The Inhibitory Effect of local *T.bovei* Volatile Oil Against ESBL- *E.coli* and *Klebsiella pneumoniae* Isolated From Patients with Urinary Tract Infections

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Abstract

This is the first research in Iraq to look in the *T. bovei* volatile oil's ability to prevent the development of ESBLs. To find successful treatment- combinations for "ESBL- producing" bacteria's emergent infections, we are evaluating antibacterial properties of phytochemical substances against bacteria that produce ESBL and investigating the effects of phytochemical substances in the presence of antibiotics. The volatile oil of the studied plant was extracted by steam distillation. Oil compounds were detected after extracting the volatile oil from *Thyme bovei* using a Gas chromatography–mass spectrometry (GC-MS). "REMA" was used to determine the MIC of the volatile oil and antibiotic solutions, with minor modifications. The checkerboard approach was used in 96 well microplates to see whether there was a synergy relationship between the volatile oil and antibiotics. *T.bovei* volatile oil had an inhibitory influence on β - Lactamase, according to the findings. Antimicrobial properties Thymol, p-cymene, and "Linalool" were the key constituents contained in GC-MC of *Thymus* volatile oil, which has already been shown to have antibacterial efficacy. The combination of *T.bovei* volatile oil and antibiotics had a synergistic effect on *E.coli* and *K.pneumoniae* producing ESBL growth in the current study.

Keyword : *T. bovei , Volatile Oil , ESBL, Urinary Tract Infections*

Introduction

Bacterial antimicrobial resistance is a worldwide issue that necessitates production new antibacterial agents. As a result of this issue, scientists are increasingly focusing on natural products in order to develop better treatments for multidrug-resistant microbial strains [(1)]. Antibiotic-resistant bacteria would be more successful if plant extracts with target sites were used [(2, 3)]. As a result, essential oils have been used to treat a variety of infectious diseases due to their high concentration of bioactive compounds with anti-oxidant and antimicrobial properties [(4)].

The most common bacterial infections in humans are urinary tract infections (UTIs). They're also the most popular source of both population- and hospital-acquired infections [(5)]. Many conventional antibiotics, such as third-generation cephalosporin's, are ineffective against UTIs caused by *Enterobacteriaceae* that produce ESBLs [(6)]. The evolution of ESBLs, on the other hand, has given these enzymes a new weapon in their arsenal. As a result, ESBLs producers are becoming more prevalent, and ESBLs producer strains are increasing morbidity, morality, and health-care costs [(7)].

Materials and Methods

Clinical isolates

Clinical *E.coli* and *K.pneumoniae* were collected from urinary tract infection (UTI) patients. Traditional Morphological methods and biochemical analysis, as well as automated systems such as Api 20E and Vitek 2, were used to identify them. They were classified as ESBL- producing *E. coli* and *K. pneumoniae* using

screening test, as well as various phenotypic and Genotypic approaches according to Al-Quhli, and Al Maeni (2021) [8, 9].

Collection and preparation of medical plant

In July 2019, the leaves of *Thyme bovei* were brought from Hadethia city's local plantations. The leaves were cleaned with tape water to remove any dirt that had adhered to their surfaces, and then washed with purified water once more.

Volatile oil extraction

The volatile oil of the studied plant was extracted by steam distillation using a Clevenger-type apparatus [10]. After extracting the volatile oil for *Thyme bovei*, oil compounds were identified by a GC-MS, in Baghdad; Ministry of Ecology.

Determination of minimum inhibitor concentration (MIC)

The Resazurin Microtitre-plate Assay (REMA) was used for determining the MIC of the volatile oil and antibiotic solutions with slight modifications. In aseptic conditions, to all wells of microtitre-plates, a hundred µl Mueller-Hinton Broth was added, followed by transferring the first row of the 96 well plates with a hundred µl material test (volatile oil). Pipette 100 µl of the substance measure in serially decreasing concentrations from the first row to the other rows "(1/2, 1/4, 1/8, 1/16, 1/32, 1/64, 1/128, and 1/256)" was used to conduct serial dilutions. Every well was filled with ten microliters of bacterial suspension containing "1×10^8 CFU/ml". They were wrapped loosely in para-film and incubated at "35±2°Cfor 18-24" hours to ensure that the bacteria did not get dehydrated. Following the incubation period, ten microliters of "resazurin- solution" (Alamar- blue) was added to each well, and after another 24 hours, the plate was re-incubated. to observe color change. The findings were visually examined by looking at the color variations in resazurin, with changes from purple to pink, red, or colorless being considered positive. The MIC value was determined as the lowest concentration that caused no change in resazurin color [11].

