International Journal of Research Studies in Biosciences (IJRSB)
Volume 5, Issue 12, 2017, PP 6-11
ISSN No. (Online) 2349-0365
DOI: http://dx.doi.org/10.20431/2349-0365.0512002
www.arcjournals.org

The Effect of *Thymus vulgaris* on the Formation of Biofilm from Uropathogenic *Escherichia* Coli in Venoclysis Tubes

Flores-Encarnación M.[1*], Hernández-Ramírez L.F.[1], Nava-Nolazco R.M.[1], Díaz-Escalona M.[1], Ramos-Maya E.P.[1], Cabrera-Maldonado C.[2]

[1]*Laboratorio de Microbiología Molecular y Celular. Facultad de Medicina. Benemérita Universidad Autónoma de Puebla. Puebla, Puebla, México.*

[2]*Depto. de Microbiología. Facultad de Ciencias Químicas. Benemérita Universidad Autónoma de Puebla. Puebla, Puebla, México.*

***Corresponding Author*: *Flores-Encarnación M,** *Laboratorio de Microbiología Molecular y Celular. Facultad de Medicina. Benemérita Universidad Autónoma de Puebla. Puebla, Puebla, México.*

Abstract: *The resistance of bacteria to antibiotics has been a current topic of public health. Currently, it is known that a variety of bacteria are resistant to antibiotics. Therefore, alternative strategies are being sought that inhibit bacterial growth and that function as bactericides for the treatment of bacterial infections. Some extracts and essential oils from plants seem to be the solution because they have powerful bactericidal properties, they are easy to obtain, they do not pollute the environment, the production cost is low. It has been reported also the bacterial antibiofilm activity from plant products or extracts rich in bioactive compounds and that they act on a variety of pathogenic bacteria. This work shows the effect of essential oil of Thymus vulgaris on the formation of biofilm from uropathogenic E. coli in venoclysis tubes.*

Keywords: *Thymus vulgaris, venoclysis, biofilm, E. coli, tubes.*

1. Introduction

The fighting to bacterial infections has been a topic of interest over time (Nazzaro *et al.*, 2013). With the discovery of antibiotics it seemed that the problem to infectious bacterial diseases was solved. However, the resistance of bacteria to antibiotics has been a current topic of public health (Ventola, 2015). Currently, it is known that a variety of bacteria are resistant to antibiotics (Sengupta *et al*., 2013). In that context, studies are being carried out in different parts of the world in the search for substances of plant origin which be used to fight infections caused by bacteria (Nazzaro *et al*., 2013). Since ancient times, essential oils have been used in traditional medicine. The essential oils are substances produced by aromatic plants as secondary metabolites; they are obtained from plant materials as flowers, leaves, fruits, branches, seeds, bark, by different methods (Bakkali *et al*., 2008; Burt, 2004; Citarasu, 2010; Cowan, 1999). One of the advantages of using essential oils as an alternative therapeutic is that it has a wide range of antimicrobial activity. They are composed of a mixture of compounds as terpenoids, alcohols, aldehydes and other (Diao *et al.*, 2013; Flores-Encarnación *et al*., 2016*c*). It has been reported the bacterial antibiofilm activity from plant products or extracts rich in bioactive compounds (Chmit *et al*., 2014). The bacterial biofilms are communities surrounded by an exopolymer matrix adhered to surfaces (Costerton *et al*., 1999; Flores-Encarnación *et al*., 2014*a*). About 70% of human bacterial infections involve biofilms. The bacterial biofilms are cause of infections related with medical devices, for example: vascular catheters, prosthetic joints and others (Bjarnsholt, 2013; Donlan, 2011; Flores-Encarnación *et al.*, 2014*a*; Flores-Encarnación *et al.*, 2014*b*). The present study the effect of *T. vulgaris* on the formation of biofilm from uropathogenic *E. coli* in venoclysis tubes was studied.

2. Material and Methods

2.1. 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.

2.2. Bacterial Strain

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

2.3. Culture Conditions

The trypticase soy broth was used for bacterial culture. Test strains that had been cultured at 37°C for 18-24 h in trypticase soy broth were seeded crosswise in a Petri dish containing trypticase soy agar, the plate was incubated at 37°C for 24 hours.

