



## The Effect of Thyme (*Thymus vulgaris*) Extract on the Expression of *norA* Efflux Pump Gene in Clinical Strains of *Staphylococcus aureus*

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### Abstract

The NorA efflux pump considered as one of the contributors to antibiotic resistance in *Staphylococcus aureus* strains. One of the challenges of the researchers is finding natural plant compounds with the ability to inhibit the pumps. The aim of this study was to investigate the inhibitory effect of thyme (*Thymus vulgaris*) extract on NorA efflux pump in ciprofloxacin-resistant strains of *S. aureus*. Here, by using polymerase chain reaction (PCR), the presence of *norA* efflux pump was identified in 10 clinical and standard ciprofloxacin-resistant strains of *S. aureus*. The extract of thyme (*T. vulgaris*) was prepared using ethanol solvent and the effect of the extract against *norA* efflux pump was investigated by ethidium bromide method. In the following, after exposure to sub minimum inhibitory concentration of the extract, *norA* gene expression was evaluated using real-time polymerase chain reaction. Finally, we used the gas chromatography mass spectrometry (GC-mass) method to characterize the compounds with antibacterial properties. The results of PCR showed that all strains had a *norA* efflux pump, and ethidium bromide phenotypic method indicated that thyme extract had an inhibitory effect on all resistant strains with *norA* efflux pump. The *norA* gene expression in ciprofloxacin-resistant strains also decreased in sub minimum inhibitory concentration of the extract. By using gas chromatography- mass spectrometric, five chemical compounds of thymol, carvacrol, indole, 2-methoxy-4-methylphenol and quinic acid were determined as the dominant compounds of thyme (*T. vulgaris*) extract. Due to the antiefflux pump effect of thyme extract, it seems that this plant extract can be used as a good antibacterial component in the pharmaceutical industry of Iran.

**Key words:** Ciprofloxacin; Efflux pump; GC-mass; *Staphylococcus aureus*

### Introduction

*Staphylococcus aureus* is a Gram-positive bacterium and one of the most opportunistic human infectious. *S. aureus* cause a wide variety of diseases from food corruption to life-threatening diseases such as potentially lethal bacteremia and endocarditis. In addition, by the emergence of methicillin-resistant *S. aureus* strains (MRSA) in the early 1980s that showed resistance to many antibacterial agents, this pathogen has become a major challenge for medical communities (Grundmann *et al.*, 2006). *S. aureus* shows many different mechanisms of resistance towards antibiotics that some of them are known and characterized in recent years. The

efflux pumps excrete the drug or other noxious agents from the cell and prevent intracellular accumulation at the threshold required for the activity of the drugs (Saiful *et al.*, 2008; Kosmidis *et al.*, 2012). It found that, the efflux pumps play an important role in *S. aureus* resistance to antibiotics and biocides (Costa *et al.*, 2013). However, a few studies have conducted to characterize the excretion mechanism of toxic substances into the environment based on the efflux systems for this pathogen. The NorA efflux pump is chromosomally encoded and one of the most important member of Facilitator Super Family (MFS) efflux system in *S. aureus*. Various studies have shown that NorA can pump a wide

range of compounds, such as hydrophobic fluoroquinolones including norfloxacin, ciprofloxacin, ethidium bromide, and quaternary ammonium compounds out of the cell (Yoshida *et al.*, 1990; Truong-Bolduc *et al.*, 2005).

Recently several articles have been published about the presence of different types of efflux pumps and their gene expression patterns in *S. aureus* clinical strains. Couto *et al.* (2008) indicated that the *norA* gene has a basic level expression within the cells that cause a little resistance to antibiotic compounds and by increasing the expression of *norA*, the fluoroquinolones resistance also increases. The overexpression of the *norA* efflux pump gene in the presence of the hexahydroquinoline derivative has been also reported by Pourmand *et al.* (2014). In other study, the reduction of Minimum Inhibitory Concentration (MIC) of ethidium bromide in the presence of inhibitor carbonyl cyanide 3-chlorophenylhydrazone (CCCP), indicating the responsibility of efflux pumps for antibiotic resistance (Le Loir *et al.*, 2003).

