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Inhibition of Gram Negative Bacterial Growth and Biofilm Formation by Alpha Thujone

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Abstract: *The increasing resistance of microorganism to antibiotics in the last decades, drove microbiologists, botanists, and natural-products chemists to search for phytochemicals that could be used for treatment of infectious diseases. Essential oils, which are derived from plants, such as thujone, have been recognized to display many biological activities many of which needed more exploration. This study was designed to test the ability of alpha thujone to inhibit the growth and biofilm formation of clinical bacterial isolates. The agar dilution method used to detect the antibacterial effect of alpha thujone, revealed that at high concentrations (30 mg/ml and 15 mg/ml) alpha thujone showed some antibacterial activity on some of the Gram-negative isolates tested. It was evident, however, and after using standard tests for detection of the inhibition of biofilm formation, that alpha thujone, at low concentrations (3.8 mg/ml) demonstrated an inhibitory effect on the biofilm formation of most of the different bacterial strains tested. This previously uncovered characteristic of alpha thujone, may prove to be very useful in preventing infections by primary pathogens.*

***Key words*:** Essential oils, alpha thujone, antibacterial agents, bacterial biofilms, inhibition of biofilm formation

I. Introduction

Over time, antibiotics were able to spare millions of lives [1]. Nowadays, the ability of microorganisms to resist antibiotic therapy is ever so increasing and is currently being considered as a major threat to human health and patients' lives; making treatments, to once curable infections, more difficult or even impossible [2].

Bacterial resistance against antimicrobial agents is attainable by many methods, the most common of which is the release of enzymes that work on targeting functional sites in these antimicrobials to reduce their effect, the extended spectrum beta lactamases (ESBLs), of which, are an example [3, 4].

What makes the problem worse is the ability of many bacterial strains to form biofilms. These rigid dome-like polysaccharide shells encompassing several micro-colonies, toil on securing core masses by limiting the penetrative abilities of antibiotics and other serum constituent that may have antibacterial effects; structures that modify the mode and state of bacterial growth from a planktonic to a sessile one [5,6].

Many natural products, mainly plants, were used, for years, in the treatment of medical conditions, as some were proved to possess antibacterial effects [7,8,9] and ability to inhibit biofilm formation ([10]. The essential oils, a major component of these products, were particularly focused on [11].

It was demonstrated that a primary constituent of the essential oils derived from a variety of plants including wormwood (*Artemisia absinthium*), mugwort (*Artemisia vulgaris*), clary (*Salvia sclarea*), tansy (*Tanacetum vulgare*), and yellow cedar (*Thuja occidentalis*) was thujone [12]. Thujone, in its alpha and beta forms, was used in traditional medicine in the treatment of many conditions including irritable bowel syndromes, warts, and acne [11,13].

Nevertheless, thujone was reported not to be harmless. In fact, alpha-thujone, the more potent isoform of this compound did cause neurotoxic side-effects once orally administered to mice and rats elucidated through convulsions [14,15,16]. Alpha thujone's LD_{50} associated with such acute toxic conditions was 134 mg/kg in mice and 180 mg/kg body weight in rats [17]. Human intoxication by alpha-thujone did show similar outcomes upon oral consumption, but dose-effect comparisons were shown to be uncertain [18,19].

The main objective of this study was to examine the effect of several concentrations on the growth and biofilm formation of several strains of clinically significant Gram negative bacteria.

II. Material and Methods

2.1. Bacterial isolates

The bacterial isolates used in the study are clinical isolates provided by the Clinical Microbiology Laboratory of the Lebanese American University Medical Center- Rizk Hospital (LAUMC- RH). The test organisms used were

2 isolates of each of following organisms: *Pseudomonas aeruginosa*, *Escherichia coli, Klebsiella pneumoniae, Proteus mirabilis,* and *Citrobacter koseri*. The strains were chosen to be strong biofilm formers (as demonstrated by the method described in section 2.3.3 below).

