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Evaluation of antibiofilm activity of *Thymus syriacus* essential oil against clinically isolated MDR bacteria

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Abstract: Finding alternative strategies to confront bacterial resistance is an urgent need. Biofilm-forming bacteria have become a serious problem in medicine and industry. Bacteria can use biofilm as a mechanism of resistance against antibacterial drugs and avoid the immune system. The aim of this study was to evaluate the antibacterial activity of *Thymus syriacus* (*T. syriacus*) essential oil in a solid and liquid medium and to study its antibiofilm formation activity. The *T. syriacus* essential oil was extracted from the aerial parts of the plants. The minimum inhibitory concentrations (MIC) measurements were used to identify The antibacterial activity of the essential oil against multidrug-resistant strains *Pseudomonas aeruginosa* (*P. aeruginosa*), *Klebsiella pneumonia* (*K. pneumonia*), *Streptococcus pneumonia* (*S. pneumonia*) isolated clinically from blood infections. The microtiter plate was used in order to quantify biofilm formation by bacteria. The minimum inhibitory concentration (MIC) against the three clinically isolated strains (*P. aeruginosa*, *K. pneumonia*, *S. pneumonia*) were (3.12, 1.56, 3.12 µL/mL) respectively. The formation of biofilm by (*P. aeruginosa*, *K. pneumonia*, *S. pneumonia*) was reduced up to (43%, 50%, 60%) respectively, when the essential oil was applied at MIC concentrations for each strain. The observed antibacterial activity of *T. syriacus* essential oil was significant against

antibacterial-resistant strains and antibiofilm formation activity was identified. The novelty of this study is we confirmed that the essential oil of *T. syriacus* exhibited not just antibacterial properties but also antibiofilm formation effect. More studies are needed in order to continue studying this oil and evaluate its other medicinal properties and toxicity.

Keywords: antibacterial, antibiofilm, natural product, medicinal plants, essential oil, *T. syriacus*

1. Introduction

The abuse of antimicrobial agents in poultry farming is one of the main causative factors of bacterial resistance [1, 2]. Likewise, irrational use of antibiotics in healthcare [3] renders antibacterial agents ineffective against bacteria such as *Legionella pneumophila* [4] and *Mycobacterium tuberculosis* [5, 6]. Multidrug-resistant (MDR) *Acinetobacter baumannii* strains are the main cause of urinary tract infections in the elderly with a biofilm-forming ability which is more difficult to treat [7]. Furthermore, the resistance problem can be caused by other bacteria like Methicillin-resistant *Staphylococcus aureus* (MRSA) and *S. pneumoniae,* and Gram-negative bacteria like *K. pneumoniae*, *P. aeruginosa, Escherichia coli* (*E. coli*)*, and Mycobacterium tuberculosis* (*Mtb*) [8, 9].

Biofilm is a self-enclosed population of bacterial cells [10] Biofilm producing organisms have a different pattern of growth rates, gene expression and metabolism with a far more resistant to antibacterial agents than not biofilm-forming bacteria [10, 11]. Bacterial biofilms are a serious problem in clinical practice and industries, and numerous efforts have been made to eradicate the bacterial biofilm [12]. Many studies have been conducted in order to understand the mechanism of biofilm formation and how the resistant genes and exopolysaccharides matrix expressed during biofilm formation, which can increase the biofilm antibiotic resistance and become a serious threat making treating such infections more difficult [13]. Even though infectious control using nanomaterials and nanotechnology has been well researched, their application is still in the developmental stage [14-16]. Therefore, finding alternative strategy in order to face this serious emerging problem and increase the activity of other antibacterial agents is an urgent need [17]. Natural products like essential oils have been studied for a long time to verify their antibacterial effect [18-20]. It was suggested that essential oils affect bacterial proliferation and contain many compounds with different mechanisms like damaging cell membrane, increasing its permeability, damaging cytoplasmic membrane, cell lysis, leakage of intracellular material, inhibiting efflux pump mechanism of antibiotics rendering them more efficient [21-24].