Study the synergism between volatile oil and some antibiotics on bacterial growth using checkerboard technique

The checkerboard approach was used in 96 well microplates to see whether there was a synergy relationship between the volatile oil and antibiotics [12]. The checkerboard assay was planned so that the two antimicrobials to be examined were serially diluted against each other in a cross fashion on two standard 96-well plates. The first antimicrobial "volatile oil" is serially diluted vertically, while the second antimicrobial "antibiotic" is serially diluted horizontally in this manner. The assay was created in such a way that the MIC calculated from the REMA assay for volatile oils could be used [13]. The standard technique for assaying hydrolyzed Beta-lactam antibiotics is similar to Sargent's description [14].

Results and Discussion

The volatile oil was extracted by steam distillation GC-MC; 100 g of leaves were placed in a Clevenger flask with 500 ml distilled water, and after 3 hours of extraction, 1 ml of *T. bovei* volatile oil was collected. The major compounds in *T.bovei* volatile oil were beta-ocimene, 3,7-Dimethyl-1,3,7-octatriene, Sabinene hydrate, Y-Terpinene, Linalool, Thyme camphor, p-cymene, p-thymol, thymol, Carvacrol, . We also found the existence of other active substances, noting that they were the same active substances found in *T.vulgaris* in the previous analysis [15].

T.bovei volatile oil had an inhibitory effect on β - Lactamase, according to the findings. In the absence of *T.bovei* volatile oil, *E. coli* β -lactamase activity was (0.003640) u/ml, while in the presence of *T.bovei* volatile oil, *E. coli* β -lactamase activity was (0.001115) u/ml.*K.pneumoniae* β-Lactamase activity was (0.004443) U/ml in all isolates without *T.bovei* volatile oil, while the β-lactamase activity of *K.pneumoniae* isolates was (0.001918) U/ml in the presence of *T.bovei* volatile oil. Antimicrobial properties Thymol, p-cymene, and

Linalool were key constituents contained in GC-MC of Thymus volatile oil, which is already known to have antibacterial activity [16].

REMA is characterized by its simplicity, low cost, speed, performance, and dependability. This is a colorization process focused on the oxidation and reduction of resazurin, which is used to test the sensitivity of medicines, antibiotics, plants, and bacteria. The ability to calculate a small volume of plant extracts separates this approach from other conventional methods. A blue reduction pigment (Resazurin) that is widely used as chemical proof is not harmful to cells in the media. Resazurin is a bacterial growth indicator that can be used without a spectrophotometer to assess bacterial growth in a small amount of solution in microliter-plates [17]. The MIC of *T. bovei* volatile oil against growth of bacteria–producing ESBLs was determined in this study and the findings are shown in the tables (1 and 2). Figure (2) showed MICs values of volatile oil, where blue color represent indicate to inhibited of bacterial growth by volatile oils due to not reduce the resazurin, while the pink and red colors were due to the reduction of Resazurin to resorufin by the bacteria [18].

Table (1). The MIC of *T. bovei* volatile oil for the growth of *E. coli*-producing ESBLs isolates.

No. of isolate	Minimum Inhibitory Concentration (MIC) T. bovei volatile oil (Titer)
E. coli 1	32
E. coli 2	128
E. coli 3	128
E. coli 4	128
E. coli 5	8
E. coli 6	128
E. coli S	256

Table (2) The MIC of *T. bovei* volatile oil for the growth of *K. pneumoniae*-producing ESBLs isolates.

No. of isolate	Minimum Inhibitory Concentration (MIC) T. bovei volatile oil (Titer)
K. pneumoniae 1	8
K. pneumoniae 2	32
K. pneumoniae 3	128
K. pneumoniae 4	32
K. pneumoniae 5	8
K. pneumoniae 6	32
K. pneumoniae S	256

Where in *E.coli*, the isolates 1 and 5were more resistant than isolates 2, 3, 4 and 6 which its resistance to *T. bovei* volatile oil was less. In *K.pneumoniae* , the isolate 3 was less resistant than the isolates 1,2,4,5 and 6. While the stander isolates of *E.coli* and *K.pneumoniae* were not resistant to *T.bovei* oil. Table (1) and (2) shows that *T.bovei* oil has a good effect on bacterial isolates. The ability of volatile oil in inhibiting the growth were due to phenolic compounds that Disrupting the cytoplasmic membrane, the proton motive force, electron flow, active transport, and cellular coagulation are all disrupted [19].