2.2. Antimicrobial effect of alpha thujone:

The antibacterial activity of alpha thujone was tested using the agar dilution method [20]. Five sets of Mueller-Hinton agar (MHA) plates were prepared as recommended by the manufacturer. Alpha thujone was added to the sterilized and cooled but still melted MHA to reach final concentration of 30, 15, 7.5, 3.75 and 1.875 mg/ml of alpha thujone respectively. The turbidity of the test organisms (2 strains of each of: *P. aeruginosa, E. coli, K. pneumoniae, P. mirabilis* and *C. koseri*) was adjusted to match that of a 0.5 McFarland standard in trypticase soy broth (TSB). The prepared sets of plates were then seeded by each of the test organisms using a sterile swab. The plates were incubated at 35°C for 24 h after which they were checked for the growth of these organisms.

2.3. Effect of the ethanol alpha- thujone on biofilm formation

2.3.1. Preparation of the alpha-thujone ethanol solutions:
A stock alpha-thujone (Sigma-Aldrich), was diluted using 80% ethanol, to solutions with alpha thujone concentrations of: 30, 15, 7.5, 3.75 and 1.875 mg/ml.

2.3.2. Preparation of the bacterial isolates
From fresh agar plates, each of the test organisms was used to inoculate a 10 ml trypticase soy broth (TSB) tube with 1% glucose. The inoculated TSB tubes were left in the incubator at 35°C for 24 h after which, the culture tubes were diluted 100 times with fresh media.

2.3.3. Effect of alpha thujone on biofilm formation

To detect the ability of biofilm formation by the isolates and its possible inhibition by the ethanol solution of alpha-thujone, a method, slightly modified from that suggested by Mathur et al. (2006), was used [21]. The ethanol solution was added to the test wells of the 96 well flat-bottom tissue culture plates and the plates were left to dry in the incubator under aseptic conditions. The amounts of alpha thujone tested were adjusted to be: 7.6, 3.8, 1.9, 0.95 and 0.475 mg/mls respectively. Two hundred µl of sterile TSB were added to the wells of the plates with 10 µl of the diluted cultures (previous section) and incubated at 35°C for 24 h. The contents of the wells were then gently discarded by repeated soft tapping, after which the wells were washed with phosphate buffered saline (PBS, pH of 7.2) several times. Then, 0.2% sodium acetate was added to fix any biofilms that may have formed and a 0.1% solution of crystal violet was finally added to stain the biofilms, when present. Excess stain was then removed with deionized water and the plates were left to dry. The optical densities were later determined by using a microplate auto-reader at 570 nm wavelength. To have a precise result, each of the test samples (and controls) was performed in 16 wells. The reported optical densities in the study were the averages of the 16 readings of each sample. In order to be able to measure the effect of alpha-thujone on the biofilm formation of the tested strains, the negative control readings, in all micro-titer plates, were deducted from the reported results.

III. Results:

The effect of the different concentrations of alpha-thujone on the growth of the test strains is shown in Table 1. It was evident that the major reduction of the growth of these strains occurred only at relatively high concentrations of alpha thujone, namely at 30 and 15 mg/ml. Tables 2, 3, 4, 5 and 6 show the average optical density (O.D.) readings, at 570nm wavelength, of the different isolates tested in this study, reflecting their ability to form biofilms under the effect of **7**.6, 3.8, 1.9, 0.95 and 0.475 mg/ml of alpha thujone respectively. Figures 1-5 are graphic representations of the results shown in Tables 2-6 respectively. Table 7 summarizes the effect of the different concentrations of alpha thujone on the formation of biofilms by the tested strains.

Table 1. The effect of the different concentrations of alpha-thujone on the growth of the test strains -: no growth, +: weak growth, ++: moderate growth, +++: confluent growth.

Isolate	Control - no alpha-thujone	30 mg/ml alpha-thujone	15 mg/ml alpha-thujone	7.5 mg/ml alpha-thujone	3.75 mg/ml alpha-thujone	1.875 mg/ml alpha-thujone
Citrobacter koseri (isolate 1)	+++	-	+	++	++	+++
Citrobacter koseri (isolate 2)	+++	+	+	++	++	+++
Klebsiella pneumoniae (isolate 1)	+++	_	+	++	+++	+++
Klebsiella pneumoniae (isolate 2)	+++	+	+	++	++	+++
Pseudomonas aeruginosa (isolate 1)	+++	+	++	++	++	+++
Pseudomonas aeruginosa (isolate 2)	+++	+	+	++	++	++++
Proteus mirabilis (isolate1)	+++	_	_	+	+	++
Proteus mirabilis (isolate 2)	+++	+	++	+++	+++	+++
Escherichia coli (isolate 1)	+++	+	++	+++	+++	+++
Escherichia coli (isolate 2)	+++	+	++	++	+++	+++