The genus *Thymus* is also known locally in Syria as Zattar [25]. It has numerous species and varieties native to southern Europe and Asia [26]. *Thymus* genus has been claimed as a source of primary material for pharmaceutical industry used in medicine and cosmetics [25]. Research and studies about *Thymus syriacus* (*T. syriacus*) are scarce and more studies are

needed to verify its medical application and characteristics. The chemical composition of essential oil could be variable. *Thymus* spp. essential oil is characterized by the presence of high concentration of isomeric phenolic monoterpenes thymol and or carvacrol [27]. The concentrations of thymol and carvacrol, the main components of the thyme essential oil range from (3-60%) of the total essential oil [28] and the other component are in trace amount. The antimicrobial activity of the essential oil and its mechanism of action may vary depending on the percentage of composition of its ingredients [29]. It was reported that the antibacterial activity of *Thymus* essential oil has been widely used in food and cosmetics. Moreover, many studies have suggested the application of essential oil in antibacterial therapy and found a role for essential oil against biofilm-forming properties of some microorganisms [30]. Despite some reports about the antibacterial activity of *Thymus* essential oil [31, 32], further evidence is desired to verify the antimicrobial activity of *T. syriacus* essential oils formulations. Our study aimed to evaluate the bactericidal, bacteriostatic, antibiofim activity of *T. syriacus* essential oil against various clinically isolated drug-resistant bacteria.

2. Materials and Methods

2.1. Preparation of essential oil

T. syriacus were prepared after harvesting the plant from the rural parts of Damascus during the month of May. The leaves of the plant were dried at 25°C. After drying, steam distillation was used to extract the essential oil. Chloroform was used to separate the oil and water from the isotropic mixture. Then the chloroform was evaporated, and we collected the essential oil. Finally, a yield of 1.5% from the *T. syriacus* essential oil was obtained.

2.2. Bacterial strain isolation and culturing

The blood samples were isolated from University Children's Hospital in Damascus. In order to identify the bacterial species, bacterial DNA was directly extracted from the blood using TMBOSIS MOLYSIS immediately after extracting DNA genomic from 2-3 mL blood taken with EDTA (Molzym GmbH & CO KG-Germany). The PCR was based on 16s rDNA (Eurofins Genomics - Ebersberg) . In addition, conventional microbiological methods was also used for the same isolates. Three bacterial strains were isolated and identified *S. pneumoniae*, *P. aeruginosa*, *K. pneumoniae*.

2.3. Diffusion disc bacterial antibiotic sensitivity

We performed the antimicrobial susceptibility for each strain (*S. pneumoniae*, *P. aeruginosa*, *K. pneumoniae*) by using three commonly used antibiotics (vancomycin, ampicilin, amikacin, gentamicin) using Kirby-Bauer disk (Bioanalyse) diffusion methods. The inhibition zone of each antibiotic for each strain was measured and bacterial strains were determined as susceptible or resistant strains [2, 33].

2.4. Evaluation of the antibacterial effect of T. syriacus essential oils on solid medium

Agar-well diffusion method was used to determine the antibacterial activity. The bacteria were cultured on Mueller–Hinton Agar medium (BiomerieuxFrance). Then, 6 mm of wells were cut through the agar using a sterile cork borer. The agar was then removed, leaving empty wells that were filled with 20 µL of *T. syriacus* essential oil. The plates were incubated at 37°C for 24h.

