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The Effect of Essential Oil of *Thymus vulgaris* on the Growth of Bacterial Environmental and Clinical Isolates

Flores-Encarnación M.[1*], Acosta-Báez, L.A.[1], Aguilar-Gutiérrez G.R.[2], Cabrera-Maldonado C.[3], Gallardo-Soto H.M.[1], Sánchez-Herrera A.J.[1]

[1]*Laboratorio de Microbiología Molecular y Celular. Biomedicina, Facultad de Medicina.*

Benemérita Universidad Autónoma de Puebla. Puebla, Puebla, México.

[2]*Centro de Investigación sobre Enfermedades Infecciosas. Instituto Nacional de Salud Pública. Cuernavaca, Morelos. México.*

[3]*Dpto. de Microbiología. Facultad de Ciencias Químicas. Benemérita Universidad Autónoma de Puebla. Puebla, Puebla, México.*

Corresponding Author:** ***M. Flores-Encarnación, *Laboratorio 421, Edificio EMA1. Biomedicina. Facultad de Medicina. Benemérita Universidad Autónoma de Puebla. 13 sur 2702, Col. Volcanes, C.P. 72410. Puebla, Puebla, México.*

Abstract: *Bacteria have developed resistance mechanisms to evade the effect of antibiotics. The antibiotic resistance is a global problem. Currently, the antimicrobial properties of plant extracts and essential oils and the research of alternatives for treatment of infectious diseases are being studied. It has been observed that essential oils inhibits the growth of pathogenic microorganisms. In the present study, the effect of T. vulgaris on growth of bacterial environmental and clinical isolates was studied.*

Keywords: *Essential oil, Thymus vulgaris, bacteria, antibiotic resistance, bacteria.*

1. INTRODUCTION

The essential oil of *Thymus vulgaris* as an antioxidant agent for a long time has been used (Kulisic *et al*., 2005). Some of its bacteriostatic and bactericidal properties also have also been studied (Borugă *et al*., 2014). It has been reported that *T. vulgaris* inhibits the growth of some pathogenic microorganisms such as *Candida albicans*, *Legionella* sp., *Mycobacterium* sp., *Pseudomonas aeruginosa*, *Vibrio parahaemolyticus*, *V. fluvialis*, *Streptococcus pneumoniae*, *S. mutans*, *Enterococcus faecalis*, *Staphylococcus aureus*, among others (Billinger *et al*., 2009; Djeghboub *et al*., 2018; Oliveira *et al*., 2017; Oramadike and Ogunbanwo, 2017; Rojas-Armas *et al*., 2015; Wang *et al*., 2014). Some of these bacteria have shown to be resistant to the action of antibiotics and they are also capable for forming biofilm on both abiotic and biotic surfaces (Fair and Tor, 2014; Flemming *et al*., 2016). Biofilms are a population of bacterial cells that grown attached to a surface involved in exopolysaccharide matrix, which protects them from attack by antibiotics or immune system (Costerton *et al*., 1999; Flores-Encarnación *et al*., 2014). In this context, it has been reported the presence of pathogenic bacteria forming biofilm inside water pipes commonly used, as well as in catheters of patients hospitalized for long periods of time and who suffer from chronic urinary tract infections (Abdallah *et al*., 2011; Tsvetanova and Najdenski, 2017). A common problem in water distribution networks is the formation of bacterial biofilms inside them (Ashbolt, 2015; Chaves-Simoes and Simoes, 2013; Flores-Encarnación *et al*., 2016b; Richards *et al*., 2015; Zhang *et al*., 2012). Bacterial growth in the water pipes has been of interest in many countries, especially because the pathogenic microorganisms to humans can grow forming biofilm (Chaves-Simões *et al*., 2013; Costerton, 1999; Flores-Encarnación *et al*., 2016b; Mahapatra *et al*., 2015). Availability of potable water devoid of pathogens is fundamental to public health, hovewer water systems are not sterile; they can contain a variety of opportunistic pathogens established as part of the drinking water microbiome (Szewzyk *et al*., 2000; Wang *et al*., 2014). It has been proven that hypochlorite has little effect on biofilms. However, chlorine dioxide dosed at a continuous low level, ozone and UV disinfection have been reported for removal and prevention of biofilm from water systems (Mahapatra *et al*., 2015). In

the present study, the effect of *T. vulgaris* on growth of bacterial environmental and clinical isolates was studied.