Table 2. The average optical density (O.D.) readings at 570nm wavelength of the different isolates tested in this study reflecting their ability to form biofilms under the effect of **7**.6 mg/ml of alpha thujone. Ck: *Citrobacter koseri,* Ec: *Escherichia coli*, Pm: *Proteus mirabilis*, Pa: *Pseudomonas aeruginosa* and Kp*: Klebsiella pneumoniae*

Well content	**7.6 t + Ck1**	**Ck1**	**7.6 t + Ck2**	**Ck2**	**7.6 t + Ec1**	**Ec1**
readings	0.4693405	0.41731	0.611044	0.479433	0.50359825	0.470668
Control elimination	0.1129239	0.060893	0.2546274	0.123016	0.14718166	0.114252
Well content	**7.6 t + Ec2**	**Ec2**	**7.6 t + Pm1**	**Pm1**	**7.6 t + Pm2**	**Pm2**
readings	0.5936331	0.605538	0.456455	0.489741	0.62269963	0.544136
Control elimination	0.2372165	0.249121	0.1000384	0.133325	0.26628303	0.187719
Well content	**7.6 t + Pa1**	**Pa1**	**7.6 t + Pa2**	**Pa2**	**7.6 t + Kp1**	**Kp1**
readings	0.5612565	0.573431	0.656243	0.567242	0.49757225	0.434139
Control elimination	0.2048399	0.217015	0.2998264	0.210825	0.14115566	0.077723
Well content	**7.6 t + Kp2**	**Kp2**	**7.6 t**	**control**		
readings	0.5309688	0.493972	0.3064166	0.356417		
Control elimination	0.1745522	0.137555	-0.0501	0		

Figure 1. Graphic representation of the data in Table 2, reflecting the ability of the different test strains to form biofilms under the effect of **7**.6 mg/ml of alpha thujone. X axis: samples tested; Y axis: The average optical density (O.D.) readings at 570 nm wavelength. Ck: *Citrobacter koseri*, Ec: *Escherichia coli*, Pm: *Proteus mirabilis*, Pa: *Pseudomonas aeruginosa* and Kp*: Klebsiella pneumoniae*.

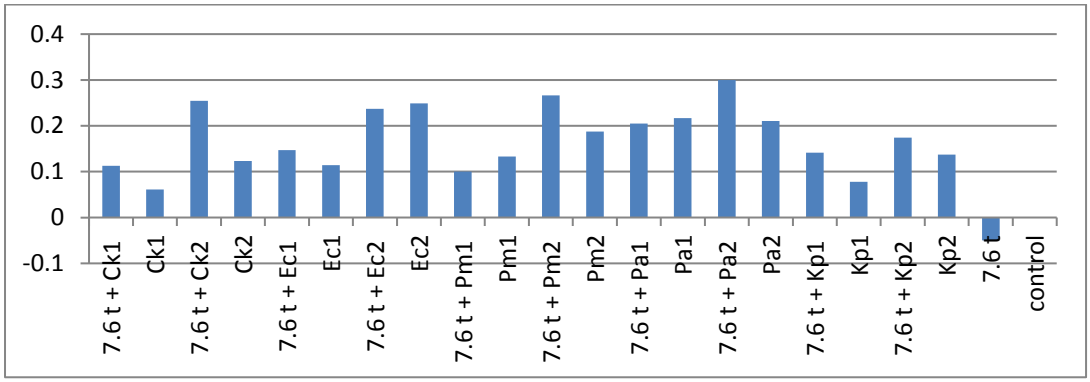

Table 3. The average optical density (O.D.) readings at 570nm wavelength of the different isolates tested in this study reflecting their ability to form biofilms under the effect of 3.8 mg/ml of alpha thujone. Ck: *Citrobacter koseri*, Ec: *Escherichia coli*, Pm: *Proteus mirabilis*, Pa: *Pseudomonas aeruginosa* and Kp*: Klebsiella pneumoniae*