In addition to the rapid emergence of antibiotic resistant strains, which can cause new infections and increase morbidity, mortality and health-care cost (Cohen, 1992); the use of antibiotics affects the human's health due to their effect on the body's natural flora. The concerns about such facts have led the researchers to attempt to discover the genetic mechanisms of bacterial resistance and seeking to find good antibiotics alternatives (Czaplewski *et al.*, 2016). In the recent decades, plants have been a valuable source of natural products for maintaining human health and the use of herbal compounds and herbal extracts has been considered as natural alternatives to antibiotics. The results of various studies have confirmed the antimicrobial properties of essential oil components such as phenolic compounds (Jansen *et al.*, 1987) and tannin (Saxena *et al.*, 1994) which synthesized in secondary metabolism of plants. For instance, Kurlenda *et al.* (2012) studied the antimicrobial effect of nettle (*Urtica dioica*) on *S. aureus* species and showed this plant component has greater effect on gram-negative bacteria than gram-positive bacteria. Clove (*Syzygium aromaticum*) has been used for centuries as food preservative and for many medicinal purposes.

Its antimicrobial potential was established when its essential oil extracts killed many gram positive and gram-negative bacteria (Gislene *et al.*, 2000). Generally, plant compounds have their antimicrobial properties through mechanisms such as cell wall decomposition, cytosolic acidity increase, cell membrane damage, cell-to-cell leakage, impaired transmission of proton and impaired in vital enzymes such as ATPase. In addition to the antimicrobial properties, as indicated by Mohadjerani *et al.* (2016) some plant extracts such as *Platyclus orientalis* with antioxidant activity might be helpful in preventing or stopping the progress of various oxidative stress-related diseases.

Thyme (*T. vulgaris*) is one of the most valuable medicinal plants that its oil and water extracts have bactericidal and bacteriostatic effect for many microorganisms (Deans and Ritchie, 1987). Cosentino *et al.* (1999) confirmed that the antimicrobial properties of thyme essential oils are mainly related to their high phenolic content such as carvacrol and thymol. The genus *Thymus* (thyme) consists of about 215 species of herbaceous perennials and subshrubs that are well adapted to hot and dry climates. Common thyme (*T. vulgaris*) is a low-growing herbaceous plant that is rich in substances that have various effects, including anti-inflammatory, anti-tumor, anti-ulcer, antioxidant, antimalarial, indigestion, antiproliferative and gallbladder contraction. This plant genus is mostly composed of terpenes and flavonoids. More than 160 flavonoids from this genus have been extracted.

However, the effect of thyme (*T. vulgaris*) extract on the expression of *norA* gene has not been studied. Therefore, in this study, we investigate the role of thyme (*T. vulgaris*) extract on *norA* gene expression in antibiotic resistance strains of *S. aureus*.

## Materials and Methods

### Bacterial isolates

Here, the study was conducted with ciprofloxacin-resistant clinical strains of *S. aureus* collected from different samples of hospitalized patient including blood, ulcers, skin, and urine collected at Imam Hossein, Atieh and Sarem hospitals in Tehran during 1392-1393.

Several tests used for identifying *S. aureus* isolates, including hot dyeing, catalase, coagulase, DNase, Baird-Parker agar, and fermentation of mannitol. Samples that were positive in all of the tests were isolated as *S. aureus*.

### Susceptibility determination

The Kirby-Bauer disk diffusion susceptibility test used in triplicate to determine the antibiotic susceptibility of the isolates to oxacillin (15 µg), ciprofloxacin (5 µg), tetracycline (30 µg), chloramphenicol (30 µg), gentamicin (10 µg), vancomycin (10 µg), penicillin (10 units), and Erythromycin (15 µg) disks. After 18 h of incubation (24 h for vancomycin) at 37°C, the results were interpreted as either sensitive, intermediate, or resistant according to the inhibitory zone diameters around the disks in accordance to Clinical and Laboratory Standards Institute (CLSI) guidelines (Katz *et al.*, 2004). In all experiments, *S. aureus* strain ATCC 25923 (ciprofloxacin resistant) and *Staphylococcus epidermidis* ATCC 12228 (without NorA efflux pump) used as positive and negative controls, respectively.

### Polymerase chain reaction (PCR) of *norA* genes

The presence of *norA* gene in *S. aureus* isolates were analyzed by PCR, using the primers described in Table 1A. For genomic DNA isolation, *AccuPrep*® Genomic DNA Extraction Kit (Cat. No.; K-3032, Bioneer, Republic of Korea) was used and DNA purity determined by Nanodrop spectrophotometer (Nanodrop ND-1000; NanoDrop Tech. Inc., Wilmington, DE). The PCR reaction mixture with the final volume of 20 µl was prepared for each sample (including positive and negative control) and DNA amplification was performed in a thermal cycler (Eppendorf Master Cycler Gradient, Germany) using the thermal profile listed in Table 1B.

