentrations higher than 1 mg ml^{-1}, the biofilm formation is almost completely inhibited.

To verify that the biofilm of *E. coli* is inhibited by essential oil from *T. vulgaris*, the production of exopolysaccharides was detected using calcofluor. Figure 3A shows the presence of exopolysaccharides produced by uropathogenic *E. coli* on a tripticasein soy agar plate containing 0.2% calcofluor dye and exposed to

UV light. Figure 3B shows the results obtained when *E. coli* was cultured in tripticasein soy agar plate containing 0.2% calcofluor and different concentrations of *T. vulgaris* (in well); the plate was exposed to UV light. The concentrations of the essential oil used were: 1.32, 2.64, 6.6 and 13.2 mg mL^{-1}. As shown in Figure 3B, the essential oil inhibited the growth and the production of exopolysaccharides by uropathogenic *E. coli*. Figure 3C and D show the control conditions.

Given that a commercial essential oil was used in this study, its identity was verified using a spectrophotometric scanning in the UV region of 200 to 360 nm. The absorption spectra of essential oil showed characteristic signals corresponding to the monoterpenes (thymol and carvacrol) used as reference standards (data not shown).

DISCUSSION

Essential oils had been used since ancient times due to their medicinal properties. They also provide aroma and flavor to food (Shuaib et al., 2016). Essential oils are composed of natural terpenes in addition to some other non-terpene components. It has been reported that some essential oils have antioxidative and anticancer activities. In animal models, monoterpenes have been shown to act as chemopreventive or chemotherapeutic agents (Shuaib et al., 2016). It has also been reported that essential oils have antiviral and antibacterial properties. As antibacterial agents, the essential oils act against a wide range of pathogenic bacteria including *Staphylococcus aureus, Bacillus cereus, Shigella dysenteriae, Listeria monocytogenes, Salmonella typhimurium* and *E. coli* O157:H7 (Al-Shuneigat et al., 2014; Høiby, 2017; Hussein et al., 2014; Sambyal et al., 2017; Shuaib et al., 2016; Upadhyay et al., 2013).

In the present work, the effect of *T. vulgaris* on growth and biofilm formation of uropathogenic *E. coli* was studied. It has been reported that the *Thymus* species are considered as medicinal plants due to their pharmacological and biological properties (Kon and Rai, 2012; Shuaib et al., 2016). The flowering parts and leaves of *Thymus* species have been used for different purposes, for example as an antiseptic (Mohsenipour and Hassanshahian, 2015). Thus, in the present study, the high antimicrobial activity was determined using the technique of disk diffusion in agar and antimicrobial susceptibility test discs. As observed, the growth of uropathogenic *E. coli* was completely inhibited (bactericidal effect) at concentrations of the essential oil tested (using both methodologies); however, the antimicrobial susceptibility test was the best method because *T. vulgaris* diffused more easily in the agar. The high antimicrobial activity of *T. vulgaris* has been reported against Gram-negative bacteria such as *E. coli*, *Salmonella enteritidis*, *Salmonella choleraesuis, S. typhimurium, Vibrio cholerae*, *Proteus mirabilis*, *P. vulgaris*, *Pseudomonas aeruginosa* and Gram-positive bacteria as *S. 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). Mohsenipour and Hassanshahian (2015) reported that the *T. vulgaris* extracts using the test of disc diffusion had high ability to inhibit the growth of *P. aeruginosa* and *S. aureus* and low inhibition efficiency on *E. coli* and *B. cereus*. These authors also reported that *T. vulgaris* extracts had low diffusion in solid media when compared with broth media and higher concentration of extract was necessary on solid media to observe the same inhibitory effect than in broth media (Mohsenipour and Hassanshahian, 2015). In the present work, similar results were observed when high concentration of essential oil was used for determination of biofilm in well polystyrene plates stained with violet crystal. The low solubility of the essential oil used at high concentration in broth media led to the esterification of the oil. As mentioned earlier, the minimum inhibitory concentration of *T. vulgaris* was calculated at approximately 4 µg mL^{-1} which was consistent with the inhibitory effect observed on agar plates with low concentration assayed. It has been reported that the hydrophobicity of the essential oils produce changes in bacterial membrane structure and wall structures. Alteration of the cell permeability, disturbance to respiration, modification of bacterial quorum sensing, potassium leakage from cells, effects on membrane potential (proton translocation), changes in pH gradient and ATP production of bacterial cell bacterial lipid membrane, lead to the lysis and death of bacteria (O'Bryan et al., 2015).

On the other hand, the effect of essential oil of *T. vulgaris* on biofilm formation of uropathogenic *E. coli* was determined. The results indicated that formation of biofilm decreased with increase in the concentration of esterified essential oil and at concentrations higher than 1 mg mL^{-1}, the biofilm formation was almost completely inhibited. Although, the minimum inhibitory concentration of *T. vulgaris* was calculated as 4 µg mL^{-1}, to inhibit the *E. coli* biofilm, a higher concentration was required because the essential oil was esterified. Microbial biofilms organized aggregations of cells attached to a substratum and surrounded by a self-produced extrapolymeric substance (EPS) matrix (Ta and Arnason, 2016).

In this work, the production of exopolysaccharides from uropathogenic *E. coli* was detected. The results indicated that uropathogenic *E. coli* exopolysaccharide production was inhibited by *T. vulgaris* using calcoflour dye. Al-Shuneigat et al., (2014) reported that *T. vulgaris* had an inhibitory effect on the formation of biofilm in several bacterial strains and that *E. coli* was the most sensitive, while *P. aeruginosa* was the most resistant for both planktonic and biofilm growth. Hussein et al. (2014) tested different plant extracts (including *T. vulgaris*) and they reported that the most efficient plant extract in inhibition of biofilm formation from *E. coli* was *T. vulgaris*;

Borago officinalis was the least efficient plant extract in inhibition of biofilm formation. It has been reported that thymol and carvacrol (components of the essential oil of *T. vulgaris*) produced the inhibition of biofilm formation in *S. aureus* and *S. epidermidis* against planktonic and biofilm strains (Ta and Arnason, 2015). The results indicated that uropathogenic *E. coli* exopolysaccharide production was inhibited by *T. vulgaris* using calcoflour dye, and also indicated that thymol and carvacrol (monoterpenes) had a strong inhibitory effect (data not shown) on the uropathogenic *E. coli* biofilm formation. These results are in agreement with previous results, which demonstrated that thymol and carvacrol are effective against biofilms of Gram-positive and negative bacteria (Ta and Arnason, 2016; Upadhyay et al., 2013). The thymol inhibited the formation of *L. monocytogenes* biofilms and genes critical to biofilm development were down regulated at concentration of 0.5 mM. Similar results were observed using 0.65 mM of carvacrol by inhibiting biofilm from *L. monocytogenes* (Upadhyay et al., 2013).

CONCLUSION

Antibiotic resistance remains a serious clinical problem; thus, it stimulates studies for search of new methods for treatment of infectious diseases using essential oils and plant extracts with antimicrobial activity. This study demonstrated the strong effect of *T. vulgaris* essential oil on the growth and biofilm formation of uropathogenic *E. coli*. This substance could be used as an alternative for the treatment of bacterial infections. However, the cytotoxic effect it has on humans, has not yet been determined

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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