Well content	**3.8 t + Ck1**	**Ck1**	**3.8 t + Ck2**	**Ck2**	**3.8 t + Ec1**	**Ec1**
readings	0.4045054	0.41731	0.4293337	0.479433	0.48574333	0.470668
Control elimination	0.0480888	0.060893	0.0729171	0.123016	0.12932674	0.114252
Well content	**3.8 t + Ec2**	**Ec2**	**3.8 t + Pm1**	**Pm1**	**3.8 t + Pm2**	**Pm2**
readings	0.6325467	0.605538	0.5040972	0.489741	0.51361763	0.544136
Control elimination	0.2761301	0.249121	0.1476806	0.133325	0.15720103	0.187719
Well content	**3.8 t + Pa1**	**Pa1**	**3.8 t + Pa2**	**Pa2**	**3.8 t + Kp1**	**Kp1**
readings	0.514832	0.573431	0.5167763	0.567242	0.39116457	0.434139
Control elimination	0.1584154	0.217015	0.1603597	0.210825	0.03474798	0.077723
Well content	**3.8 t + Kp2**	**Kp2**	**3.8 t**	**control**		
readings	0.5029893	0.493972	0.2943877	0.356417		
Control elimination	0.1465727	0.137555	-0.0620289	0		

Figure 2. Graphic representation of the data in Table 3, reflecting the ability of the different test strains to form biofilms under the effect of 3.8 mg/ml of alpha thujone. X axis: samples tested; Y axis: The average optical density (O.D.) readings at 570 nm wavelength. Ck: *Citrobacter koseri*, Ec: *Escherichia coli*, Pm: *Proteus mirabilis*, Pa: *Pseudomonas aeruginosa* and Kp*: Klebsiella pneumoniae*.

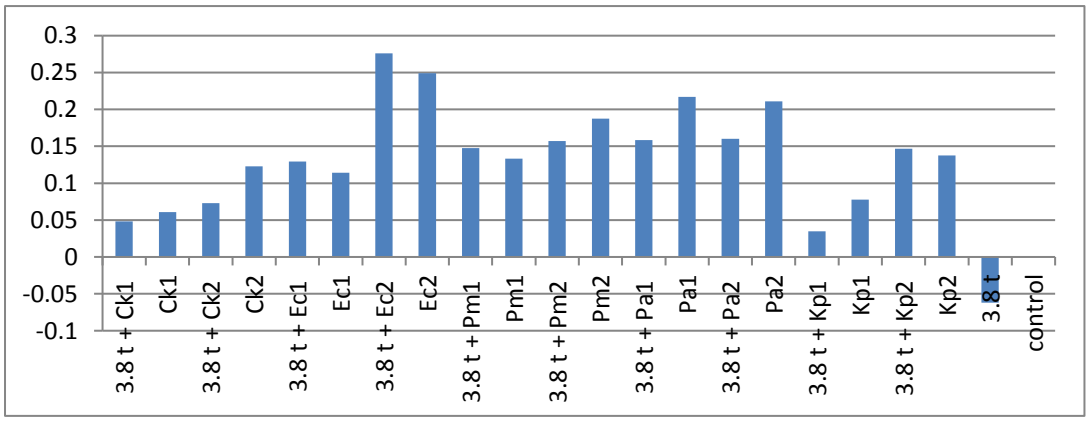

Table 4. The average optical density (O.D.) readings at 570nm wavelength of the different isolates tested in this study reflecting their ability to form biofilms under the effect of 1.9 mg/ml of alpha thujone. Ck: *Citrobacter koseri*, Ec: *Escherichia coli*, Pm: *Proteus mirabilis*, Pa: *Pseudomonas aeruginosa* and Kp*: Klebsiella pneumoniae*.

Well content	**1.9 t + Ck1**	**Ck1**	**1.9 t + Ck2**	**Ck2**	**1.9 t + Ec1**	**Ec1**
readings	0.431938	0.41731	0.40145913	0.479433	0.49459225	0.470668
Control elimination	0.07552141	0.060893	0.04504253	0.123016	0.13817566	0.114252
Well content	**1.9 t + Ec2**	**Ec2**	**1.9 t + Pm1**	**Pm1**	**1.9 t + Pm2**	**Pm2**
readings	0.563985625	0.605538	0.521306	0.489741	0.606342	0.544136
Control elimination	0.207569035	0.249121	0.16488941	0.133325	0.24992541	0.187719
Well content	**1.9 t + Pa1**	**Pa1**	**1.9 t + Pa2**	**Pa2**	**1.9 t + Kp1**	**Kp1**
readings	0.533324429	0.573431	0.55208063	0.567242	0.3849035	0.434139
Control elimination	0.176907838	0.217015	0.19566403	0.210825	0.02848691	0.077723
Well content	**1.9 t + Kp2**	**Kp2**	**1.9 t**	**control**		
readings	0.59788775	0.493972	0.33625821	0.356417		
Control elimination	0.24147116	0.137555	-0.0201584	0		