### Plant collection and extraction

We prepared the thyme (*T. vulgaris*) plant used in this study from the Iranian biosphere reserve and maintained in optimum conditions after drying. The dried thyme material was subjected to preparation of bioactive extracts by maceration method. The 40 g of dried powder leaves added into 300 ml 80% ethanol and placed in a shaking water bath (Clifton Range®) at constant speed of 90 rpm, for 24 hours. After incubation time, supernatant was collected as thyme extract and filtered with Whatman No.1 filter paper. The filtrates then were evaporated in incubator at 37 °C.

**Table 1.** Characteristics of PCR amplification and qPCR for *norA* gene.

<b>A) Oligonucleotide primers used in PCR and RT-PCR.</b>			
Primers	Sequence (5' →3')	Target Size (bp)	Ref.
NorA-Fw	TTCACCAAGCCATCAAAAAG	620	Couto <i>et al.</i> , 2008
NorA-Rv	CTGCCTTCTCCAGCAATA		
NorA-RT(Fw)	ATGGTCAAGCCAGACAGAG	112	Ding <i>et al.</i> , 2008
NorA-RT(Rv)	CGTGTTTTCAACATTTAATGCAA		
Gmk-RT(Fw)	TATCAGGACCATCTGGAGTAGG	122	Ding <i>et al.</i> , 2008
Gmk-RT(Rv)	CATCAACTTCACCTTCACGC		
<b>B) PCR thermocycling conditions.</b>			
1	Initial denaturation	94 °C	5 minutes
2	Denaturation	94 °C	30 seconds
3	Annealing	60 °C	30 seconds
4	Extension	72 °C	60 seconds
5	Final extension	72 °C	5 minutes
Cycle (2-4)	30		

### Susceptibility determination

After identifying ciprofloxacin resistant strains, the Minimum Inhibitory Concentration (MIC) was determined by the dilution method in 96-well plates for thyme extract. The experiment carried out in triplicate with interpretation in

accordance with CLSI guidelines (Wayne, 2011). The extract was poured into the 96-well dilution plates at concentrations of 62.5 to 4000 µg/ml and transferred to the Muller Hinton Broth (MHB) (Merck Co., Germany) culture medium to achieve a final volume of 100 µl. The 50 µl of microbial culture equal to a 0.5 McFarland

standard was added to the all well plates to achieve a final inoculum of  $5 \times 10^5$  cfu/ml. The plates incubated for 18 h at 37 °C in ambient air. The MIC was recorded as the lowest dilution showing no bacterial growth. In this study, free bacterial extract and extract contained *S. aureus* (strain ATCC 25923) used as negative and positive control, respectively.

The ciprofloxacin-resistant formulations were studied for MIC testing. MIC-based assay in accordance with CLSI was performed by dilution method in 96-well plates for ciprofloxacin and ethidium bromide. Briefly, the ethidium bromide solution is poured into the well A and Mueller Hinton broth culture were added to achieve a final volume of 100  $\mu$ l. The wells B-H used to make sequential dilution relative to well A (0.5 to 128  $\mu$ g/ml). All wells were added with 50  $\mu$ l of ciprofloxacin-resistant strain culture equal to a 0.5 McFarland concentration.

#### **Treatment of the efflux pump inhibitor**

To determinate the role of efflux pump in the ciprofloxacin-resistant phenotypes in *S. aureus* isolates, the MIC of ciprofloxacin evaluated in the presence of 20  $\mu$ g/mL efflux pump inhibitor carbonyl cyanide 3-chlorophenylhydrazone (CCCP). The CCCP was added to each wells of plates containing 0.5 to 128  $\mu$ g/mL ciprofloxacin. Then, MICs were determined again and compared them with and without CCCP. One of the wells containing CCCP and not containing ciprofloxacin was used as control. Decrease of ciprofloxacin MIC after the CCCP addition is considered to be the positive criterion for the presence of efflux pump in *S. aureus* isolates.

