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Sajjad Jafari
Zanjan University of Medical Sciences
Mina Shirmohammadpour
Zanjan University of Medical Sciences
Arman Taran
Zanjan University of Medical Sciences
Bahman Mirzaei
dr.bahman.m@gmail.com
Zanjan University of Medical Sciences

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Synergistic antimicrobial effect of thymol with ceftazidime and ampicillin against *Escherichia coli*

Sajjad Jafari[1,2], Mina Shirmohammadpour[1,3,4], Arman Taran[1,3], Bahman Mirzaei[1]*

[1]Department of Microbiology and Virology, Faculty of Medicine, Zanjan University of Medical Sciences, Zanjan, Iran

[2]Department of Microbiology, Faculty of Medicine, Urmia University of Medical Sciences, Urmia, West Azerbaijan, Iran

[3]Student Research Committee, Department of Microbiology and Virology, Faculty of Medicine, Zanjan University of Medical Sciences, Zanjan, Iran

[4]Department of Microbiology, School of Medicine, Tehran University of Medical Sciences, Tehran, Iran

*Corresponding Author's Email: dr.bahman.m@gmail.com

Abstract

Introduction: Using essential oils in combination with antibiotics helps to combat antibiotic resistance. Thymol is a major phenolic compound found in many essential oils. This study aimed to compare the antimicrobial, anti-biofilm, and cytotoxic effects of antibiotics with thymol.

Material and Methods: The antimicrobial effects of thymol, ampicillin, ceftazidime, and their combinations were examined against *Escherichia coli (E. coli)* ATCC 25922 by the MIC-MBC method. Then, the antimicrobial effects of compounds were evaluated against twenty clinical strains. The chemical bonds, functional groups, and molecular interactions of the mentioned compounds were investigated with an FT-IR spectrometer. The kinetics, biofilm inhibition, hemolysis, and MTT assays were also conducted.

Results: The antimicrobial activity of thymol/ampicillin and thymol/ceftazidime was strain-dependent; thymol/ampicillin caused lower MIC and MBC against E. coli ATCC 25922, while thymol/ceftazidime showed greater antimicrobial activity against clinical isolates. FT-IR analysis of thymol, antibiotics, and their combinations revealed characteristic absorption bands corresponding to the functional groups present in the individual compounds. Changes in the position and intensity of some peaks in the combination spectra may indicate intermolecular interactions between thymol and antibiotics. The biofilm inhibition effect of thymol/ceftazidime (75.51%) was greater than that of thymol/ampicillin (62%). The rate of bactericidal activity of thymol/ceftazidime was greater. The cytotoxic effects of compounds on red blood cells (RBCs) and human foreskin fibroblast (HFF) were lower than Triton X-100 (0.1%).

Conclusion: Based on *in vitro* laboratory results, thymol/ceftazidime appears to be a better therapeutic option than thymol/ampicillin against *E. coli* bacteria. After *in vivo* investigations, this combination can be used as a new drug.

Keywords: Essential oils, *Escherichia coli*, Thymol, Ceftazidime, Ampicillin

1. Introduction

Escherichia coli (E. coli) is a rod-shaped Gram-negative bacterium of the Enterobacteriaceae family and is a common commensal of the human gastrointestinal tract. However, the transition of pathogenic *E. coli* strains to extraintestinal sites is the most common cause of infectious diseases, including septicemia, neonatal meningitis, and urinary tract infections (UTIs) (1, 2).

Over the past ten years, increasing concerns for public health have arisen due to the rise in antibiotic resistance in Enterobacteriaceae isolates. The World Health Organization (WHO) identifies antimicrobial-resistant *E. coli* as a major global health concern due to its contribution to both nosocomial and community-acquired infections (3). Strains of this bacterium that produce extended-spectrum beta-lactamases (ESBLs), plasmid-mediated AmpC (pAmpC), and carbapenemases are resistant to third-generation cephalosporins and penicillins (4). A rise in resistance in *E. coli* isolates has also been reported in Iran (5).

Numerous investigations are being conducted on how to make antibiotics more effective and combat antibiotic resistance. This includes several studies on the potential benefits of using essential oils (EOs) in combination with antibiotics. EOs contain several molecules with antibacterial properties, including thymol, a major phenolic compound found in many EOs (6).

The primary monoterpene, thymol (2-isopropyl-5-methylphenol), is naturally present in thyme *(Thymus vulgaris)* EO (7). Thymol has the potential to enhance the concentrations of C16 and C18 saturated fatty acids while simultaneously reducing the levels of C18 unsaturated fatty acids. Additionally, it influences the enzymes responsible for synthesizing these fatty acids through competitive and non-competitive inhibition mechanisms. Furthermore, thymol disrupts the integrity of the bacterial cytoplasmic membrane, leading to the collapse of the membrane potential and proton motive force. Consequently, the loss of this electrochemical gradient impairs ATP production by ATP synthase, which ultimately disrupts essential cellular functions, including cell division (8, 9). Thymol possesses immunomodulatory, antioxidant, anti-inflammatory, and anticancer properties. EOs can also prevent the formation of biofilms created by Gram-negative bacteria, such as *E. coli* (10, 11).

We investigated the in vitro antibacterial synergism of thymol in combination with ampicillin and ceftazidime against *E. coli* American Type Culture Collection (ATCC) 25922 and twenty clinical strains to address the prevalence of bacterial infections and antibiotic resistance.

2. Materials and methods

2.1 Antimicrobial compounds

Thymol, with a purity of 97%, was provided by Glaxir Pars Company (Mashhad, Iran) as a dry powder. Ampicillin and ceftazidime were provided by Dexir Company (Tehran, Iran) as dry powders. All materials were stored at -20°C. Thymol was diluted to a concentration of 4096 µg/mL using distilled water and ethanol 97%. Ampicillin and ceftazidime were diluted to a concentration of 1024 µg/mL using distilled water.

2.2 Bacterial strains

E. coli strain from the American Type Culture Collection (ATCC) 25922 was obtained from the Microbiology Laboratory of Zanjan University of Medical Sciences. Twenty clinical strains from patients suffering from UTIs caused by *E. coli* were obtained from laboratories and hospitals in Tehran Province. To identify these strains, microbiology tests were conducted, including Sulfide-Indole-Motility (SIM), Triple-Sugar-Iron (TSI), Methyl-Red (MR), Voges-Proskauer (VP), citrate, gelatin liquefaction test, and lysine decarboxylase (LDC) (12). The susceptibilities of the strains to several antibiotics were investigated (Table 1); some strains were multidrug resistant (MDR), and some were extensively drug resistant (XDR). The bacteria were cultured in MHB (Mueller-Hinton Broth, Aria Teb Company, Tehran, Iran) medium at 37°C, and the prepared suspension was stored at 4°C in 0.01 M $MgSO_4$ solution until use.