Figure 3. Graphic representation of the data in Table 4, reflecting the ability of the different test strains to form biofilms under the effect of 1.9 mg/ml of alpha thujone. X axis: samples tested; Y axis: The average optical density (O.D.) readings at 570 nm wavelength. Ck: *Citrobacter koseri*, Ec: *Escherichia coli*, Pm: *Proteus mirabilis*, Pa: *Pseudomonas aeruginosa* and Kp*: Klebsiella pneumoniae*.

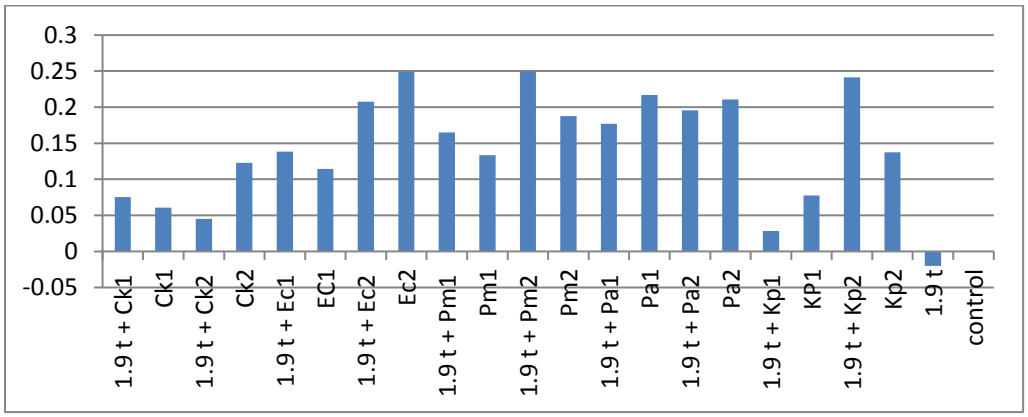

Table 5. The average optical density (O.D.) readings at 570nm wavelength of the different isolates tested in this study reflecting their ability to form biofilms under the effect of 0.95 mg/ml of alpha thujone. Ck: *Citrobacter koseri*, Ec: *Escherichia coli*, Pm: *Proteus mirabilis*, Pa: *Pseudomonas aeruginosa* and Kp*: Klebsiella pneumoniae*.

Well content	**0.95 t + Ck1**	**Ck1**	**0.95 t + Ck2**	**Ck2**	**0.95 t + Ec1**	**Ec1**
readings	0.416093875	0.41731	0.37524813	0.479433	0.490638333	0.470668
Control elimination	0.059677285	0.060893	0.01883153	0.123016	0.134221743	0.114252
Well content	**0.95 t + Ec2**	**Ec2**	**0.95 t + Pm1**	**Pm1**	**0.95 t + Pm2**	**Pm2**
readings	0.523095143	0.605538	0.52814313	0.489741	0.624039	0.544136
Control elimination	0.166678552	0.249121	0.17172653	0.133325	0.26762241	0.187719
Well content	**0.95 t + Pa1**	**Pa1**	**0.95 t + Pa2**	**Pa2**	**0.95 t + Kp1**	**Kp1**
readings	0.526681	0.573431	0.568543	0.567242	0.397814	0.434139
Control elimination	0.17026441	0.217015	0.21212641	0.210825	0.04139741	0.077723
Well content	**0.95 t + Kp2**	**Kp2**	**0.95 t**	**control**		
readings	0.615335143	0.493972	0.29315294	0.356417		
Control elimination	0.258918552	0.137555	-0.0632637	0		

Figure 4. Graphic representation of the data in Table 5, reflecting the ability of the different test strains to form biofilms under the effect of 0.95 mg/ml of alpha thujone. X axis: samples tested; Y axis: The average optical density (O.D.) readings at 570 nm wavelength. Ck: *Citrobacter koseri*, Ec: *Escherichia coli*, Pm: *Proteus mirabilis*, Pa: *Pseudomonas aeruginosa* and Kp*: Klebsiella pneumoniae*.