#### **RNA extraction and complementary DNA (cDNA) production**

RNA extraction from ciprofloxacin-resistant *S. aureus* strain after treatment with subMIC extract concentration performed using High Pure RNA Isolation kit (Roche Co., Germany) according to the manufacturer's instructions. We evaluated the quantity and quality of total RNA using UV spectroscopy and agarose gel electrophoresis methods. The RNA was DNase

treated using DNase I (Fermentas, Sinagen Co., Iran) to remove remaining genomic DNA and complete removal of contaminating DNA was confirmed by PCR. Reverse transcription was performed with the Revert Aid™ First Strand cDNA Synthesis Kit (Fermentas, Sinagen Co., Iran) using 1  $\mu$ g RNA.

#### **Real-time PCR**

To study whether expression of the *norA* gene in *S. aureus* strains was modified after treatment by sub inhibitory concentrations of thyme extract, we used real-time PCR using primers described in Table 1A. RT-PCR was performed in triplicate using a Power SYBR Green PCR Master Mix (Applied Biosystems Co., UK) on a StepOne ABI real-time PCR equipment (Applied Biosystems, Foster City, CA). The cDNA was used as a template in 20  $\mu$ l reactions including 10  $\mu$ l Power SYBR®Green PCR Master Mix (Applied Biosystems) and 2 pmol of each primer. The qPCR cycling was performed at 95 °C for 10 min, followed by 40 cycles at 95 °C for 20 s and 40 s at 60 °C and finally a melting stage to determine the unspecific PCR product or possible primer dimers. Triplets of a negative control (resistant strain without herbal extract) were included in all qPCR runs, and *gmk* (guanylate kinase) gene was used as an endogenous control. The relative expression of *norA* efflux pump gene was determined using  $\Delta\Delta C_T$  method.

#### **Statistical analysis**

The susceptibility results between the resistant and sensitive strains in disk diffusion compared by chi-square and fisher exact test. The differences between the expressions of target genes were determined by Tukey's HSD post-hoc test between control and treated samples. All values were expressed as the mean  $\pm$  standard error of the mean and differences were considered as statistically significant if the p-value was  $\leq 0.05$ . This study was carried out as completely randomized designs with two replications. We calculate  $\Delta\Delta C_T$  for each sample, and then, using SPSS 16 software (SPSS Inc., Chicago, IL) and the one-way ANOVA method, the expression change rate of each

sample was analyzed.

### Gas chromatography- mass spectrometric analysis (GC-MS)

The GC-MS analysis was performed using an Agilent Technologies GC systems with GC-7890B/MS-5977A model (Agilent Technologies, Santa Clara, CA, USA) equipped with HP DB5 capillary column with dimensions of 30 mm × 0.25 mm ID × 0.25 μm film. The GC oven initial temperature was 50°C for 2 min then raised to 280°C with increasing rate of 5°C/min, and kept for 2 min. Injection port and detection temperature were ensured at 250°C and 280°C, correspondingly. Flow rate of helium as carrier gas was 5 mL/min. The samples volume injected was 2 μl in split mode as 10:1 and mass spectra were taken at 70 eV of the mass range of 35–400 (Heidari *et al.*, 2018). Interpretation on mass spectrum of GC-MS was done using the database

of National Institute Standard and Technology. The mass spectrum of unknown components was compared with the spectrum of the identified components saved in these libraries to determine the name, molecular weight, and structure of the components of the tested materials.

## Results

### Disk diffusion test

Based in the results of antibiogram test for 30 strains of *S. aureus*, the highest resistance observed for penicillin (60%), tetracycline (60%), and erythromycin (53%), while the lowest resistance was related to vancomycin (93% susceptible) and chloramphenicol (86% susceptible) antibiotics (Table 2). In general, the antibiotic resistance in the strains isolated from urine samples and ulcers was higher than other strains.

**Table 2.** The number of resistant and sensitive *S. aureus* strains to different antibiotics in disk diffusion test (Values expressed are averages of three replicates).