Table 1: A list of antibiotic resistance and clinical strains of *E. coli* bacteria obtained from urine samples

Antibiotics	Ciprofloxacin	Cefixime	Ceftriaxone	Trimethoprim (sulfamethoxazole)	Piperacillin	Ampicillin	Nitrofurantoin	Amikacin	Gentamicin	Meropenem	Imipenem	Amoxicillin
***E. coli* 1**	S	S	S	R	R	R	S	S	S	S	S	R
***E. coli* 2**	I	S	S	S	R	R	S	S	S	S	S	R
***E. coli* 3**	R	R	R	R	R	R	S	S	S	I	R	R
***E. coli* 4**	R	S	S	S	I	R	I	S	S	R	I	R
***E. coli* 5**	S	S	S	R	S	S	S	S	S	S	I	S
***E. coli* 6**	I	S	S	S	R	R	S	S	S	S	I	R
***E. coli* 7**	R	R	R	R	R	R	S	S	S	S	S	R
***E. coli* 8**	R	R	R	R	R	R	S	S	S	S	S	R
***E. coli* 9**	S	R	R	R	R	R	S	S	S	S	S	R
***E. coli* 10**	S	S	S	R	R	R	S	S	S	S	S	R
***E. coli* 11**	R	R	R	S	R	R	S	S	S	R	R	R
***E. coli* 12**	S	S	S	S	S	R	R	S	S	S	S	R
***E. coli* 13**	S	R	R	R	R	R	S	S	S	S	S	R
***E. coli* 14**	R	R	R	R	R	R	S	S	R	S	S	R
***E. coli* 15**	S	S	S	R	R	R	S	S	S	S	S	R
***E. coli* 16**	S	S	S	R	R	R	S	S	S	S	S	R
***E. coli* 17**	S	S	S	R	R	R	S	S	S	S	S	R
***E. coli* 18**	S	S	S	R	R	R	S	S	S	S	S	R
***E. coli* 19**	S	S	S	R	R	R	S	S	S	S	S	R
***E. coli* 20**	S	S	S	R	R	R	S	S	S	S	S	R

2.3 *In vitro* antimicrobial assay

The antimicrobial properties of thymol, ampicillin, and ceftazidime were evaluated separately and in combination using the broth microdilution method to determine the minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) using a 96 well-microtiter plate. 100µl of MHB was added to each well, while 100µl of compounds was added to the row A. Then, two-fold serial dilutions serial dilutions were performed by transferring 100 µL from one well to the next. 100 µl of *E. coli* ATCC 25922 suspension containing 10^6 CFU/mL (Colony Forming Unit/mL) was added to each well, resulting in a final bacterial inoculum of 5×10^5 CFU/mL. The final concentration of thymol in the well is 1024 µg/mL, the final concentration of ampicillin and ceftazidime is 256 µg/mL.

The growth control consisted of the bacterial suspension and MHB medium without any antimicrobial agent, while the negative control consisted of the antibiotic and MHB medium without bacteria. The samples were then incubated for 24 hours at 37°C.

The MIC well and all wells containing higher antimicrobial concentrations that showed no visible bacterial growth were subcultured onto Mueller–Hinton agar (MHA) plates for MBC determination.

The well that corresponded to the MIC and all the wells containing higher concentrations were cultured onto MHA medium (Mueller Hinton Agar, Aria Teb Company, Tehran, Iran) to determine the MBC of thymol, ampicillin, and ceftazidime. The MIC was defined as the lowest concentration of the antimicrobial agent that completely inhibited visible bacterial growth after incubation. The MBC was defined as the lowest concentration that resulted in no bacterial colony growth following subculture onto MHA plates (13). The experiment was conducted in triplicate.

2.4 Checkerboard assay

To evaluate the potential synergy between thymol and antibiotic (either ampicillin or ceftazidime) in four different combinations, the checkerboard method was utilized (Table 2 and 3). Thymol was diluted in columns 1 to 7, while antibiotic was diluted in rows A to G on a 96-well microtiter plate. Then, the bacterial suspension (McFarland standard) was inoculated into each well of the microtiter plate, and incubated at 37°C for 24 hours. The experiment was conducted in triplicate.

Table 2: Examining the effects of thymol and ceftazidime separately and in combination on *E. coli* ATCC 25922

Compound	Thymol	Ceftazidime	A1		A2		A3		A4	
			Thymol	Ceftazidime	Thymol	Ceftazidime	Thymol	Ceftazidime	Thymol	Ceftazidime
Concentration (µg/mL)	1024	256	1024	256	1024	128	512	256	512	128

Table 3: Examining the effects of thymol and ampicillin separately and in combination on *E. coli* ATCC 25922

Compound	Thymol	Ampicillin	A1		A2		A3		A4	
			Thymol	Ampicillin	Thymol	Ampicillin	Thymol	Ampicillin	Thymol	Ampicillin
Concentration (µg/mL)	1024	256	1024	256	1024	128	512	256	512	128

To determine the type of interaction between compounds the fractional inhibitory concentration index (FICI) was calculated via the following formula (14):

$$FICI=FIC_A+FIC_B$$

$$FIC_A = \frac{MIC\ of\ drug\ A\ in\ the\ combination}{MIC\ of\ drug\ A\ alone}$$

$$FIC_B = \frac{MIC\ of\ drug\ B\ in\ the\ combination}{MIC\ of\ drug\ B\ alone}$$

According to the standards provided by the European Committee on Antimicrobial Susceptibility Testing (15), an FICI less than 0.5 indicates synergy, between 0.5 and 1 indicates additive (partial synergy), between 1 and 4 indicates indifference, and more than 4 indicates opposing effects (antagonism) (16, 17).

2.5 Fourier transform infrared (FT-IR) spectroscopy

The regular deconvolution method was applied to analyze the infrared spectra of Thymol, ceftazidime, ampicillin, thymol/ampicillin (A1 concentration ratio), and the thymol/ceftazidime combination (A1 concentration ratio). Each compound was ground into a uniform powder and then mixed with spectroscopic grade potassium bromide (KBr) in an appropriate ratio. The mixtures were carefully homogenized and compressed into transparent pellets using a hydraulic press under vacuum to minimize moisture interference. The prepared KBr pellets were subsequently placed in

the sample holder, and the FT-IR spectra were recorded using a Bruker TENSOR 27 spectrometer, while confirmation measurements were performed on a Thermo Scientific (USA) FT-IR instrument spectral deconvolution was then conducted to resolve overlapping bands and identify functional group interactions (18, 19).

2.6 Antibiofilm assay

Three to five colonies of the bacteria were suspended in 5 mL of tryptone soy broth (TSB, Aria Teb Company, Tehran, Iran) containing 1% glucose. The bacterial suspension was adjusted to approximately 10^6 CFU/mL using the McFarland standard. The mixture was incubated for 24 h at 37°C. A 96-well microtiter plate was filled with 100 µL of TSB. 10 µL of thymol, ceftazidime, ampicillin, thymol/ampicillin (A1 concentration ratio), and the thymol/ceftazidime combination (A1 concentration ratio) was then added to the first row, and TSB was subsequently added until the total volume reached 200 µl. Serial dilutions were conducted in TSB with 1% glucose. A negative control contained TSB without bacteria, while a growth control contained TSB with bacteria. The samples were incubated for 24 hours at 37°C. Then, the wells were washed three times with phosphate-buffered saline (PBS), 200 µl of 99% methanol was added to each well and incubated at room temperature for 5 min. Methanol was removed, 200 µl of 1% crystal violet dye was added to each well, and incubated at room temperature for 5 min. The wells were washed three times with PBS after the dye was removed. Following the injection of 200 µl of 96% ethanol, the optical density (OD, BioTek Instruments, Winooski, VT, USA) of each well was measured at a wavelength of 590 nm (20, 21).The experiment was conducted in triplicate. The degree of biofilm inhibition was calculated via the following formula (22):

$$\%\ Inhibition = \left[\frac{OD_{Negative\ control} - OD_{Sample}}{OD_{Negative\ control}}\right] \times 100$$

2.7 Time-kill assay

Thymol, ceftazidime, ampicillin, thymol/ampicillin (A1 concentration ratio), and thymol/ceftazidime (A1 concentration ratio) underwent a serial dilution in a 96-well microtiter plate. 100µl of MHB was added to each well, while 100µl of compounds was added to the row A.

Then, two-fold serial dilutions serial dilutions were performed by transferring 100 µL from one well to the next. 100 µL of *E. coli* ATCC 25922 suspension (10^6 CFU/mL) was added to each well, resulting in a final bacterial inoculum of 5×10^5 CFU/mL, and incubated at 37°C. 10 µL of each well was cultured in MHA every 6, 12, 18, or 24 h. A negative control contained MHA without bacteria, while a growth control contained MHA with bacteria. The count and lethality kinetics were evaluated after a 24 h cultivation period. The killing activity was characterized by the absence of colonies growth in MHA after 24 h (23). The experiment was conducted in triplicate.