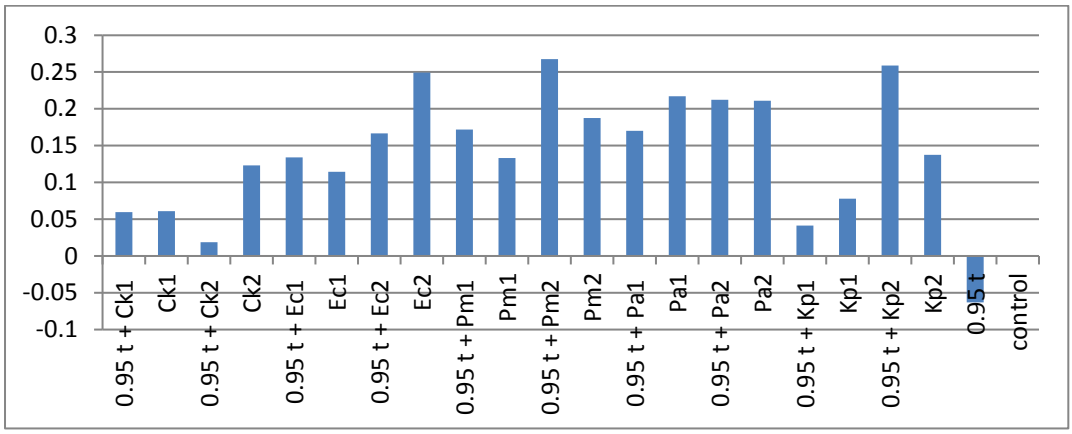

Table 6. The average optical density (O.D.) readings at 570nm wavelength of the different isolates tested in this study reflecting their ability to form biofilms under the effect of 0.475 mg/ml of alpha thujone. Ck: *Citrobacter koseri*, Ec: *Escherichia coli*, Pm: *Proteus mirabilis*, Pa: *Pseudomonas aeruginosa* and Kp*: Klebsiella pneumoniae*.

Well content	**0.475 t + Ck1**	**Ck1**	**0.475 t + Ck2**	**Ck2**	**0.475 t + Ec1**	**Ec1**
readings	0.478332	0.41731	0.4428285	0.479433	0.457713375	0.470668
Control elimination	0.12191541	0.060893	0.08641191	0.123016	0.101296785	0.114252
Well content	**0.475 t + Ec2**	**Ec2**	**0.475 t + Pm1**	**Pm1**	**0.475 t + Pm2**	**Pm2**
readings	0.530272625	0.605538	0.5737375	0.489741	0.78414225	0.544136
Control elimination	0.173856035	0.249121	0.21732091	0.133325	0.42772566	0.187719
Well content	**0.475 t + Pa1**	**Pa1**	**0.475 t + Pa2**	**Pa2**	**0.475 t + Kp1**	**Kp1**
readings	0.648851	0.573431	0.58688163	0.567242	0.462142625	0.434139
Control elimination	0.29243441	0.217015	0.23046503	0.210825	0.105726035	0.077723
Well content	**0.475 t + Kp2**	**Kp2**	**0.475 t**	**control**		
readings	0.499087857	0.493972	0.29441538	0.356417		
Control elimination	0.142671267	0.137555	-0.0620012	0		

Figure 5. Graphic representation of the data in Table 6, reflecting the ability of the different test strains to form biofilms under the effect of 0.475 mg/ml of alpha thujone. X axis: samples tested; Y axis: The average optical density (O.D.) readings at 570 nm wavelength. Ck: *Citrobacter koseri*, Ec: *Escherichia coli*, Pm: *Proteus mirabilis*, Pa: *Pseudomonas aeruginosa* and Kp*: Klebsiella pneumoniae*.