Antibiotic	Sensitive		Intermediately sensitive		Resistant	
	Number	Percent±0.1	Number	Percent±0.1	Number	Percent±0.1
Oxacillin sodium	14	46.7	0	0.0	16	53.3
Vancomycin	28	93.3	2	6.7	0	0.0
Ciprofloxacin	18	60.0	5	16.7	7	23.3
Penicillin	12	40.0	0	0.0	18	60.0
Erythromycin	14	46.7	0	0.0	16	53.3
Tetracycline	12	40.0	0	0.0	18	60.0
Gentamicin	14	46.7	0	0.0	16	53.3
Chloramphenicol	26	86.6	2	6.7	2	6.7

Total number of strains: 30

### Screening of *norA* efflux pump gene

The gene that coded for the NorA efflux pump in isolated strains were screened by PCR, using a paired of the primers that amplify a 620 bp fragment. The *norA* gene was observed in 12 strains that are resistant to ciprofloxacin.

### Susceptibility determination

The MIC values of thyme extracts against ciprofloxacin-resistant *S. aureus* represented in Table 3. Different strains exposed to the 62/5-4000 μg/ml concentration of the herbal extract within 24 hours. The results showed the MIC values of ciprofloxacin resistant strains range

from 62.5 to 250 μg/ml. In addition, results indicated that most of the isolates became less resistant (2 to 4 folds) to ciprofloxacin in the presence of CCCP as efflux pump inhibitor.

### Real-time PCR of *norA*

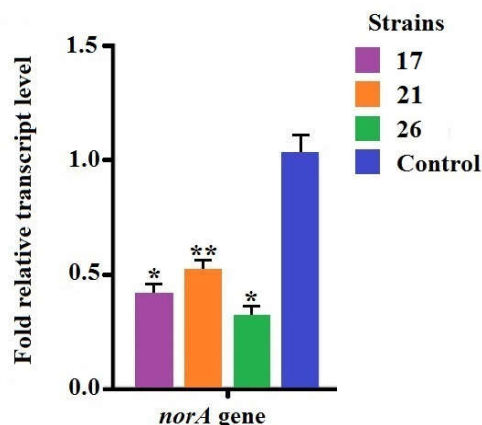
The relative expression of the *norA* gene in three ciprofloxacin-resistant strains of *S. aureus*, evaluated in the presence of thyme extracts by qRT-PCR analysis. Based on the results, the reduction in the expression of the *norA* gene had a statistically significant difference compared to *gmk* gene expression (P <0.05) (Fig. 1, Table 4).

**Table 3.** The MIC of thyme (*T. vulgaris*) extract in ciprofloxacin resistant strains of *S. aureus*.

Strain number	MIC ( $\mu\text{g/ml}$ )
1	125
5	62.5
9	62.5
17	62.5
21	125
26	250
27	125
ATCC 25923	500
ATCC 12228	125

### GC/MS analysis

The results of our previous study (Heidari *et al.*, 2018) showed *Thymus vulgaris* leaf ethanolic extract contain 42 different compounds (Fig 2, Table 5) among which, the dominant constituents were quinic acid (12.7%), carvacrol (6.47%), indole (3.48%), thymol (3.04%) and 2-Methoxy-4-methylphenol (10.01%).



**Fig 1.** The *norA* transcription. The expression of *norA* decrease more than two fold in ciprofloxacin-resistant strains in the presence of thyme extracts (n = 3, \*= P < 0.01, \*\*= P < 0.05). Resistant *S. aureus* strain in the absence of thyme extract used as negative control.

**Table 4.** The *norA* gene expression of three ciprofloxacin resistant strains in the presence of thyme extracts. The *gmk* (guanylate kinase) gene used as an endogenous control.

Strain	Change the gene expression		
	<i>norA</i>		<i>gmk</i>
17	0.42±0.35	↓	1
21	0.52±0.29	↓	1
26	0.32±0.34	↓	1
ATCC 25923 (Control)	1.01±0.78	↓	1

### Discussion

Inappropriate and irrational use of antibiotics has led to the emergence and spread of antibiotic resistant bacteria. There are several mechanisms for bacterial resistance to antibiotics, among which efflux pumps decrease the intracellular concentration of the antibiotics by extruding them outside the cell (Savjani *et al.*, 2009; Costa *et al.*, 2011). Efflux systems are found in almost all bacterial species such as *S. aureus* (Li and Nikaido, 2009; Saiful *et al.*, 2008). The multidrug efflux pump NorA is one of the most important efflux systems in *S. aureus*. The *norA* is a chromosomal gene that its expression significantly increased in the presence of antibiotics. In recent years, the researchers have tried to find suitable alternatives for antibiotics. In between, the use of herbal compounds and herbal extracts has been considered as a natural

alternative to antibiotics due to their inhibitory effects on the expression of resistance genes as well as their function as inhibitors on resistance proteins such as efflux pumps. Thyme is one of the plants that its extracts are known to possess some antimicrobial agents include flavones, terpenes, thymol, eugenol, aliphatic alcohols, and glycosides of phenolic monoterpenoids that are used in herbal medicine (Costa *et al.*, 2011; Kurlenda *et al.*, 2012).