2. MATERIALS AND METHODS

2.1. Source of Material

In this study a commercial essential oil of *T. vulgaris* was used. As reference commercial essential oils of peppermint, eucalyptus and clove were used. All were obtained from a flavour and fragrance company at Puebla, México.

2.2. Bacterial Strains

For this study, *Pseudomonas aeruginosa* (10 isolations: P1-P10), *Escherichia coli* (5 isolations: E1-E5), *Citrobacter freundii* (3 isolations: C1-C3) and *Klebsiella oxytoca* (5 isolations: K1-K5) environmental bacteria were used. The bacterial samples forming biofilm were previously isolated and identified from domestic water pipes commonly used at Puebla municipality, México (Flores-Encarnación *et.al*, 2016b). 10 strains of uropathogenic *E. coli* from clinical isolates also were used. The strains were provided by some diagnostic laboratories at Puebla city. The identity of uropathogenic *E. coli* strains was confirmed by using the microbial biochemical tests described by Fernández-Olmos *et al*., (2010). Bacterial strains were stored at -40°C in tryptic soy broth with 20% glycerol until analysis. As reference *E. coli* CFT073 strain was used.

2.3. Culture

The tripticasein soy broth was used for bacterial culture. For that, a total of 125 µL of each bacterial strain was inoculated in 5 mL of tripticasein soy broth and incubated overnight at 37°C during 24 hours (preculture). The growth in plate was assayed on tripticasein soy agar plates. Bacteria were inoculated in cross groove on tripticasein soy agar plates and it was incubated at 37°C for 24 hours.

2.4. Effect of *T. vulgaris*

The effect of essential oil was determined using the technique of disk diffusion in agar using discs impregnated with essential oil of *T. vulgaris*. Briefly, tripticasein soy agar plates containing 20 mL of medium were prepared. Sterile Petri dishes (60x100 mm) were used. Plates were inoculated by cross-striation with bacterial strains. Each inoculum contained approximately $1x10^6$ CFU mL^{-1}. Then, sterile filter paper disks (5 mm diameter) were placed on the surface of tripticasein soy agar plates. Different concentrations of the essential oil were added: 1.3, 2.6, 3.9, 6.5 and 13 µg. The agar plates were incubated at 37°C for 24 hours. As a comparison, the assay also was done using peppermint, eucalyptus and clove essential oils. Different concentrations (1 to 10 µg) of the essential oil were added: peppermint, eucalyptus and rosemary. The analyses were conducted in triplicate.

2.5. Antibiotic Sensitivity Test

As reference, the antibiotic sensitivity test of uropathogenic *E. coli* strains was done. For this, the antibiotic diffusion plate technique was used. The discs with antibiotics used: ampicillin (10µg), oxacillin (1µg), chloramphenicol (30µg) and furazolidone (100µg) (BBL, Sensi-Disc, Oxoid discs). The bacteria was incubated overnight 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. The antibiotic sensitivity test for bacteria forming biofilm in domestic water pipes was determined in a previous work (Flores-Encarnación *et.al,* 2016a).

3. RESULTS

In this study, 30 bacterial strains were used which 20 strains were recovered from biofilm formed inside water pipes. The bacterial strains forming biofilm were previously isolated and identified from domestic water pipes commonly used at Puebla. The remaining 10 were strains of uropathogenic *E. coli* from clinical isolates (patients with urinary tract infection). The latter were provided by diagnostic laboratories of Puebla city. The Table 1 shows bacterial strains from environmental and clinical isolates. The identity of bacterial strains was confirmed by using the microbial biochemical tests as described in Material and Methods. As seen in Table 1, *P. aeruginosa*, *E. coli*, *Citrobacter*