2.4. Antimicrobial Activity Assay

The antimicrobial activity assay 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 (100 mm) were used. Plates were inoculated by cross-striation with uropathogenic *E. coli*. Each inoculum contained approximately $1x10^6$ 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 of the essential oil were placed in each well: 13.2, 19.8, 26.4, 39.6 and 59.4 mg. The agar plates were allowed to stand for about 20 minutes at room temperature. The plates were then incubated at 37°C for 24 hours. The effect of essential oil of *T. vulgaris* on uropathogenic *E. coli* growth was also tested using antimicrobial susceptibility test discs. For that, the plates were inoculated by cross-striation with uropathogenic *E. coli*. 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: 0.66, 1.32, 2.64, 6.6 and 13.2 mg. The agar plates were incubated at 37°C for 24 hours. The analyses were conducted in triplicate.

2.5. Detection of Biofilm

The biofilm was detected using crystal violet and calcofluor white stains according to the methodology modified by Flores-Encarnación *et al*., (2016*a,b,c*). For this, the uropathogenic *E. coli* strain was precultured during 24 hours in tripticasein soy broth. Then, *E. coli* was cultivated in a Petri dish (diameter 100 mm) containing tripticasein soy broth and sterile venoclysis tubes of length 15 mm. The Petri dishes were incubated at 37°C for 4 to 7 days in humidity chamber. After that time, venoclysis tubes was removed and each tube was washed with distilled water. The biofilm was detected staining the venoclysis tubes with 0.1% violet crystal for 20 min at room temperature. On the other hand, staining with calcofluor white was used for to check the formation of biofilm by producing of exopolysaccharides from biofilm matrix. For that, venoclysis tubes were staining with 0.2% calcofluor dye for 20 min at room temperature. Subsequently, the venoclysis tubes were irradiated with UV light. A Petri dish containing tripticasein soy broth and sterile venoclysis tubes was used as negative control.

2.6. The Effect of *T. Vulgaris* on Biofilm Formation

In order to know the effect of *T. vulgaris* on biofilm formation, the Petri dishes containing tripticasein soy broth and sterile venoclysis tubes were prepared as was described in the previous section. Before placing the venoclysis tubes, they were immersed in the essential oil of *T. vulgaris* for 1 to 20 min and then placed in the culture broth. The Petri dishes were incubated at 37°C for 4 to 7 days in humidity chamber. The biofilm was detected staining the venoclysis tubes with violet crystal and calcofluor white.

3. RESULTS

In this study the antibacterial activity was determined using the agar diffusion and antimicrobial susceptibility disc tests. In the first case, tripticasein soy agar plates were inoculated with uropathogenic *E. coli* and different concentrations of the essential oil were added in each well as in Materials and Methods was described. The results obtained were shown in Fig. 1. Fig. 1A shows the strong inhibitory effect on the growth of uropathogenic *E. coli* in all tested concentrations of the essential oil of *T. vulgaris*. As can be seen in Fig. 1A, the trypticasein soy agar surface lacked bacterial growth and the surface of the agar acquired a bright appearance. These results were obtained at high concentrations of the essential oil: 13.2 to 59.4 mg. Therefore, lower concentrations of

essential oil were tested: 0.66 to 13.2 mg. For that, the antimicrobial susceptibility test discs were used as in Materials and Methods was described. The results obtained were shown in Fig. 1C. As shown in Fig. 1C, at the lowest concentrations of essential oil used, the growth of uropathogenic *E. coli* was also completely inhibited. The bright appearance of the trypticasein soy agar surface was also observed. The Fig. 1B and Fig. 1D showed the growth of uropathogenic *E. coli* in the absence of essential oil. 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.

On the other hand, the effect of *T. vulgaris* on the biofilm formation of uropathogenic *E. coli* in venoclysis tubes was tested. The venoclysis tubes were incubated and prepared as mentioned in the Materials and Methods section. The results obtained were shown in Fig. 2. As shown in Fig. 2A, staining with violet crystal showed the formation of biofilm from uropathogenic *E. coli* in the venoclysis tubes. This same result was confirmed when the venoclysis tubes were irradiated with UV light, which showed the production of exopolysaccharides in the biofilm of uropathogenic *E. coli* (Fig. 2D). As shown in Fig. 2B and Fig. 2C, the *T. vulgaris* inhibited the formation of biofilm when the venoclysis tubes were exposed for 1 and 20 min, respectively. The production of exopolysaccharides in the *E. coli* biofilm was also inhibited by the essential oil, as could be demonstrated using calcofluor white staining (Fig. 2E and Fig. 2F). When the venoclysis tubes without bacterial growth were exposed to UV light, was no observed fluorescence emission; the same happened when they were impregnated with the essential oil and were exposed to UV light (data not shown). The results obtained indicated that *T. vulgaris* showed a potent inhibitory effect on biofilm formation of uropathogenic *E. coli* at the concentrations of the essential oil tested.