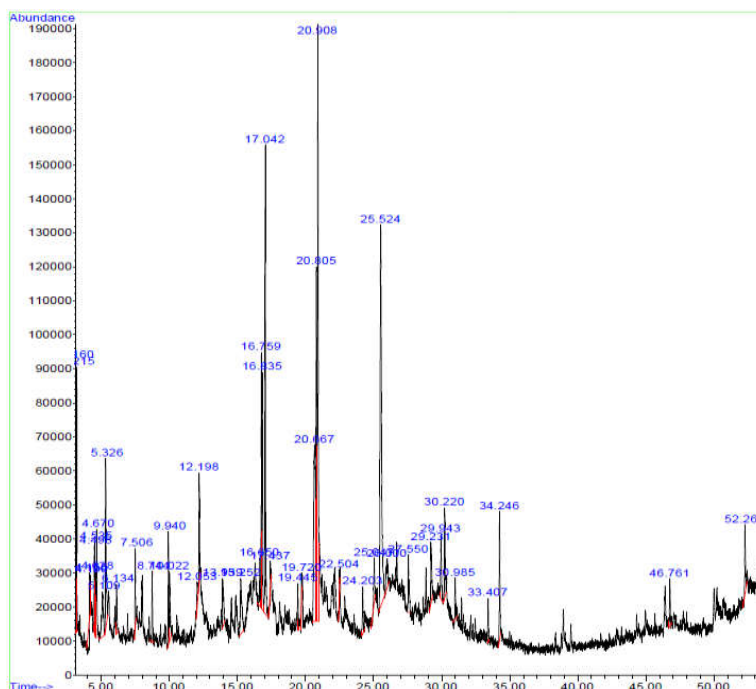
Here, in order to investigate the mechanism of resistance to ciprofloxacin and its relationship with the efflux pump performance, the screening of *S. aureus* strains for the presence of *norA* gene performed by PCR. The functions of NorA efflux pump checked by MIC ethidium bromide methods, in conjunction with CCCP efflux pump inhibitor. Etidium bromide is a common substrate of the efflux pump, which used in most studies as a positive control to measure the

activity of the efflux pump system. As indicated in the results, in the presence of CCCP, the amount of ethidium bromide MIC decreases, which is consistent with the results of some other studies and indicating that the efflux pump is

responsible for antibiotic resistance. The results of this study showed that antimicrobial compound of thyme at a concentration of 100 µg/ml could reduce the MIC of antibiotics in the presence of ethidium bromide.

**Table 5.** Chemical constituents of *Thymus vulgaris* extract, based on GC-MS analysis (Heidari *et al.*, 2018).

No.	Compound identified	Retention Time (min)	Percentage (%)
1	Hexadecanoic acid	34.24	1.99
2	Silane	19.446	0.64
3	DL-Glyceraldehyde	4.191	0.38
4	Carbonodithioic acid	5.324	2.78
5	Thiazolidine	4.500	0.73
6	N-Methylthioacetamide	4.535	0.75
7	Hexadecanoic acid, 3-hydroxy-, methyl ester	4.626	0.49
8	2-Furanmethanol	4.672	1.36
9	2-Methyl-3-(methylthio)-1-propene	5.107	1.79
10	Myrcenol	16.648	0.71
11	3-Furanmethanol	6.137	0.71
12	2,4-Dihydroxy-2,5-dimethyl-3(2H)-furan-3-one	7.504	0.83
13	Benzene, 1-methyl-2-(1-methylethyl)-	8.746	0.90
14	2-Methyl-5-methyleneoxane	9.942	1.34
15	Phenol 4-(3-hydroxy-1-propenyl-2-methoxy)	29.231	1.32
16	Loliolide	29.946	1.32
17	4H-Pyran-4-one, 2,3-dihydro-3,5-dihydroxy-6-methyl-	12.196	1.49
18	1,2-Benzenediol	13.942	1.08
19	Methyl 3-hydroxydodecanoate	15.252	1.90
20	Thymine	10.022	1.39
21	Thymol	16.757	3.04
22	Indole	16.837	3.48
23	Carvacrol	17.043	6.47
24	Benzeneethanamine, 2,5-dimethoxy-alpha,4-dimethyl	52.26	1.18
25	Syringic acid	30.988	0.60
26	Terpinolene	19.721	0.25
27	Sucrose	20.665	6.54
28	2-Hydroxy-5-methylbenzaldehyde	20.802	7.52
29	2-Methoxy-4-methylphenol	20.911	10.10
30	Hexasiloxane	22.502	0.17
31	Thiophenol	24.201	0.74
32	Hydrazinecarbothioamide	25.048	0.81
33	Amyl Nitrite	4.157	0.46
34	D-Mannose	25.998	1.11
35	Cyclododecasiloxane	27.549	1.29
36	2,2-dimethoxybutane	3.161	2.78
37	3-Methoxy-5-methylphenol	46.76	0.69
38	2,3-Dimethylhydroquinone	30.221	1.66
39	Xylitol	12.053	0.50
40	1,2-Benzenediol bis(trimethylsilyl) ether	33.408	0.57
41	4-Vinylguaiaicol	17.438	0.03
42	Quinic acid	25.523	12.73