freundii and *Klebsiella oxytoca* were the bacteria recovered from the interior of water pipes (environmental strains), as soon as the clinical isolates (from urinary tract infection) included 10 strains of uropathogenic *E. coli*. On the other hand, the effect of essential oil of f *T. vulgaris* was determined using the technique of disk diffusion on agar as described in Material and Methods. So, the discs were impregnated with different concentrations of essential oil: 1.3, 2.6, 3.9, 6.5 and 13 µg. The results obtained were shown in Fig. 1. The Fig. 1A shows the surface of a tripticasein soy agar plate where bacterial strains from environmental isolates were cultured, observing that the growth was completely inhibited for the different concentrations of *T. vulgaris* used. The same effect was observed when *E. coli*, *C. freundii* and *K. oxytoca* strains were subjected to essential oil. In the case of *P. aeruginosa*, one of the strains was not as sensitive to the presence of *T. vulgaris*. The use of 1.3 to 3.9 µg of essential oil caused the formation of growth inhibition halos (data not shown). As mentioned earlier, 10 strains of uropathogenic *E. coli* from clinical isolates also were assayed. The results obtained were shown in Fig. 1B. As can be seen in Fig. 1B, the trypticasein soy agar surface lacked bacterial growth and the surface of the agar acquired a bright appearance. In all strains of uropathogenic *E. coli* a strong inhibitory effect of growth was observed. The results obtained indicated that *T. vulgaris* showed a potent inhibitory effect on the growth of uropathogenic *E. coli* at the concentrations of the essential oil tested. In all cases, the results obtained indicated a bactericidal effect because the growth was not recorded (data not shown). As a comparison, the effect of thyme, peppermint, eucaliptus and rosemary essential oils was determined using an uropathogenic *E. coli* CFT 073 strain. The results obtained were shown in Fig. 2. As can be seen, thyme essential oil showed the greatest inhibitory effect on growth of uropathogenic *E. coli* (Fig. 2A). The essential oils of peppermint, eucalyptus and rosemary showed a lower inhibitory effect on the growth of uropathogenic *E. coli* at the concentrations tested (Fig. 2B, 2C, 2D). On the other hand, the antibiotic sensitivity test was done (as reference). In previous work, the sensitivity to antibiotics of some strains forming biofilm in water pipes was determined (data not shown). In the present study, the antibiotic sensitivity for uropathogenic *E. coli* strains (from urinary tract infection) was done. The diffusion plate technique was made using discs with ampicillin, oxacillin, chloramphenicol and furazolidone as described in Material and Methods. The results obtained shown that all uropathogenic *E. coli* strains were resistant to ampicillin and oxacillin and they were sensitive to chloramphenicol and furazolidone. In the antibiotic sensitivity tests, the growth inhibition zone obtained using chloramphenicol and furazolidone discs measured 22 to 30 mm in diameter for the concentrations tested (30 and 100 µg, respectively) (data not shown). However, in none of the cases a strong inhibition of bacterial growth was observed, as it happened using the essential oil of *T. vulgaris*.

Table1. *The bacterial strains from environmental and clinical isolates.*

Bacterial strain	From
Pseudomonas aeruginosa 1	Forming biofilm in water pipes
Pseudomonas aeruginosa 2	Forming biofilm in water pipes
Pseudomonas aeruginosa 3	Forming biofilm in water pipes
Pseudomonas aeruginosa 4	Forming biofilm in water pipes
Pseudomonas aeruginosa 5	Forming biofilm in water pipes
Pseudomonas aeruginosa 6	Forming biofilm in water pipes
Pseudomonas aeruginosa 7	Forming biofilm in water pipes
Pseudomonas aeruginosa 8	Forming biofilm in water pipes
Pseudomonas aeruginosa 9	Forming biofilm in water pipes
Pseudomonas aeruginosa 10	Forming biofilm in water pipes
Escherichia coli 1	Forming biofilm in water pipes
Escherichia coli 2	Forming biofilm in water pipes
Escherichia coli 3	Forming biofilm in water pipes
Escherichia coli 4	Forming biofilm in water pipes
Escherichia coli 5	Forming biofilm in water pipes
Citrobacter freundii 1	Forming biofilm in water pipes
Citrobacter freundii 2	Forming biofilm in water pipes
Citrobacter freundii 3	Forming biofilm in water pipes
Klebsiella oxytoca 1	Forming biofilm in water pipes
Klebsiella oxytoca 2	Forming biofilm in water pipes
Klebsiella oxytoca 3	Forming biofilm in water pipes

Uropathogenic *E. coli* 1	Urinary tract infection
Uropathogenic *E. coli* 2	Urinary tract infection
Uropathogenic *E. coli* 3	Urinary tract infection
Uropathogenic *E. coli* 4	Urinary tract infection
Uropathogenic *E. coli* 5	Urinary tract infection
Uropathogenic *E. coli* 6	Urinary tract infection
Uropathogenic *E. coli* 7	Urinary tract infection
Uropathogenic *E. coli* 8	Urinary tract infection
Uropathogenic *E. coli* 9	Urinary tract infection
Uropathogenic *E. coli* 10	Urinary tract infection