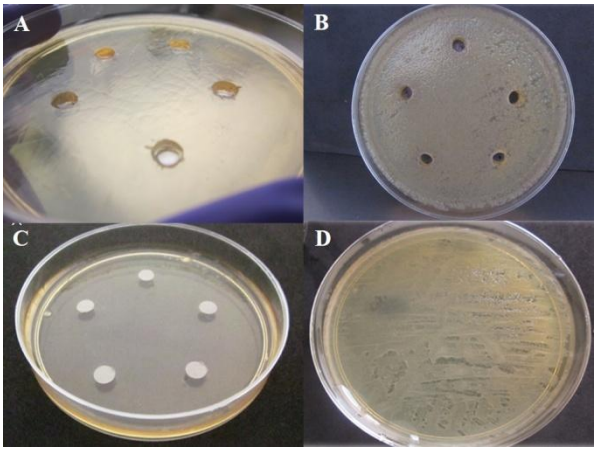

Fig. 1. *The antibacterial activity of T. vulgaris on the growth of uropathogenic E. coli. A. The agar diffusion test. B. Control condition. C. The antimicrobial susceptibility disc test. D. Control condition*

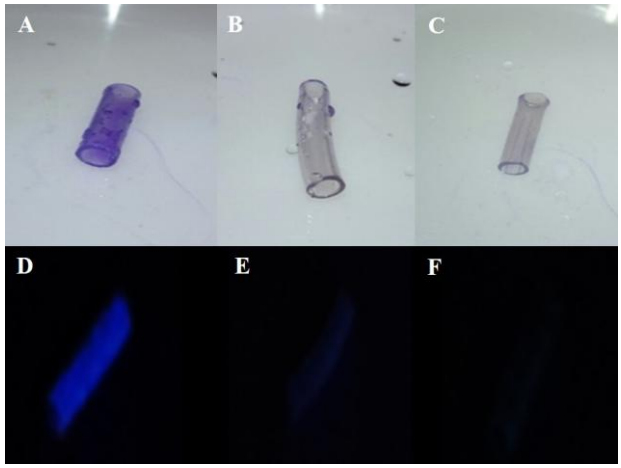

Fig. 2. *The effect of T. vulgaris on the biofilm formation of uropathogenic E. coli in venoclysis tubes. A. Formation of biofilm from uropathogenic E. coli staining with violet crystal. B. Inhibition of biofilm formation using T. vulgaris for 1 min. C. Inhibition of biofilm formation using T. vulgaris for 20 min. D. Presence of exopolysaccharides in the biofilm of uropathogenic E. coli. E. Inhibition of exopolysaccharides production using T. vulgaris for 1 min. F. Inhibition of exopolysaccharides production using T. vulgaris for 20 min*