**Fig 2.** GC-MS chromatogram of *Thymus vulgaris* extract (Heidari *et al.*, 2018).

Real-time PCR used to verify expression of the *norA* gene after treatment by sub-inhibitory concentrations of thyme extract. The results indicated that the thyme plant reduced the expression of the *norA* gene in the *S. aureus*. The decrease in the MIC of ciprofloxacin and ethidium bromide, along with the reduction of *norA* gene expression, in the presence of thyme (*T. vulgaris*) extract showed that the drug efflux systems are associated with resistance to ciprofloxacin in *S. aureus* isolates. These findings confirm the results of Huet *et al.* (2008) and De Kievit *et al.* (2001), which showed the overexpression of multidrug efflux pump in the presence of antibiotic and biocides. The reduction of the *norA* gene expression cause poor performance of the efflux pump and a high effect of antibiotic and other disinfectants, so thyme (*T. vulgaris*) extract is an appropriate antibacterial drug. These results are in agreement with findings of other researchers. Sienkiewicz *et al.* (2012) confirmed the antimicrobial activity of thyme essential oil against multidrug resistant clinical bacterial strains (*Staphylococcus*, *Enterococcus*, *Escherichia*, and *Pseudomonas* genus). Tintino *et al.* (2016) investigated the inhibitory effects of tannic acid on the

expression of NorA efflux pump in strains of *S. aureus*.

The chemical composition of the thyme (*T. vulgaris*) extract was detected by gas chromatography Mass spectrometry (GC-MS). The antibacterial activity of some thyme (*T. vulgaris*) extract compounds that we mentioned in this study, have been reported in other studies: Quinic acid (Gohari *et al.*, 2010), 2-Hydroxy-5-methylbenzaldehyde (Tukmechi *et al.*, 2010), Indole (Matsuda *et al.*, 1990), Thymol (Cosentino *et al.*, 1999, Marchese *et al.*, 2016), carvacrol (Cosentino *et al.*, 1999), Cyclododecasiloxane (Moustafa *et al.*, 2013; Patil *et al.*, 2014), Benzene, 1-methyl-2-(1-methylethyl)- (Deryabin *et al.*, 2015) and Hexasiloxane (Ghebleh *et al.*, 2014). It was experimentally demonstrated that not even a single component isolated from thyme (*T. vulgaris*) extract showed a high activity against pathogenic bacteria. It seems the antimicrobial action of thyme (*T. vulgaris*) extract is complicated and could be due to the synergistic effect between flavonoids, hydroxyl acids, and sesquiterpenes (Chouhan *et al.*, 2017).