2.5. Determination of the Minimum Inhibitory Concentration (MIC) and Minimum Bactericidal Concentration (MBC) of T. syriacus essential oils

In order to evaluate the MIC and MBC of *T.syriacus* essential oils, the three bacterial strains (*P. aeruginosa, K. pneumoniae, S. pneumoniae)* were cultured on LB broth medium (Tmmedia, India) with DMSO 10% (Sigma Aldrich) at 37C°. A series of decreasing concentrations of the essential oil (100, 50, 25, 12.5, 6.25, 3.125, 1.56, 0 µL/mL) were prepared and inoculated with approximately $5x10^3$ CFU/mL and incubated at 37 C° for 24h. Microplate Alamar Blue Assay was used to identify the *T. syriacus* essential oil MIC [34]. In addition, in order to identify the MIC and MBC concentration, CFUs counting was used and aliquots were cultured on LB agar medium (Tmmedia, India) and incubated for 24h at 37 C°. Each essay was assayed in triplicate.

2.6. Measuring and quantifying biofilms

The quantification of biofilm mass was performed by a microtiter plate [35]. The three strains (*P. aeruginosa, K. pneumoniae, S. pneumoniae*) were cultured in LB broth at 37°C to reach OD_{600}=1, then diluted 1:100 in LB broth . 1 mL of the bacterial culture was incubated in 24 microtiter plate at 37°C. In order to evaluate the antibiofilm activity of *T. syriacus* essential oil. The essential oil was added at two concentration MIC and MBC concentrations at different time points (24h, 48h, 72h). The medium was removed and each well was left to dry at room temperature. Then, 500µL of crystal violet 1% (Sigma Aldrich) was added to each well and incubated for 10 minutes. In the next step, each well was washed with distilled water three times then let to dry at room temperature. Finally, 1 mL of ethanol 95% was added to each well and 100µL from each condition was transferred to 96 well microtiter plate. In order to quantify the biofilm mass, OD_{570} was measured to quantify the amount of crystal violet-stained biofilm. Each condition was assayed in triplicate.

2.7. Statistical analysis

Microsoft Excel (2010) and Graphpad Prism software (GraphPad Prism 6 software, Graphpad Software Inc, CA, USA) were used to analyze the data. All data were conducted as the mean ± standard deviation and analyzed by one-way or two-way ANOVA comparison tests followed by appropriate correction, as specified in the caption under each figure. All experiments were performed in triplicates.

3. Results

3.1. T. syriacus essential oil antibacterial activity on solid medium cultured with clinically isolated bacteria

The studying of antibacterial activity of the *T. syriacus* on solid medium was verified by using three clinically isolated multidrug-resistant (MDR) bacteria (*P. aeruginosa, K. pneumoniae, S. pneumoniae*). The three strains involved in the study have different antibiotic sensitivity profiles. *P. aeruginosa* showed resistance against (gentamicin and ampicillin) and sensitivity against (amikacin and vancomycin), *K. pneumoniae* showed resistance against (ampicillin, vancomycin and gentamycin) and sensitivity against amikacin, while *S. pneumoniae* exert resistance against (gentamicin, ampicillin and amikacin) and sensitivity against vancomycin. In order to verify the antibacterial activity on solid medium, the inhibiting zone on the solid medium was measured at different concentration of the essential oils as described in material and methods and the observed results are reported in Table 1. The obtained results showed good activity of *T. syriacus* essential oil at concentrations (25% - 50% - 100%) and moderate or intermediate activity at 10% concentration against the studied bacterial strains.

Table 1. *T. syriacus* essential oils antibacterial activity on solid medium (Mueller-Hinton agar). The three different multidrug-resistant (MDR) bacterial strains (*P. aeruginosa, Klebsiella pneumoniae, S. pneumoniae*) were cultured on solid medium and different concentrations of *T. syriacus* essential oil were applied (100%, 50%, 25%, 10%, 5%). Different results were obtained by measuring zone inhibition in mm after 24 h incubation at 37 C° for each strain.