2.8 *In vitro* hemolytic activity

5 mL of fresh human blood was mixed with 0/5 mL of sodium citrate dihydrate so that the blood could not clot. After centrifugation at 500×g for 10 min, the plasma was removed, and the samples were washed twice with PBS. Then, 2 mL of fresh blood was mixed with 48 mL of PBS to prepare 2% RBCs. Thymol, ceftazidime, ampicillin, thymol/ampicillin (A1 concentration ratio), and thymol/ceftazidime (A1 concentration ratio) underwent a serial dilution in a 96-well microtiter plate, and 100 µl of 2% RBCs was added to each well. After 24 h incubation at 37°C, the absorbance values were measured at 570 nm with an ELISA (Enzyme-linked immunosorbent assay) Reader (Agilent, USA). The experiment was conducted in triplicate. The hemolysis percentage of each compound was calculated via the following formula (24):

$$\text{Hemolysis (\%)} = \left[\frac{OD_{sample} - OD_{blank}}{OD_{control} - OD_{blank}}\right] \times 100$$

Triton X-100 (0.1%) was used as the positive control, and PBS as the negative control.

2.9 *In vitro* MTT assay

Cytotoxicity of thymol, ceftazidime, ampicillin, thymol/ampicillin (A1 concentration ratio), and thymol/ceftazidime (A1 concentration ratio) against a human foreskin fibroblast (HFF) cell line (Pasteur Institute, Tehran, Iran) evaluated using the MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay. Initially, 3×10^4 cells were cultured in a 96-well microtiter plate with 100 µL of 90% Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin (256 µg/mL) (Dexir Company, located

in Tehran, Iran). The cells were subsequently cultured at 37°C for 24 h in a humidified atmosphere containing 5% CO_2. Control groups included untreated cells, medium plus MTT without cells (blank), and wells containing the tested compounds plus MTT without cells to account for possible absorbance interference. Mentioned compounds underwent a serial dilution in the microplate, and incubated at 37°C for 24 h. Then, 20 µL of MTT stock solution (5 mg/mL) was added to each well, resulting in a final concentration of 0.83 mg/mL. After 4 h incubation at 37°C, the supernatant was removed, and 100 µL of dimethyl sulfoxide (DMSO) was added to each well. The microplate was shaken for 15 min to allow the cells to release insoluble purple formazan. Finally, the absorbance values were measured at 570 nm via a microplate spectrophotometer (model ELx808 made by BioTek). The experiment was conducted in triplicate. The percentage of cytotoxicity was calculated via the following formula (25):

$$\text{Cytotoxicity (\%)}=100\text{-}\textit{Cell viability}\ (\%)$$

$$\text{Cell viability (\%)}=\left[\frac{OD_{test}-OD_{blank}}{OD_{control}-OD_{blank}}\right]\times 100$$

Peptide-free cell suspensions and Triton X-100 (0.1%) were used as negative and positive controls, respectively.

3. Results

3.1. Antimicrobial activity of thymol and antibiotics on *E. coli* ATCC 25922

3.1.1. Thymol

MIC of 256 µg/mL, and MBC of 512 µg/mL was reported (Table 4).

3.1.2. Antibiotics

MIC and MBC of ampicillin were 2 and 4 µg/mL, while the MIC and MBC of ceftazidime were 8 and 16 µg/mL, respectively (Table 4).

3.1.3. Combinations

Combinations of thymol and antibiotics (ampicillin or ceftazidime) showed four times lower MIC and MBC compared to either drug alone. The MIC ranges for thymol/ceftazidime were 4-16 µg/mL for thymol and 2-4 µg/mL for ceftazidime, while the MBC ranges were 8-32µg/mL for thymol and 4-8 µg/mL for ceftazidime. The MIC ranges for thymol/ampicillin were 2-64 µg/mL for thymol and 1-8 µg/mL for ampicillin, while the MBC ranges were 4-128 µg/mL for thymol and 2-16 µg/mL for ampicillin. As a result, thymol/ampicillin caused lower MIC and MBC compared to thymol/ceftazidime against *E .coli* ATCC 25922 (Tables 4 and 5).

Table 4: The outcomes of the thymol and ceftazidime individual and combination MIC and MBC tests on *E. coli* ATCC 25922

Compound		Thymol	Ceftazidime	A1		A2		A3		A4	
				Thymol	Ceftazidime	Thymol	Ceftazidime	Thymol	Ceftazidime	Thymol	Ceftazidime
MIC (µg/mL)		256	8	16	4	16	2	4	2	4	2
MBC (µg/mL)		512	16	32	8	32	4	8	4	8	4
FICI	Value	-	-	0.56		0.31		0.27		0.27	
	Effect	-	-	Addition		Synergistic		Synergistic		Synergistic	

Table 5: The outcomes of the thymol and ampicillin individual and combination MIC and MBC tests on *E. coli* ATCC 25922

Compound		Thymol	Ampicillin	A1		A2		A3		A4	
				Thymol	Ampicillin	Thymol	Ampicillin	Thymol	Ampicillin	Thymol	Ampicillin
MIC (µg/mL)		256	2	8	2	64	8	2	1	2	1
MBC (µg/mL)		512	4	16	4	128	16	4	2	4	2
FICI	Value	-	-	1		4.25		0.51		0.51	
	Effect	-	-	Addition		Antagonism		Addition		Addition	

3.2.Antimicrobial susceptibility assay

Antibacterial activity of thymol/ampicillin (A1 concentration ratio), and thymol/ceftazidime (A1 concentration ratio) were evaluated against clinical strains of *E. coli*. The MIC ranges for thymol/ceftazidime were 8-1024 µg/mL for thymol and 4-128 µg/mL for ceftazidime, while the MBC ranges were 16-1024 µg/mL for thymol and 4-256 µg/mL for ceftazidime. The MIC ranges for thymol/ampicillin were 128-512 µg/mL for thymol and 32-128 µg/mL for ampicillin, while the MBC ranges were 256-1024 µg/mL for thymol and 64-256 µg/mL for ampicillin (Table 6). As

a result, thymol/ampicillin caused lower MIC and MBC compared to thymol/ceftazidime against clinical strains of *E .coli*, result in better antimicrobial effect.

Table 6: Antibacterial activity of A1 (thymol/ceftazidime) and A1 (thymol/ampicillin) compounds on clinical strains of E. coli obtained from patients suffering from UTI using MIC and MBC assays

E. coli **clinical strains**	**Thymol/Ceftazidime**		**Thymol/Ampicillin**	
	MIC (µg/mL)	**MBC (µg/mL)**	**MIC (µg/mL)**	**MBC (µg/mL)**
***E. coli* 1**	128/32	256/64	>1024/256	>1024/256
***E. coli* 2**	1024/128	1024/256	>1024/256	>1024/256
***E. coli* 3**	>1024/256	>1024/256	>1024/256	>1024/256
***E. coli* 4**	64/4	64/4	>1024/256	>1024/256
***E. coli* 5**	1024/128	1024/128	>1024/256	>1024/256
***E. coli* 6**	>1024/256	>1024/256	>1024/256	>1024/256
***E. coli* 7**	1024/128	1024/128	>1024/256	>1024/256
***E. coli* 8**	512/64	512/64	>1024/256	>1024/256
***E. coli* 9**	64/4	128/8	>1024/256	>1024/256
***E. coli* 10**	64/4	128/8	>1024/256	>1024/256
***E. coli* 11**	512/128	512/128	>1024/256	>1024/256
***E. coli* 12**	512/128	512/128	>1024/256	>1024/256
***E. coli* 13**	>1024/256	>1024/256	512/128	1024/256
***E. coli* 14**	>1024/256	>1024/256	>1024/256	>1024/256
***E. coli* 15**	256/64	512/128	128/32	256/64
***E. coli* 16**	256/64	512/128	256/64	512/128
***E. coli* 17**	8/4	16/8	>1024/256	>1024/256
***E. coli* 18**	512/128	512/128	512/128	1024/256
***E. coli* 19**	>1024/256	>1024/256	>1024/256	>1024/256
***E. coli* 20**	>1024/256	>1024/256	>1024/256	>1024/256

3.3. Evaluation of synergy via the checkerboard assay

In thymol/ceftazidime, compound A1 exhibited an additive effect (FICI=0.56), whereas compounds A2, A3, and A4 exhibited synergistic effects with FICI values of 0.31, 0.27, and 0.27, respectively.