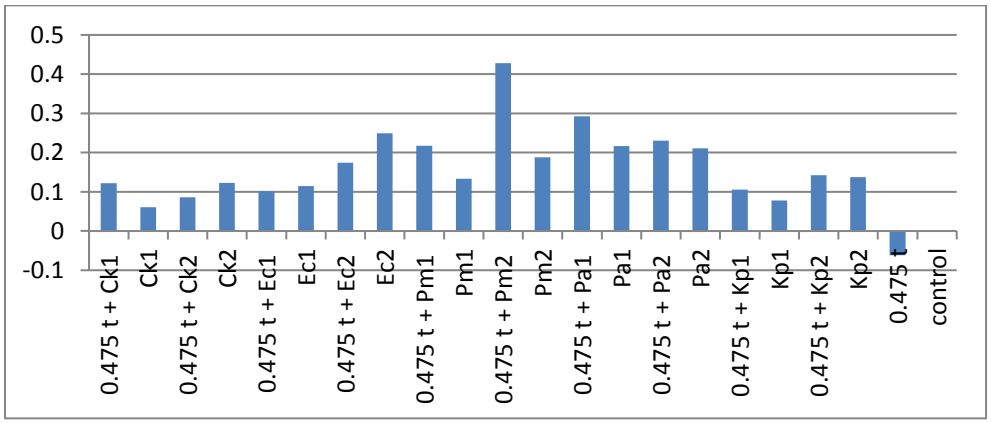

Table 7. Summary of alpha thujone antibiofilm effect at different concentrations against all bacterial isolates tested in this study. Ck: *Citrobacter koseri*, Ec: *E. coli*, Pm: *Proteus mirabilis*, Pa: *Pseudmonas aeuroginosa*, Kp*: K. pneumoniae*. +: Inhibitory effect on biofilm formation. -: No inhibitory effect on biofilm formation.

	Ck1	Ck2	Ec1	Ec2	Pm1	Pm2	Pa1	Pa2	Kp1	Kp2
7.6t	-	-	-	+	+	-	+	-	-	-
3.8t	+	+	-	-	-	+	+	+	+	-
1.9t	-	+	-	+	-	-	+	+	+	-
0.95t	-	+	-	+	-	-	+	-	+	-
0.475t	-	+	-	+	-	-	-	-	-	-

IV. Discussion

Since alpha-thujone was reported to have low water solubility (407 mg/l at room temperature [22], it was decided that the standard agar dilution method [23], was the most suitable protocol to determine the antibacterial effect of alpha thujone as previously used by other investigators [24].

Testing the antibacterial effect of alpha thujone was attempted previously [25,26,27,28]. In this study, the antibacterial properties of alpha thujone, were obvious at the higher concentrations used. At a concentration of 30 mg/ml, alpha thujone was capable of completely preventing the growth of 1 strain of each of *Citrobacter koseri*, *Klebsiella pneumoniae* and *Proteus mirabilus* (Table 1). There was, however, a noticeable reduction of the growth of all of the other strains used in the study. The use of higher concentration could have stopped the growth of all the tested organisms, but it was not attempted to do so as the chemical was known to be toxic at high concentrations [16,29,30,31,32,33,34].

This, to our knowledge, is the first report about the ability of alpha thujone to interfere with the bacterial ability to form biofilms. Bacterial biofilms were found to be responsible for development of many serious and sometimes chronic infections [35]. The bacteria that are known to produce biofilms, were found to be in a sessile state after being in a planktonic state, and thus protected from what may harm them, in the surrounding environment, by a polymeric matrix [36,37]. That shield also served as a location where communication between bacteria (quorum sensing) was possible, leading to gene transfer or gene activation, that changed the dispersed organisms to become much more pathogenic to the host [38,39]. Moreover, the presence of biofilms was demonstrated to delay healing of regular and surgical wounds [40].

The ability of alpha thujone to inhibit biofilm formation by the Gram negative bacteria used in this study was obvious (Tables 2-6 and Figures 1-5). Table 7 which summarizes the results, shows that all the low concentrations of alpha thujone used, had some inhibitory effect on the ability of at least 2 of the tested strains to form biofilms. However, the maximal effect was obtained at an alpha thujone concentration of 3.8 mg/ml concentration (Table 7), where 60% of the clinical strains used were completely prevented from forming biofilms.

The addition of alpha thujone, in low concentrations, that are nontoxic, to scrubbing fluids and soap, ointments and even detergents may prove useful in inhibiting biofilm formation of potentially pathogenic bacteria and thus minimize the dangers of their initial attachment to the host tissues or exposed surfaces.

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