Zone inhibition against *S. pneumonia* in mm	**Zone inhibition against *P. aeruginosa* in mm**	**Zone inhibition against *K. pneumonia* in mm**	**Concentration of *T. syriacus* essential oil (v/v)**
5.5	8	No growth	100%
3	3.5	5	50%
4.5	3	4	25%
2	0	1.5	10%
0	0	0	5%

3.2. The antibacterial activity of T. syriacus essential oil in liquid medium culture with clinically isolated bacteria

The antimicrobial activity of *T.syriacus* essential oil was verified using three resistant bacterial strains clinically isolated (*S. pneumoniae, K. pneumoniae and P. aeruginosa*). The minimum inhibitory concentration (MIC) and the minimum bactericidal concentration (MBC) in axenic culture were identified, and the results are reported in Table 2. The observed results showed antibacterial activity of the *T. syriacus* essential oil against all three strains

and the MIC results were arranged between (1.56 µL/mL) against *K. pneumoniae* and (3.12µL/mL) against *S. pneumonie* and *P. aeruginosa.*

Table 2. *T. syriacus* essential oil minimum inhibitory concentration of (MIC) and minimum bactericidal concentration (MBC) against multidrug-resistant (MDR) strains of *S pneumoniae, K. pneumoniae and P. aeruginosa.*

Essential oil	**MIC** µL**/mL** ***S .pneumoniae***	**MBC** µL**/mL** ***S .pneumoniae***	**MIC** µL**/mL** ***K.pneumoniae***	**MBC** µL**/mL** ***K .pneumoniae***	**MIC** µL**/mL** ***P.aeruginosa***	**MBC** µL**/mL** ***P.aeruginosa***
T. syriacus	3.12	6.25	1.56	3.12	3.12	6.25

3.3. Antibiofilm formation activity of the T.syriacus essential oils against clinically isolated bacteria

Three different strains (*P. aeruginosa, K. pneumoniae, S. pneumoniae*) were used to quantify the antibiofilm activity of *T. syriacus* essential oil by incubating the three different strains in contact with different concentrations of the *T. syriacus* essential oil representing a different MIC concentration against different bacterial strains (3.12 µL/mL for *S. pneumoniae*, 3.12 µL/mL for *P. aeruginosa*, and 1.56 µL/mL for *K. pneumonia*). Furthermore, in order to verify if the MBC concentrations have different effects, we incubated the three different strains with different MBC concentrations of the *T. syriacus* essential oil (6.2 5µL/mL for *S. pneumonia*, 6.25 µL/mL for *P. aeruginosa* and 3.12 µL/mL for *K. pneumoniae*). In order to quantify biofilm masses, a microtiter plate, as described in the material and methods, was used at different time points (24h, 48h, 72h) and compared with the control group (untreated bacteria). As shown in Figure 1 (A), biofilm formation of *S. pneumoniae* (Gram-positive bacteria) was significantly reduced by *T. syriacus* essential oil at MIC and MBC concentration at (24h, 48h and 72h). The reduction was between (60 %) and (68%) at 72h post-treatment. The results in Figure 1 (B) showed that biofilm formation of *P. aeruginosa* was significantly reduced by *T.syriacus* essential oil at (24h, 48h and 72h) but the reducing effect was less than the effect against *S. pneumoniae*, especially at 24h and 48h. The reducing effect was increased from (43%) to (75%) when the MBC concentration was used at 72h post-treatment. Similarly, Figure 1 (C) showed that biofilm formation was significantly reduced by *T.syriacus* essential oil against *K. pneumoniae* at different time points(24h, 48h and 72h), but the reducing effect at 24h post-treatment was less than the effect against *S. pneumoniae*. The reducing effect was increased to (66%) after 48h post-treatment and from (50%) to (67%) when the MBC concentration was used at 72h post-treatment.