In thymol/ampicillin, compound A1 exhibited an additive effect (FICI = 1.03), compound A2 exhibited an antagonistic effect (FICI = 4.25), and compounds A3 and A4 exhibited synergistic effects (FICI = 0.51 for both) (Tables 4 and 5).

3.4. FT-IR spectroscopy

Thymol/ampicillin and thymol/ceftazidime contain peaks that ranged from 592.61 to 3442.64 cm^{-1}, which are associated with the C=O ester group (26); the C=O amide I band (27); the C–N amide II band (27); the C–O–C–polysaccharide (28); the CH stretching alkene group (29); and the OH of carbohydrates (30), proteins, and polysaccharides. Non-covalent interactions like hydrogen binding or π–π stacking between mentioned functional groups of thymol and antibiotics, could modify spatial arrangement and influence antibiotic effectiveness. The C=C conjugated and related absorption bands separates the combined and individual states, suggesting changes in the molecular environment that may be attributed to non-covalent intermolecular interactions (Fig. 1-5).

Figure 1: FT-IR results for thymol

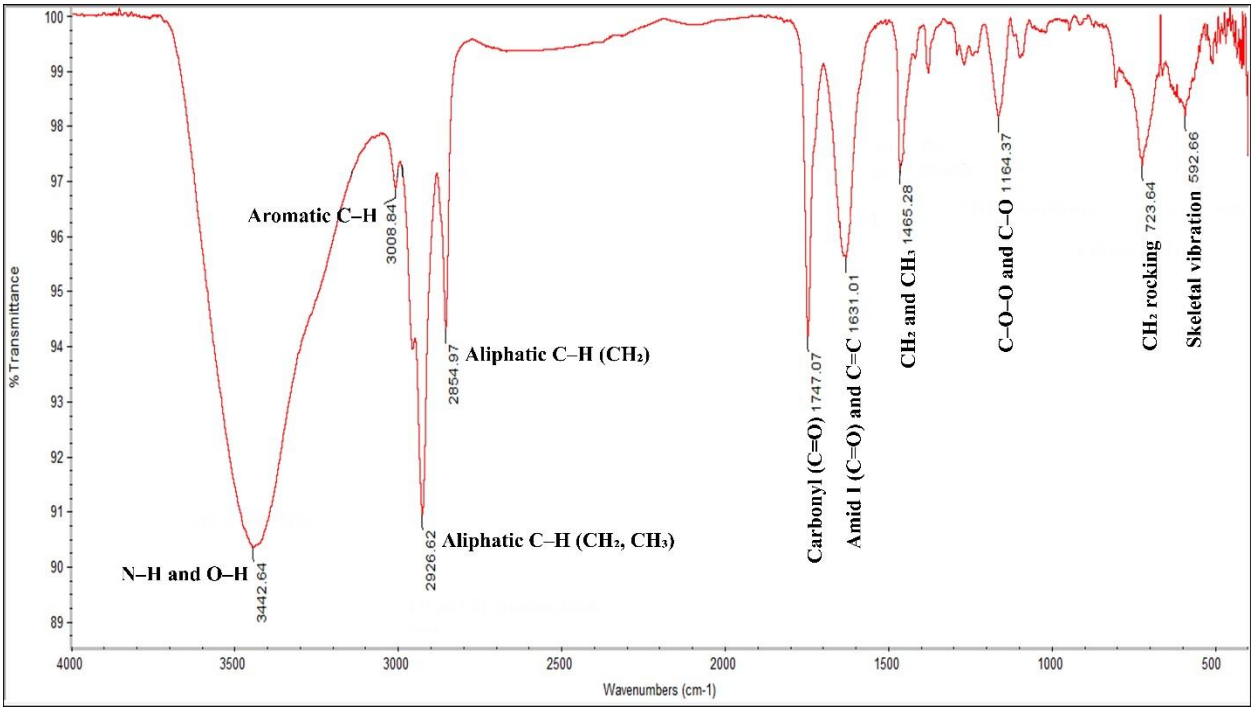


Figure 2: FT-IR results for ampicillin

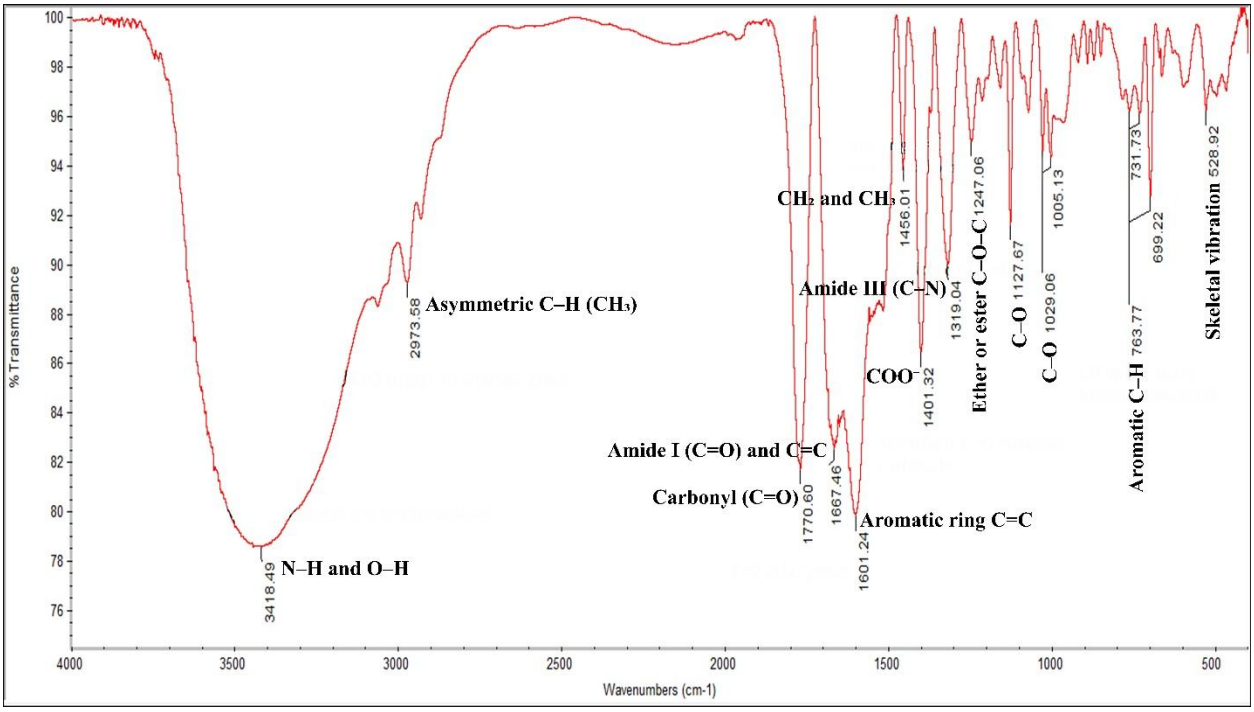


Figure 3: FT-IR results for ceftazidime

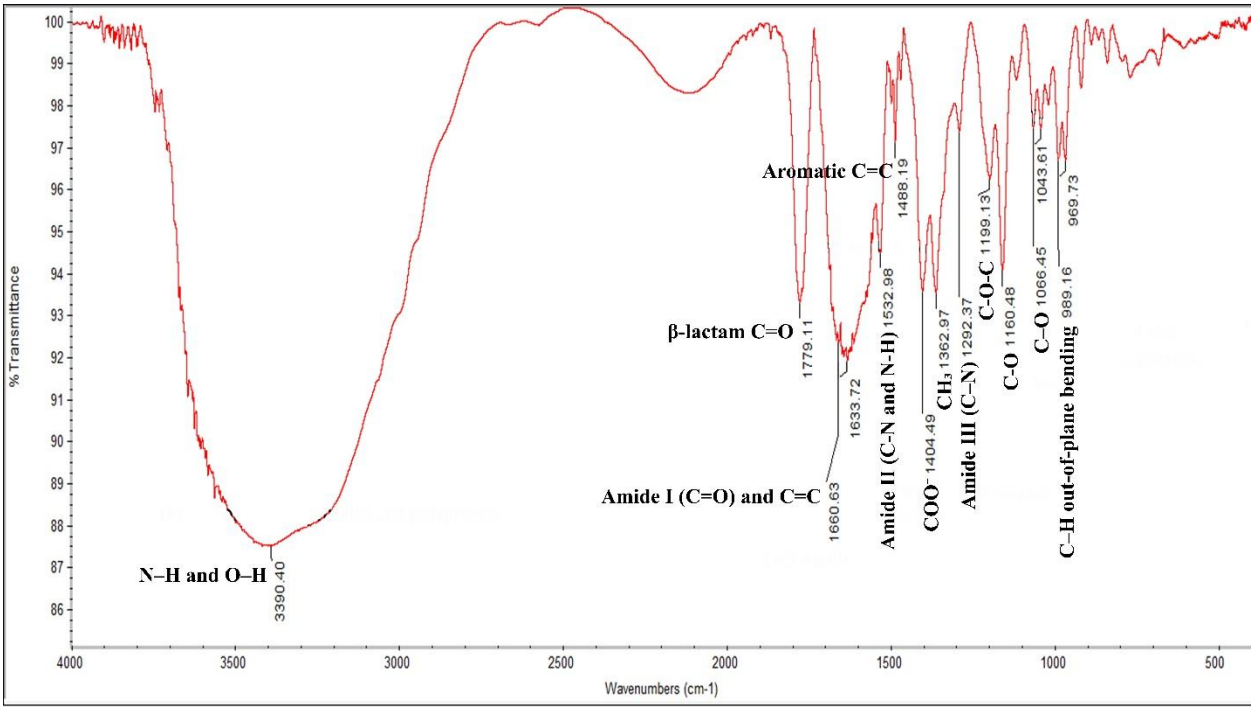


Figure 4: FT-IR results for thymol/ampicillin

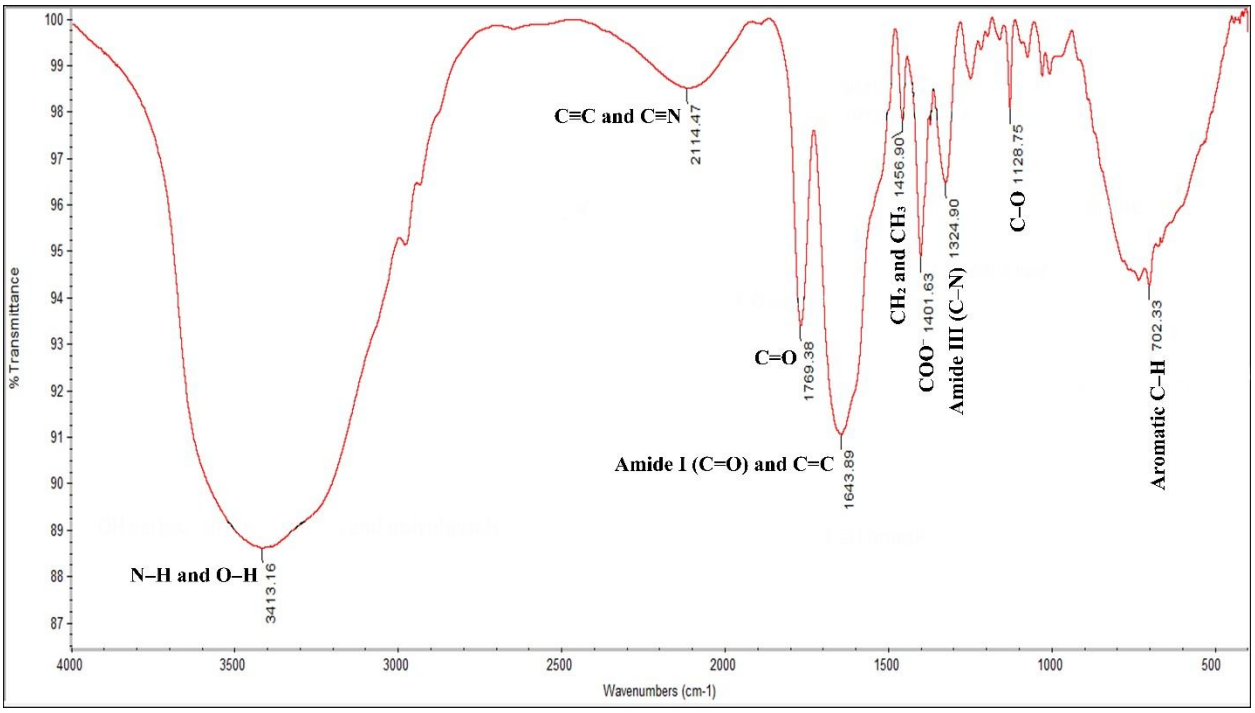
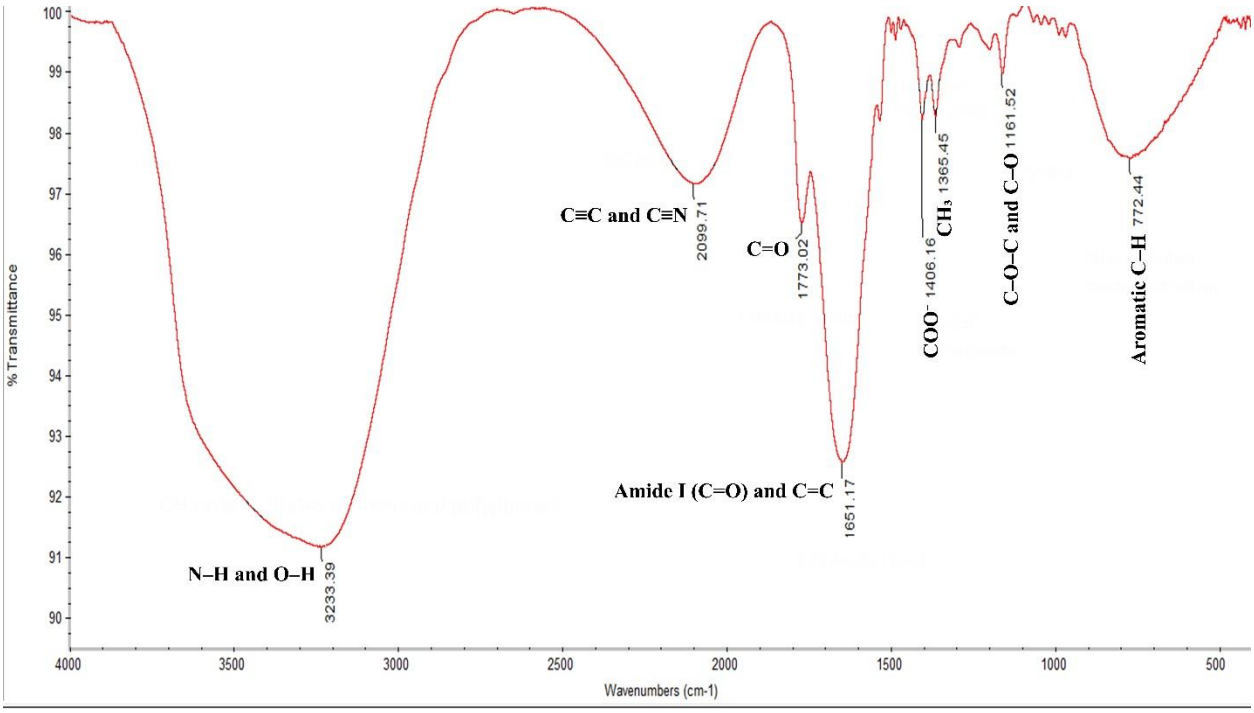