4. DISCUSSION

One of the great challenges at present is the resistance to antibiotics, which represents a serious global public health problem (Bjarnsholt, 2013; Sengupta *et al*., 2013; Ventola, 2015). Despite the development of new and varied antibiotics, the bacteria develop resistance in a short time making the treatments are not effective in their entirety. Bacteria have developed different mechanisms that have allowed them to develop even in the presence of antibiotics (Lin *et al*., 2015). One of them is the ability to form biofilm. The biofilms constitute a protected mode of growth that allows survival in a hostile environment (Costerton *et al*., 1999). The presence of biofilm gives certain advantages to bacteria: protection from the environment, resistance to the bactericidal action and host defense mechanisms (for example: the phagocytic activity) (Flores-Encarnación *et al*., 2014*a*; Kostakioti *et al*., 2013). In recent years, various substances of plant origin with bactericidal activity have been studied. Among them, plant extracts and essential oils has attracted the attention of numerous research groups around the world (Bakkali *et al*., 2008; Flores-Encarnación *et al*., 2016*c*; Nazzaro *et al*., 2013). The essential oils are present in flowers, leave, seeds, stems, fruits, roots and they contain a wide series of secondary metabolites which inhibit the growth of bacteria (Burt, 2004; Nazzaro *et al*., 2013). On the other hand, it has been reported that 1.7 million patients acquire infections during treatment in hospitals and healthcare units (Peleg and Hooper, 2010). Many of the nosocomial infections are related to the use of medical devices, for example: urinary catheters, feeding tubes and prostheses (Bertl *et al*., 2015; Siddiq and Darouiche, 2012; Talpaert *et al*., 2015). Device-associated infections are mostly originated from the formation of pathogenic biofilms on the device surface (Pickard *et al*., 2012). For that, in recent years great progresses have been made in development of anti-biofilm substances and antimicrobial functions (Chen *et al*., 2017). So, in the present study the effect of *T. vulgaris* on the formation of biofilm from uropathogenic *E. coli* in venoclysis tubes was studied. The results obtained shown that the essential oil of *T. vulgaris* inhibited completely the growth of uropathogenic *E. coli*, both at low and high concentrations. To the concentrations tested (0.66 to 59.4 mg of essential oil), the growth of the 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 *Pseudomonas aeruginosa* and *Staphylococcus aureus* but it inhibited poorly the growth of *Bacillus cereus* and *E. coli*. *T. vulgaris* extracts are more efficient in broth media than in solid media; they have low diffusion in solid media compared with broth media. Therefore, greater concentration is required in solid media compared with broth media (Mohsenipour and Hassanshahian, 2015). In our study, the essential oil of *T. vulgaris* had a potent inhibitory effect, even in solid media where the studies were carried out. The minimum inhibitory concentration was calculated at 3 micromolar (data not shown). Mohsenipour and Hassanshahian (2015) reported the minimum inhibitory concentration of *T. vulgaris* extracts 0.156 to 2.5 mg, attributing to it bacteriostatic properties. The essential oil of *T. vulgaris* contains several chemical components. Thymol, carvacrol and eugenol are the most active constituents with a wide antimicrobial spectrum. Both constituents destabilizes the bacterial cytoplasmic membrane (Bassolé *et al*. 2010; Al-Shuneigat *et al*., 2014; Soković *et al*., 2010; Ultee *et al*., 2002). Several mechanisms have been proposed to explain their mechanism of action. Hydroxyl group of eugenol may react with proteins and inhibit action of enzymes; hydrophobic thymol and carvacrol may damage the outer membrane of Gram-negative bacterial cell wall releasing lipopolysaccharides (Gómez-Estaca *et al*. 2010; Kon and Rai, 2012).

In this study was also determined the effect of essential oil on the formation of biofilm in venoclysis tubes. The results obtained indicated that *T. vulgaris* showed a potent inhibitory effect on biofilm formation of uropathogenic *E. coli* at the concentrations of the essential oil tested (Fig. 2). *T. vulgaris* inhibited the formation of biofilm when the venoclysis tubes were exposed to essential oil for 1 and 20 min (determined by violet cristal test). The violet crystal staining provides a biofilm qualitative assay because it evidences the adherence to the walls of venoclysis tubes used in this assay. The violet crystal stains bacterial organic matter attached to surfaces. The violet crystal provides a good measure of biofilm mass but it does not give a measure of biofilm viability (Welch *et al*., 2012). To verify the existence of living bacteria in biofilm, the calcofluor white stain was used. The calcofluor white is a fluorescent dye that binds in the glycosidic linkages β-(1-3) and β-(1-4); it is generally used for to observe the exopolysaccharides in biofilm (Flores-Encarnación *et al*., 2016*a*). As it was observed in

Fig. 2, the production of exopolysaccharides in the uropathogenic *E. coli* biofilm was also inhibited by *T. vulgaris*, which provided other evidence of the bactericidal effect of the essential oil and therefore, the inhibition of biofilm formation.

It has been reported that *T. vulgaris* has produced a remarkably decreased in the biofilm formation by *S. aureus* and *P. aeruginosa* in 0.2% concentration, destroying the living bacteria in the biofilm. Their research confirmed that the inhibitory effect of essential oil was more efficient than some antibiotics such as ampicillin, loxacin, and gentamicin (Kavanaugh and Ribbeck, 2012; Mohsenipour and Hassanshahian, 2015).

5. CONCLUSION

The interest in essential oils and bioactive compounds has been increasing due to cases of bacterial resistance that occur in the world. The essential oils are a possible alternative for the treatment and control of pathogenic bacteria. So, the essential oil of *T. vulgaris* provide an effective, economical and feasible alternative for combating infections.

ACKNOWLEDGEMENTS

Thank to VIEP-BUAP and PRODEP for the facilities provided for the development of this work.

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Citation: *F. Encarnación et al., "The Effect of Thymus vulgaris on the Formation of Biofilm from Uropathogenic Escherichia coli in Venoclysis Tubes", International Journal of Research Studies in Biosciences (IJRSB), vol. 5, no. 12, pp. 6-11, 2017. http://dx.doi.org/10.20431/2349-0365.0512002*