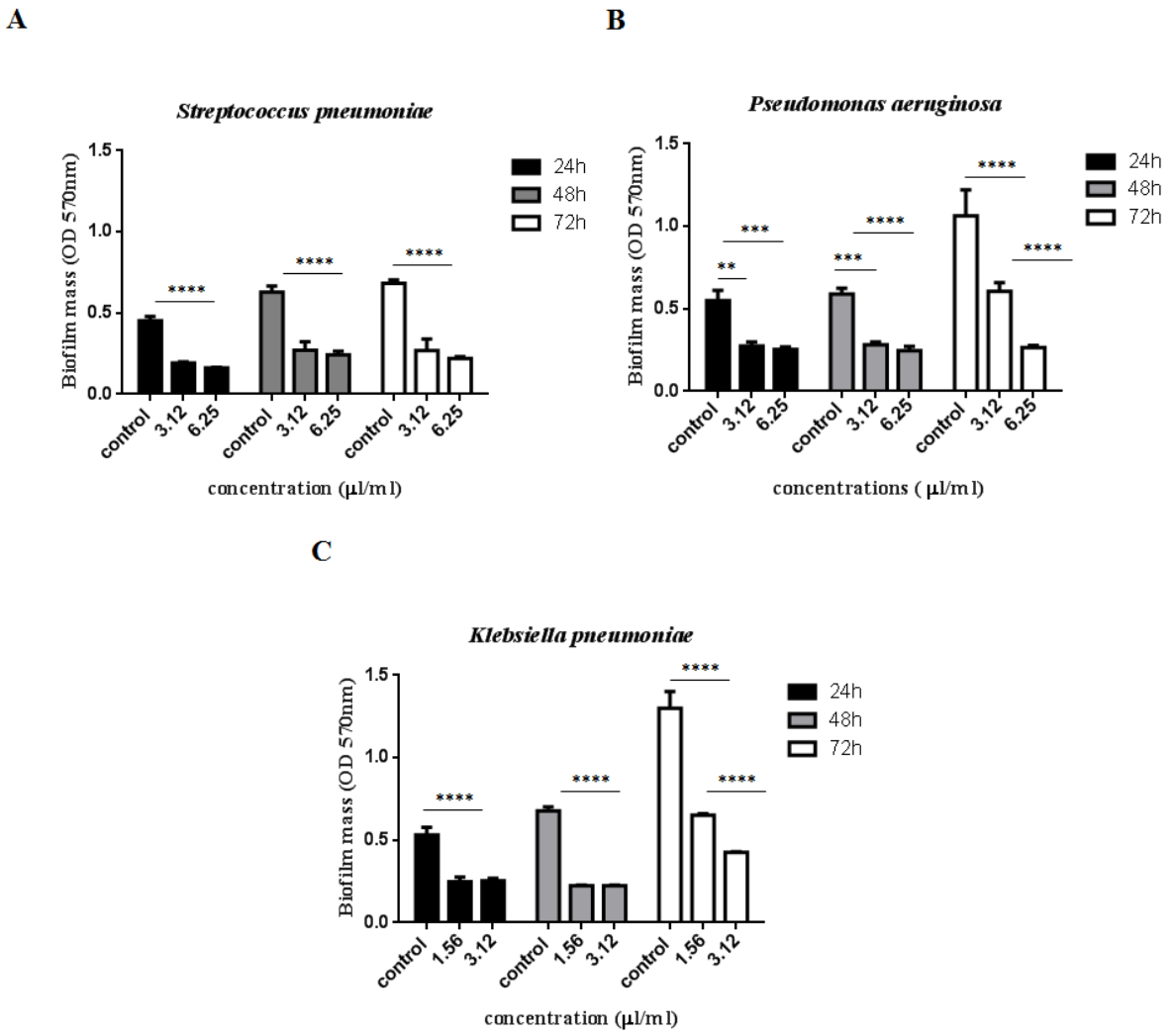

Figure 1. (A) Quantification of biofilm mass of *S. pneumoniae* after application of *T. syriacus* essential oils with different concentrations at different time points. The essential oil was added at MIC (minimum inhibitory concentration) concentration (3.12µL/mL) and MBC (minimum bactericidal concentration) concentration (6.25µL/mL). Crystal violet staining was used to quantify The biofilm mass at different time points (24, 48, 72h). The obtained results showed a significant antibiofilm activity and a significant decrease in biomass after adding essential oil. ****P<0.00001 for *T. syriacus* with MIC and MBC concentration at the three different time points (24, 48, 72h) versus control. (B) Quantification of biofilm mass of *P. aeruginosa* after treatment with MIC and MBC concentration of *T. syriacus* essential oils at different time points. The essential oil was added at MIC concentration (3.12µL/mL) and MBC concentration (6.25µL/mL). The obtained results exhibited a significant antibiofilm activity and a significant decrease in biomass after adding essential oil. **P<0.001for *T. syriacus* with MIC concentration and ***P<0.0001 with MBC at 24h post-treatment, ***P<0.0001 for *T. syriacus* with MIC concentration and ****P<0.00001 with MBC at 48h post-treatment versus control , ****P<0.00001 for *T. syriacus* with two concentration MIC and MBC at 72 post-treatment and****P<0.00001 for *T. syriacus* with MIC concentration versus MBC concentration after 72 h of treatment. (C) quantification of biofilm mass of *K. pneumoniae* after treatment with MIC and MBC concentrations of *T. syriacus* essential oils at different time points. The essential oil was added at MIC concentration (1.56µL/mL) and MBC concentration (3.12µL/mL). The obtained results showed a significant antibiofilm activity and a significant decrease in biomass after adding essential oil. ****P<0.00001for *T. syriacus* with MIC and MBC concentration versus control, ****P<0.00001for *T. syriacus* with MIC concentration versus MBC concentration after 72 h of treatment.

4. Discussions