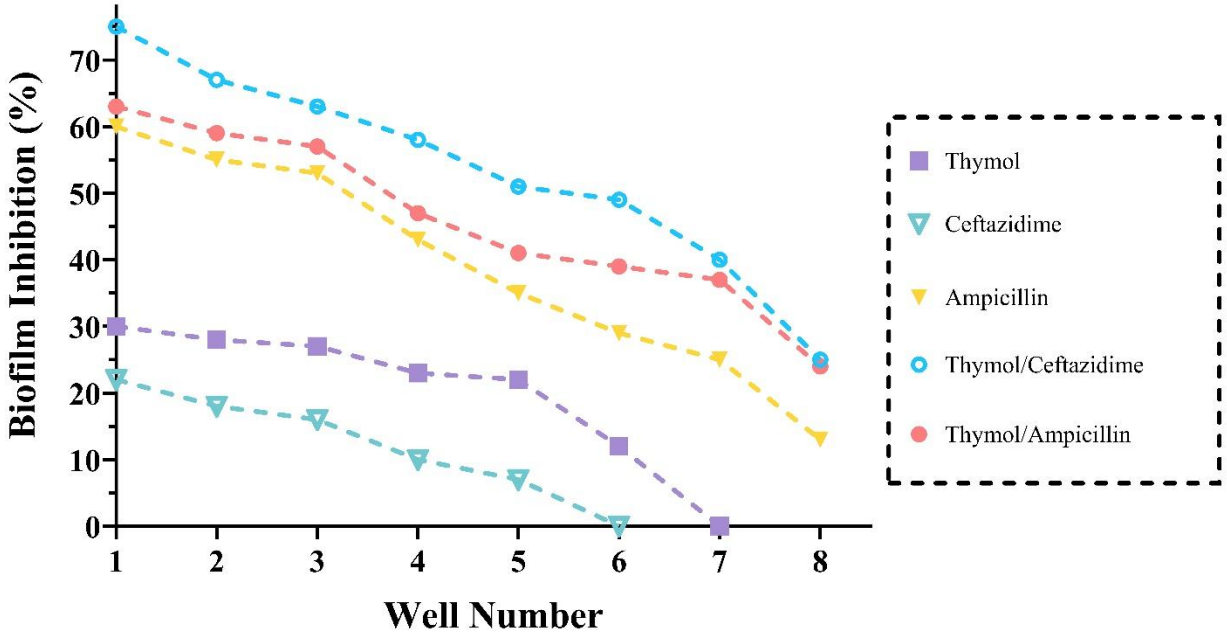
Figure 5: FT-IR results for thymol/ceftazidime



3.5.Antibiofilm evaluation

Thymol, ampicillin, and ceftazidime inhibited biofilm formation by 29.69%, 60%, and 22.14%, respectively. Whereas, The Thymol/ampicillin (A1 concentration ratio), and thymol/ceftazidime (A1 concentration ratio) combinations inhibited biofilm formation by 62% and 75.51%, respectively (Fig. 6). Biofilm inhibition by the individual compounds was lower than that of combinations, indicating that the combined treatments were more effective at inhibiting biofilm formation than the individual agents. Furthermore, the thymol/ceftazidime combination exhibited a higher biofilm inhibition rate than the thymol/ampicillin combination.

Figure 6: Antibiofilm results of thymol, ampicillin, ceftazidime, thymol/ampicillin (A1 concentration ratio), and thymol/ceftazidime (A1 concentration ratio) on *E. coli* ATCC 25922



Well numbers represent consecutive two-fold (1:2) serial dilutions of the tested compounds. Well 1 corresponds to the highest concentration: thymol (1024 µg/mL), ampicillin (256 µg/mL), ceftazidime (256 µg/mL), thymol/ampicillin (1024/256 µg/mL; A1 concentration ratio), and thymol/ceftazidime (1024/256 µg/mL; A1 concentration ratio). Wells 2-8 represent two-fold (1:2) serial dilutions of these starting concentrations.

3.6. Time-kill assay

Thymol was able to reduce the number of bacteria to zero at a concentration of 256 µg/mL in 6, 12, 18, and 24 h. Ceftazidime was able to reduce the number of bacteria to zero in the first 6 h, at the concentration of 128 µg/mL, at 12, 18, and 24 h, at the concentration of 8 µg/mL. Ampicillin was able to reduce the number of bacteria to zero at a concentration of 128 µg/mL in the first 6 h and at a concentration of 8 µg/mL at 12, 18 and 24 h.

Thymol/ceftazidime (A1 concentration ratio) was able to reduce the number of bacteria to zero in the first 6 h at a concentration of (thymol 256 µg/mL/ceftazidime 64 µg/mL), at 12 h at a concentration of (thymol 64 µg/mL/ceftazidime 16 µg/mL), and at 18 and 24 h at a concentration of (thymol 4 µg/mL/ceftazidime16 µg/mL) (Fig. 7). Thymol/ampicillin (A1 concentration ratio) was able to reduce the number of bacteria to zero in the first 6 h at a concentration of (Thymol 128 µg/mL/ampicillin 32 µg/mL), at 12, 18, and 24 h at a concentration of (thymol 32

µg/mL/ampicillin 4 µg/mL) (Fig. 8). Thymol/ceftazidime was faster and more effective in killing bacteria than thymol/ampicillin.

Figure 7: Kinetic effects comparison of thymol, ceftazidime, and combination of thymol/ceftazidime at 6, 12, 18, or 24 h on *E. coli* ATCC 25922

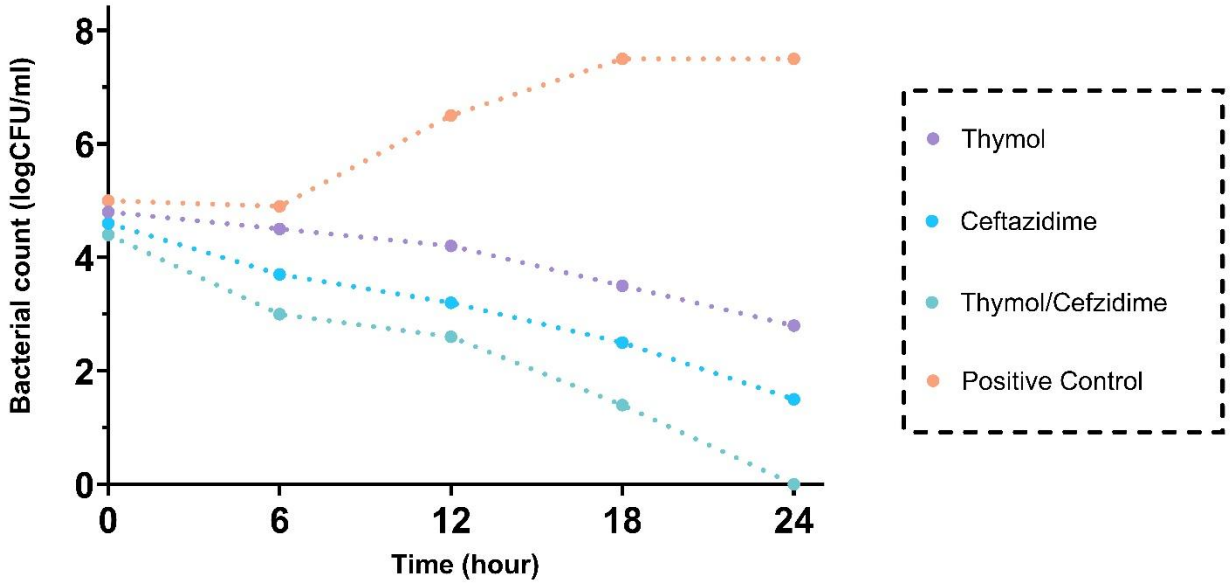
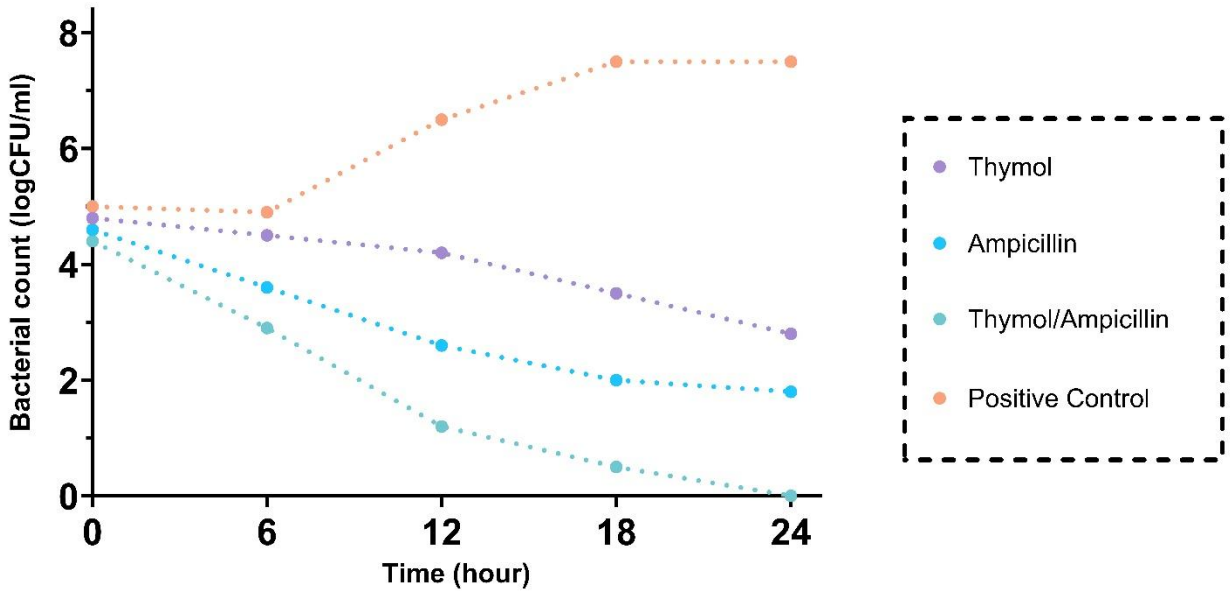