4. DISCUSSION

The essential oils have been used for flavored foods and beverages; they have been used to disguise unpleasant odors and control health problems in humans and animals (Franz *et al.*, 2010; Teles Andrade *et al*., 2014). The essentil oils are typically liquid, colored, volatile. They are synthesized by aromatic plants as secondary metabolites in several plant organs (flowers, leaves, stems, branches, seeds, roots, bark) and they are characterized by a strong odor (Bakkali *et al*., 2008). It has been reported that the essential oils have several biological properties, such as larvicidal action, antioxidant, analgesic and anti-inflammatory, fungicide and antitumor activity (Carmo *et al*., 2008; Mendes *et al*., 2010; Rajkumar *et al*., 2010; Silva *et al*., 2008; Wannes *et al*., 2010). Unfortunately, the marked increase in antimicrobial resistance among common bacterial pathogens is now threatening this therapeutic. The World Health Organization has named antibiotic resistance as one of the three most important public health threats of the 21st century (Munita and Arias, 2016). In recent years there has been increasing interest in the use of biologically active organic compounds which are extracted from plant species that have the ability to eliminate pathogenic microorganisms by themselves; this is mainly due to the resistance that microorganisms have developed to antibiotics (Daferera *et al*., 2003; Flores-Encarnación *et al*., 2016c; Marston *et al*., 2016). The antimicrobial activity of essential oils has been researched against a variety of microorganisms (López *et al*., 2005). However, the emergence of multidrugresistant bacteria poses a challenge to treating infections, so the need to find new substances with antimicrobial properties for use in the fight against these microorganisms is evident (Hemaiswarya *et al*., 2008; Pereira *et al*., 2004; Teles Andrade *et al*., 2014). In the present study, the effect of *T. vulgaris* on growth of bacterial environmental and clinical isolates was studied. For that, 20 strains were recovered from biofilm formed inside water pipes and 10 were strains of uropathogenic *E. coli* from clinical isolates. The antimicrobial activity was determined using the technique of disk diffusion in agar and antimicrobial susceptibility test discs. As it was observed, the growth of bacterial strains from environmental isolates was completely inhibited at the different concentrations of *T. vulgaris* used. The same effect was observed when *E. coli*, *C. freundii* and *K. oxytoca* strains were subjected to essential oil (Fig. 1). One of the strains of *P. aeruginosa* was not as sensitive to the presence of *T. vulgaris* where it was observed the formation of growth inhibition halos (data not shown). It has been reported that the pathogenic bacteria to humans can grow forming biofilm inside water pipes which it has been of interest in world (Flores-Encarnación *et al.*, 2016b; Mahapatra *et al.*, 2015). Bacteria forming biofilms are surrounded by a exopolymer matrix that binds them to water storage tanks and pipes (Flores-Encarnación *et al.*, 2016b). Bacteria settle on the inner surfaces of pipes and form biofilm which becomes them the source of secondary microbial contamination of water (Rożej *et al*., 2015). As mentioned earlier, the results obtained shown that the essential oil of *T. vulgaris* inhibited completely the growth from environmental isolates at low concentration. To the concentrations tested (1.3 to 13 µg of essential oil), the growth of bacteria was totally dejected. The effect was bactericidal as could be demonstrated from the reseeding that was done it not having recorded subsequent growth in a fresh culture medium (data not shown). It has been reported that *T. vulgaris* extract strongly inhibited the growth of *P. aeruginosa* and *S. aureus* but it inhibited poorly the growth of *Bacillus cereus* and *E. coli* (Mohsenipour and Hassanshahian, 2015). It has been reported several chemical components, such as thymol, carvacrol and eugenol, are the most active constituents of *T. vulgaris* which destabilizes the bacterial cytoplasmic membrane (Al-Shuneigat *et al*., 2014).