Thymus vulgaris has been used for centuries in traditional medicine [36, 37]. The emergence of resistant and biofilm-forming bacteria makes finding new antibacterial strategies an urgent need [38, 39]. Essential oils contain many compounds mixed together and could be a promising alternative strategy in front of emerging resistance bacterial infection [40-42]. In this study, we verified the antibacterial activity of *T. syriacus* essential oil extracted from the peripheral region of Damascus against resistant bacteria clinically isolated. The yield of extraction was 1.5% lower than the 2.8% yield obtained in other studies conducted in Syria [25]. The obtained results showed that *T. syriacus* essential oil have significant antibacterial activity against *P. aeruginosa* with MIC (3.12 µL/mL), which is in agreement with another study conducted on *T. syriacus* essential oil. This study verified the activity of *T. syriacus* essential oil against Gram-negative isolated bacteria and found that the MIC was (3.12 µL/mL) against *P. aeruginosa* [25]. The minimum inhibitory concentration of the *T. syriacus* essential oil against *K. pneumonia*e was (1.56 µL/mL) in contrast with another study that showed *Thymus* essential oil showed no antibacterial activity against *A. baumanni* and *K. pneumonia* [43]. It was also demonstrated in a study developed to evaluate the antibacterial effect and antibiofilm forming of *Thymus vulgaris* essential oil against clinically isolated *P. aeruginosa* that the MIC and MBC values were between 0.062 and 2% (v/v) of isolates [44]. In another study, the inhibitory effect of essential oil was also explained, and the results showed that the zone of inhibition of *Thymus vulgaris* essential oil was less than (10 mm) for *K. pneumonia*, *P. aeruginosa* and *S. pneumoniae*. The calculated MIC was (0.312 mg/mL) for *S. pneumoniae*, (0.625 mg/mL) for *P. aeruginosa* and (1.25 mg/mL) for *K. pneumonia*e which are less than our obtained results. Previous and current findings confirmed that *Thymus* essential oil is effective as an antibacterial for both Gram-negative bacteria and Gram-positive like *S. pneumoniae*. Furthermore, the antibacterial activity of *Thymus vulgaris* against other microorganism Gram-positive bacteria *Staphylococcus aureus* (*Staph. aureus*) with MIC (0.625 mg/mL) and Gram-negative bacteria *E. coli* with MIC (2.5 mg/mL) was reported [45]. In addition, the minimum inhibitory concentration of *Thymus vulgaris* essential oil against clinically isolated bacterial strains (MRSA, *S. pneumoniae*, *P. aeruginosa*, *E. coli* and *K. pneumoniae*) was (0.625, 0.312, 0.625, 2.5 and 1.5 mg/mL) respectively [45].