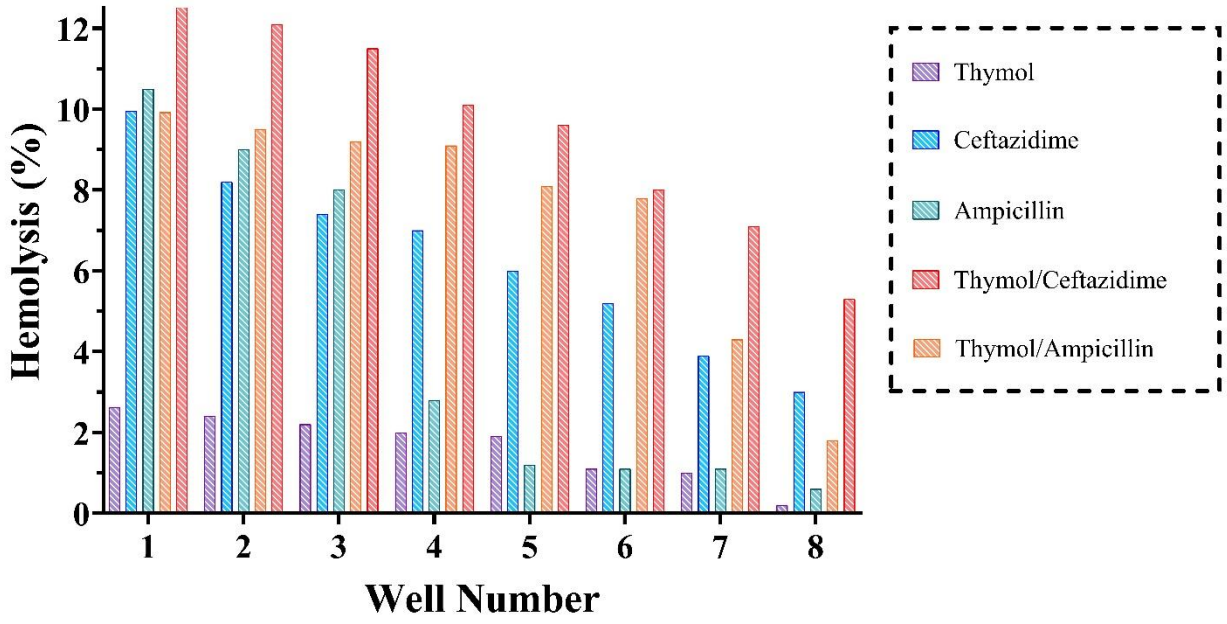
Figure 8: Kinetic effects comparison of thymol, ampicillin, and combination of thymol/ampicillin at 6, 12, 18, or 24 h on *E. coli* ATCC 25922



3.7. *In vitro* hemolysis assay

Hemolytic activities of thymol, ampicillin, and ceftazidime against RBCs were evaluated 36.12-, 9.05-, and 9.54-fold lower than Triton X-100, respectively. Since Triton X-100 induced hemolysis in 95% of RBCs, while thymol, ampicillin, and ceftazidime induced hemolysis in only 2.63%, 10.5%, and 9.96% of RBCs, respectively. Thymol/ampicillin (A1 concentration ratio), and thymol/ceftazidime (A1 concentration ratio) exhibited 9.58- and 6.83-fold lower hemolytic activity compared to Triton X-100, which induced hemolysis in 9.92% and 13.9% of RBCs, respectively (Fig. 9). Both compounds had a lower hemolytic activity compare to the positive control.

Figure 9: Hemolytic activity effect of thymol, ampicillin, ceftazidime, thymol/ampicillin (A1 concentration ratio), and thymol/ceftazidime (A1 concentration ratio) against human RBCs



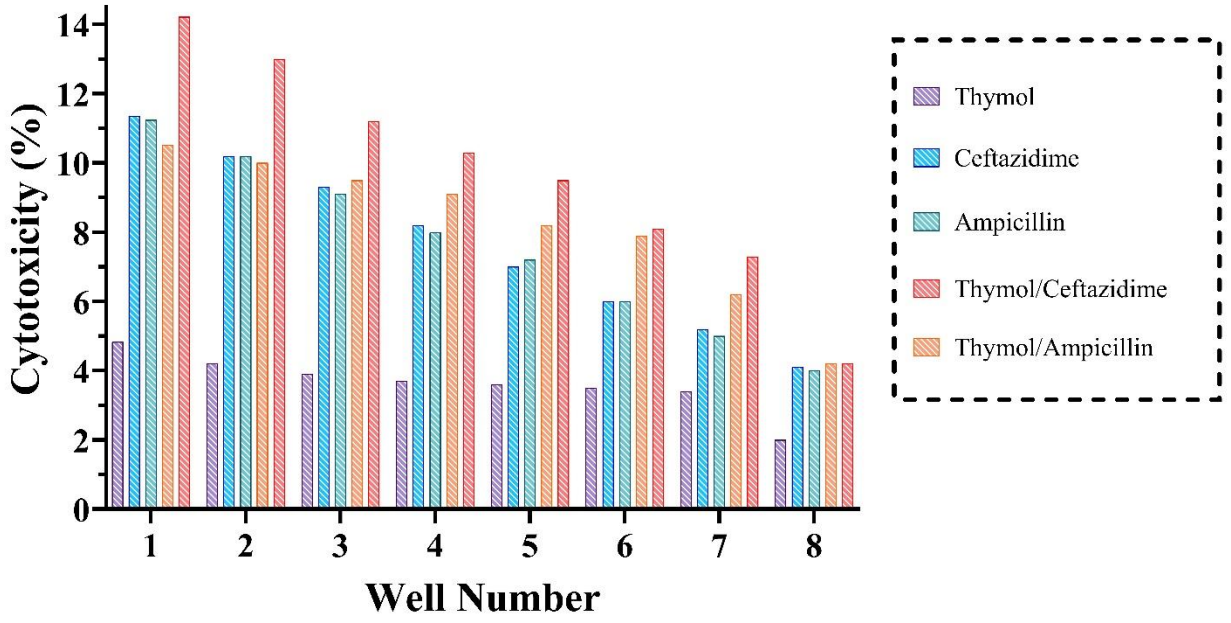
PBS and Triton X-100 (0.1%) were included as the negative and positive controls, respectively. Hemolysis percentages were calculated by to these controls; PBS representing 0% hemolysis and Triton X-100 representing 95% hemolysis. Well numbers represent consecutive two-fold (1:2) serial dilutions of the tested compounds. Well 1 corresponds to the highest concentration: thymol (1024 µg/mL), ampicillin (256 µg/mL), ceftazidime (256 µg/mL), thymol/ampicillin (1024/256 µg/mL; A1 concentration ratio), and thymol/ceftazidime (1024/256 µg/mL; A1 concentration ratio). Wells 2-8 represent two-fold (1:2) serial dilutions of these starting concentrations.