As mentioned earlier, the uropathogenic *E. coli* strains from clinical isolates also were assayed. The results shown a strong inhibitory effect on growth from all strains of uropathogenic *E. coli* tested: the

trypticasein soy agar surface lacked bacterial growth and the surface of the agar acquired a bright appearance (Fig. 1B). The results obtained shown that the essential oil of *T. vulgaris* inhibited completely the growth from clinical isolates at low concentration (1.3 to 13 µg). Biofilm formation is an important step in the pathogenicity of bacteria because biofilm increases the resistance to antibiotics as well as to the immune system, for example: cells living in biofilm are about 1000 times more resistant to antimicrobial agents (Al-Shuneigat *et al*., 2014; Costerton *et al*., 1999). It has been reported that the most common infections caused by biofilms are urinary tract infections being uropathogenic *E. coli* the infectious agent that is most frequently isolated (Flores-Mireles *et al*., 2015; Foxman *et al*., 2014; Salehzadeh and Zamani, 2018). Other infectious agent living in biofilm are related to use of catheters (by *S. epidermidis*), middle-ear infections in children (by *Haemophilus influenzae*), tooth decay (by *S. mutans*), burns, wound infection, lung infection (by *P. aeruginosa*) (Al-Shuneigat *et al*., 2014). In the present study, the antibiotic sensitivity for uropathogenic *E. coli* strains was done using ampicillin, oxacillin, chloramphenicol and furazolidone. The results shown that all uropathogenic *E. coli* strains were resistant to ampicillin and oxacillin; they were sensitive to chloramphenicol and furazolidone. However, in none of the cases a strong inhibition of bacterial growth was observed, as it happened using the essential oil of *T. vulgaris*. This result could be explained by the effectiveness of essential oil components for alter the permeability of the bacterial wall, such as phenols and monoterpenes which are responsible for the irreversible damage in the membrane and the cellular walls (Winward *et al*. 2002). It has been reported also that the essential oils have high penetrability and are more effective acting on organized biofilm than antibiotics (Al-Shuneigat *et al*., 2014). To compare the effect produced by the essential oil of *T. vulgaris* on bacterial growth, in this study the thyme, peppermint, eucaliptus and rosemary essential oils was tested using an uropathogenic *E. coli* CFT 073 strain (Fig. 2). The results shown that the thyme essential oil produced the greatest inhibitory effect on bacterial growth. The essential oils of peppermint, eucalyptus and rosemary showed a lower inhibitory effect on the growth of uropathogenic *E. coli* at the concentrations tested.

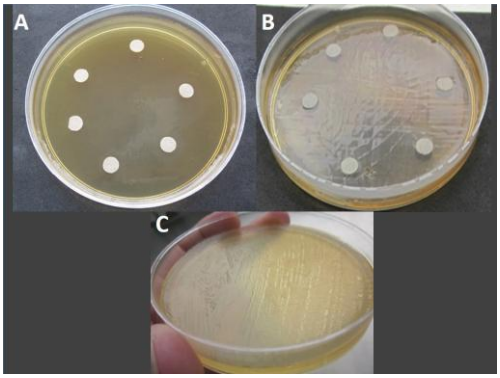

Fig1. *The effect of essential oil of T. vulgaris on the growth of bacterial environmental and clinical isolates. A. The growth was completely inhibited by T. vulgaris in strains from environmental isolates (forming biofilm in water pipes); B. All strains of uropathogenic E. coli from clinical isolates have a strong inhibitory effect on growth; C. Control condition.*

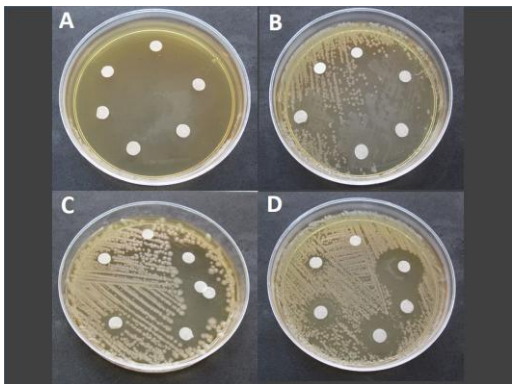

Fig2. *The effect of some essential oils on growth of uropathogenic E. coli. A. Thyme; B. Peppermint; C. Eucaliptus; D. Rosemary. In all cases, 1 to 10 µg of essential oil were used and placed in increasing concentrations in the counterclockwise direction.*

5. CONCLUSION

In the present study was possible to conclude that the essential oil of *T. vulgaris* was a potent inhibitor of growth from clinical uropathogenic *E. coli* and environmental isolates strains. Given the current problem that has generated the antibiotic resistance is necessary the research of alternatives for treatment of infectious diseases using essential oils and plant extracts with antimicrobial activity.

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