It is important to note that *Thymus vulgaris* essential oil with its major chemical compounds (Thymol, linalool, carvacrol, 1,8-cineole, eugenol, camphor, camphene, α-pinene, borneol, β-pinene) [46] have inhibiting activity against different microorganisms (*Clostridium perfringens, Listeria monocytogenes, E. coli, Salmonella typhimurium, Staphylococcus aureus, Salmonella typhi*) with MIC values (1.25, 0.04, 0.1, 0.2, 0.08 and 0.2 mg/mL) respectively [47]. The antimicrobial activity of *Thymus vulgaris* essential oil was not restricted to bacteria but also to fungi. A study showed that *Thymus vulgaris* essential oil with its major chemical compounds (thymol, *p*-cymene, carvacrol, carvacrol acetate, and linalool) have antifungal activity against *Candida albicans, Candida glabrata, Candida parapsilosis, Aspergillus fumigates* with MIC (0.031, 0.031, 0.0625, 0.016µg/mL) respectively [31]. In addition, it was documented by other studies that the antimicrobial activity of the *Thymus*

vulgaris essential oil was associated with its phenolic compound thymol and carvacrol [48]. The hydroxyl group of these compounds interacts with the cytoplasmic membrane, changes its permeability and affects the stability of the lipid bilayer, leading to disruption of the cytoplasmic membrane and leakage of intracellular components [49, 50].

In this study, we verified the activity of *Thymus syriacus* essential oils in inhibiting the biofilm formation and found a significant reduction in biofilm mass after treatment with MIC and MBC concentrations of essential oil against *P. aeruginosa, K. pneumoniae* and *S. pneumoniae* at different hours of incubation. The essential oil was more effective in reducing the biofilm mass by using MIC and MBC concentration and at three different time points with more activity against *S. pneumoniae* biofilm formed. Moreover, the antibiofilm activity of *T. syriacus* essential oil increased by augmenting the essential oil concentration at 72h post-treatment. Another study that tested against *K. pneumoniae* determined the minimum biofilm inhibitory concentration was 5 mg/mL and the biofilm reduced by 70% after 24h of treatment. At the same time, the reducing effects were 20% and 85%, respectively, by using the same concentration of *P. aeruginosa* and *S. pneumoniae*, which aligns with the current findings that the antibiofilm effect of the *Thymus syriacus* was more efficient against *S. pneumoniae* than *P. aeruginosa* and *K. pneumoniae*. The antibiofilm formation of *Thymus vulgaris* essential oil was also established against other bacteria. It was demonstrated that using 0.01% of the essential was able to reduce the biofilm formation by 53%, and this effect was increased to 76% by increasing the concentration to 0.05% [51]. Finally, our study confirmed with other studies the antibacterial and antibiofilm activity of the *Thymus* essential oil. The different origin of essential oil with different chemical compositions leads to different obtained results of MIC, and the reason that the essential oil was tested on different strains of bacteria.

5. Conclusion

In this study, we found a significant activity of *T. syriacus* essential oil against a group of multidrug-resistant bacteria. Moreover, the results of our study showed a significant effect of *T. syriacus* essential oil against bacterial biofilm formation. The findings point out that *T. syriacus* essential could be an alternative armamentarium targeting bacterial infection and effective inhibitor of biofilm formation. Its synergistic use with the existing range of antimicrobial agents could be explored too. Although this study utilised only conventional testing methodology, the findings verified the activities of *T. syriacus* essential oil against other bacterial strains and other pathogenic microorganisms like viruses and fungi. Furthermore, determining the cytotoxic effects of *T. syriacus* essential oil using different concentrations and verifying its effect using in vivo model are steps to be taken before further commercialization consideration.

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