3.8. *In vitro* MTT assay

Cytotoxic activities of thymol, ampicillin, and ceftazidime against HFF cells were evaluated 19.66-, 8.45-, and 8.36-fold lower than Triton X-100, respectively. Since Triton X-100 caused 95% cytotoxicity in HFF cells, thymol, ampicillin, and ceftazidime caused 4.83%, 11.24%, and 11.36% cytotoxicity in HFF cells, respectively. Thymol/ampicillin (A1 concentration ratio), and thymol/ceftazidime (A1 concentration ratio) exhibited 9.03- and 6.68-fold lower cytotoxic activity compared to Triton X-100, which caused 10.52% and 14.22 cytotoxicity in HFF cells, respectively (Fig. 10). Both compounds had a lower cytotoxicity effect than positive control against HFF cells.

Figure 10

Cytotoxicity effect of thymol, ceftazidime, ampicillin, thymol/ampicillin (A1 concentration ratio), and thymol/ceftazidime (A1 concentration ratio) against HFF cells



Peptide-free cell suspensions and Triton X-100 (0.1%) were included as the negative and positive controls, respectively. Hemolysis percentages were calculated by to these controls; Peptide-free cell suspensions representing 0% hemolysis and Triton X-100 representing 95% hemolysis. Well numbers represent consecutive two-fold (1:2) serial dilutions of the tested compounds. Well 1 corresponds to the highest concentration: thymol (1024 µg/mL), ampicillin (256 µg/mL), ceftazidime (256 µg/mL), thymol/ampicillin (1024/256 µg/mL; A1 concentration ratio), and thymol/ceftazidime (1024/256 µg/mL; A1 concentration ratio). Wells 2-8 represent two-fold (1:2) serial dilutions of these starting concentrations.

4. Discussion

The widespread use of antibiotics caused the emergence of MDR bacteria (31). Ampicillin and ceftazidime often use to treat infections caused by *E. coli* isolated from UTIs, but significant resistance to these drugs, even 30–90% or more, has been reported (32). There is an urgent need for new strategies to combat antibiotic-resistant bacteria, which represents a primary global health concern (33). One of these strategies is combining thymol and antibiotics (34).

In the study by Yao et al. (2022), thymol/colistin had an antimicrobial synergistic effect (FICI: 0.5) on *E. coli* (35). In a study by Noreen et al. (2018), antimicrobial effect of aqueous and ethanolic extracts of cloves, cinnamon, and garlic with ampicillin, imipenem, ciprofloxacin, norfloxacin, and nalidixic acid on *E. coli* were investigated (36). In a study by Tavakoli et al. (2022), the antimicrobial effects of a methanolic extract of *Salvia limbata* with ceftazidime on *Staphylococcus aureus* (*S. aureus*) and *Pseudomonas aeruginosa* (*P. aeruginosa*) were investigated, which had a better antimicrobial effect (37). In a study by Mitra, Hodiwala, and Kar (2024), the antimicrobial effect of an aqueous extract of *Psidium guajava* (guava) plants with ceftazidime was investigated on *E. coli* (38). In a study by Amassmoud et al. (2023), the essential oils of *Thymus pallidus* and *Thymus broussonnetii* exhibited synergistic antibacterial effects when combined with ciprofloxacin and streptomyci (39). In a study of Anwesa Bag et al. in 2011, *Terminalia chebula* fruit extract with ceftazidime had synergistic effect (40). The compounds in the phenolic plant extract induce an antimicrobial effect (41).

Combining thymol with ampicillin or ceftazidime enhanced antibacterial and antibiofilm activities, shortened bacterial killing time, and exhibited low cytotoxicity and hemolytic activity in comparison with Triton X-100 as the positive control. Based on FT-IR analysis, hydrogen binding between OH groups of thymol and the C=O or amide groups in ampicillin or ceftazidime, and π–π stacking between the aromatic rings of thymol and antibiotics might contribute to the antimicrobial and antibiofilm effects (42). FT-IR analysis demonstrated changes in the characteristic of the treated samples. However, further chemical isolation and activity-guided fractionation are necessary to determine whether these changes are associated with the observed bioactivity.

Thymol is a phenolic compound extracted from the *Thymus daenensis* subsp. *lancifolius*. Thymol, a structural isomer of carvacrol, has a phenolic hydroxyl group and high lipophilicity, which facilitates its incorporation into bacterial membranes (43). This leads to membrane disruption, increased permeability, and causes leakage of ions such as proton and potassium, leading to the

loss of membrane potential in *E. coli* (44). Ampicillin and ceftazidime inhibit peptidoglycan cross-linking by targeting penicillin-binding proteins (PBPs), leading to cell wall weakening and subsequent osmotic lysis of bacterial cells (45).

Limitations

This study was limited to *in vitro* experiments and a relatively small number of clinical isolates, which may not fully represent *in vivo* conditions. Future studies should include *in vivo* assessments, explore the molecular mechanisms underlying the synergistic effects of thymol with antibiotics, and evaluate thymol derivatives or thymol-rich plant extracts against a broader range of MDR and XDR bacterial strains to confirm and extend these findings.

Conclusion

Thymol/ceftazidime showed greater antimicrobial and antibiofilm effects than thymol/ampicillin. The cytotoxic effects of both compounds were lower than the Triton X-100 on human RBCs and HFF cells. According to the present findings, the combination of thymol and ceftazidime may represent a promising strategy against antibiotic resistance and *E. coli*-associated urinary tract infections; however, further validation through in vivo efficacy, pharmacokinetic, and safety studies is required.

Declarations

Ethical Approval and consent to participate

Informed consent was obtained from all blood donors for the collection and use of fresh human blood. This study is in compliance with the Helsinki Declaration (https://www.wma.net/policies-post/wma-declaration-of-helsinki/), and also approved by the Ethics Committee of Zanjan University of Medical Sciences (ethical code IR.ZUMS.REC.1399.441, approved in 2020).

Consent for publication

There is no limit to the publication of this study. All the authors consented to the publication of this research.

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Conflicts of interest

The authors declare that they have no conflicts of interest.

Data availability

Summary data are presented in the published article. All the data supporting the findings, including the raw experimental data, are available from the corresponding author upon reasonable request.

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Author contributions

SJ: Methodology, Project Administration, Data Curation, Resources, Writing - Original Draft, Investigating, Formal Analysis

MSh: Data Curation, Writing - Review and Editing, Investigation, Formal Analysis

AT: Writing - Review and Editing, Visualization, Investigation, Formal Analysis

BM: Conceptualization, Supervision, Writing - Review and Editing

All the authors have read and agreed to the published version of the manuscript.

Use of AI/LLM

The authors declare that no artificial intelligence (AI) or large language model (LLM) tools were used in the writing and editing.

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