A present study, showed synergistic result of combining *T.bovei* volatile oil with antibiotics on *E.coli* and *K.pneumoniae* producing ESBL growth tables (3) & (4) . A rise in bacterial resistance to antibiotics, as well as a shortage of new antibiotics on the market, necessitated the development of alternative methods to deal with infections caused by drug-resistant bacteria [20]. Among the possible solutions suggested are the creations of antibiotic alternatives and the discovery or development of adjuvants [21]. Some attempts have been made to improve or restore antimicrobial activity against multidrug-resistant bacteria. When volatile oils are added to antibiotics, the antimicrobial MIC is reduced [22]. According to Al Dossary & Al Meani (2019), Since volatile oils are multi-component in nature, compared to many traditional antimicrobials that only have a single target site, they are thought to be more promising in preventing bacterial resistance [23]. Antibiotic efficacy can be improved by enhancing antibiotic diffusion through bacterial membranes, and/or inhibiting efflux pumps, which are a common mechanism of resistance; in Gram-negative bacteria [24, 25].

Table (3). The effect of combining *T. bovei* volatile oil with antibiotics on *E. coli* –producing ESBLs growth.

Antibiotic	MIC (µg/ml) By REMA	Combination between Antibiotics & T.bovei	FICI (ΣFIC)	Outcome
Ceftazidime	62.5	0.78125 (128) titer + 7.8125 µg/ml	0.375	Synergistic
Cefpodoxime	125	0.78125 (128) titer + 31.25 µg/ml	0.5	Synergistic
Cefotaxime	125	0.78125 (128) titer + 31.25 µg/ml	0.5	Synergistic
Ceftriaxone	62.5	0.78125 (128) titer + 7.8125 µg/ml	0.375	Synergistic
Cefepime	62.5	1.5625 (64) titer + 7.8125 µg/ml	0.25	Synergistic
Aztreonam	125	0.78125 (128) titer + 31.25 µg/ml	0.5	Synergistic

Table (4). The effect of combining *T. bovei* volatile oil with antibiotics on *K. pneumoniae*–producing ESBLs growth.

Antibiotic	MIC (µg/ml) By REMA	Combination between Antibiotics & T.bovei	FICI (ΣFIC)	Outcome
Ceftazidime	62.5	1.5625 (64) titer + 7.8125 µg/ml	0.25	Synergistic
Cefpodoxime	62.5	1.5625 (64) titer + 15.625 µg/ml	0.75	Additive
Cefotaxime	125	0.78125 (128) titer + 31.25 µg/ml	0.5	Synergistic
Ceftriaxone	125	0.78125 (128) titer + 31.25 µg/ml	0.5	Synergistic
Cefepime	62.5	1.5625 (64) titer + 7.8125 µg/ml	0.25	Synergistic
Aztreonam	62.5	0.78125 (128) titer + 7.8125 µg/ml	0.375	Synergistic

Thyme is an important medicine that has been used for centuries and has been known as a rich source of bioactive substances with substantial properties that are anti-oxidant and anti-inflammatory, potentially useful in the treatment and prevention of pathological circumstances, but its popularity as an antibacterial molecule that prompted us to choose it [26].

Conclusion

In conclusion, our findings revealed that *T.bovei* volatile oil has an inhibitory effect on the growth of *"E.coli* "and "*Klebsiella pneumoniae*" - generating ESBLs, in addition inhibition activity against β-Lactamase enzyme. When combined with antibiotics including "Ceftazidime, Cefpodoxime, Cefotaxime, Ceftriaxone, Cefepime, and Aztreonam", *T.bovei* volatile oil has a synergistic effect. As well as, *T.bovei* essential oil had strong biological activities and was a potential source of various natural compounds. It's a powerful inhibitor of *E.coli* and *K.pneumoniae*-producing ESBLs isolated from UTI patients at Al-Anbar Hospital, and it's led to the development of new medicinal plant-based therapeutics.

Ethical Clearance : Taken from Al-Anbar University ethical committee

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