In general, the alcoholic extracts showed the good activity on *norA* expression, suggesting that this solvent is appropriate to extract the



mainly polar antioxidant phenolic compounds. In addition, by comparing the results of this study with findings of other researchers, it concluded the concentration of ethanol played a major role in the activity of extract component: the 80% ethanolic extracts yielded the higher total phenolic contents than the 50-60% extracts reported in other studies (Chizzola *et al.*, 2008). In 80% ethanolic extracts, total phenolic and rosmarinic acids gave higher recoveries and the lipophilic carnosic type antioxidants were better extracted with higher proportions of ethanol. In conclusion, the active phytochemicals identified in the extracts of *Thymus vulgaris* decreased the MIC of ciprofloxacin and reduced expression of *norA* efflux pump gene in *S. aureus* strains. Evaluation of the toxicity of this derivative in animal models should be considered in future studies. Furthermore, we suggest the study of the expression and performance of other efflux pumps, and comparison of the antibiotic resistance pattern in the clinical strains of *S. aureus*.

## References

- Chizzola R, Michitsch H, Franz C. 2008. Antioxidative properties of *Thymus vulgaris* leaves: comparison of different extracts and essential oil chemotypes. *J Agric Food Chem* 56: 6897-6904.
- Chouhan S, Sharma K, Guleria S. 2017. Antimicrobial activity of some essential oils-present status and future perspectives. *Medicines (Basel)* 4(3): E58. doi: 10.3390/medicines4030058.
- Cohen ML. 1992. Epidemiology of drug resistance: implications for a post-antimicrobial era. *Science* 257: 1050-1055.
- Cosentino S, Tubeerso CIG, Pisano B, Satta M, Mascia V, Arzedi E, *et al.* 1999. In-vitro antimicrobial activity and chemical composition of sardinian *Thymus* essential oils. *Lett Appl Microbiol* 29: 130-135.
- Costa SS, Falcão C, Viveiros M, Machado D, Martins M, Melo-Cristino J, *et al.* 2011. Exploring the contribution of efflux on the resistance to fluoroquinolones in clinical isolates of *Staphylococcus aureus*. *BMC Microbiol* 11: 241.
- Costa SS, Junqueira E, Palma C, Viveiros M, Melo-Cristino J, Amaral L, *et al.* 2013. Resistance to antimicrobials mediated by efflux pumps in *Staphylococcus aureus*. *Antibiotics (Basel)* 2(1): 83-99.
- Couto I, Costa SS, Viveiros M, Martins M, Amaral L. 2008. Efflux-mediated response of *Staphylococcus aureus* exposed to ethidium bromide. *J Antimicrob Chemoth* 62(3): 504-13.
- Czaplewski L, Bax R, Clokie M, Dawson M, Fairhead H, Fischetti VA, *et al.* 2016. Alternatives to antibiotics: a pipeline portfolio review. *Lancet Infect Dis.* 16(2): 239-251.
- Deans SG, Ritchie GA. 1987. Antimicrobial properties of plant essential oils. *Int J Food Microbiol* 5: 165-180.
- De Kievit TR, Parkins MD, Gillis RJ, Srikumar R, Ceri H, Poole K, *et al.* 2001. Multidrug efflux pumps: expression patterns and contribution to antibiotic resistance in *Pseudomonas aeruginosa* biofilms. *Antimicrob Agents Chemother* 45(6): 1761-70.
- Deryabin DG, Tolmacheva AA. 2015. Antibacterial and anti-quorum sensing molecular composition derived from *Quercus cortex* (Oak bark) extract. *Molecules* 20(9): 17093-17108.
- Ding Y, Onodera Y, Lee JC, Hooper DC. 2008. NorB, an efflux pump in *Staphylococcus aureus* strain MW2, contributes to bacterial fitness in abscesses. *J Bacteriol* 190(21): 7123-7129.
- Ghebleh N, Sadat Naghavi N. 2014. Antagonistic activity of bioactive compounds extracted from cyanobacterium *Oscillatoria* isolated from oil refinery waste. *Int J Mol Clin Microbiol* 4 (2): 457-462.
- Gislene GF, Paulo C, Giuliana L. 2000. Antibacterial activity of plant extracts and phytochemicals on antibiotic resistant bacteria. *Braz J Microbiol* 31: 314-325.
- Gohari AR, Saeidnia S, Mollazadeh K, Yassa N, Malmir M, Shahverdi AR. 2010. Isolation of a new quinic acid derivative and its antibacterial modulating activity. *Daru* 18(1): 69-73.
- Grundmann H, Ires-de-Sousa M, Boyce J, Tiemersma E. 2006. Emergence and

- resurgence of methicillin-resistant *Staphylococcus aureus* as a public-health threat. *Lancet* 368: 874-885.
- Heidari Z, Salehzadeh A, Sadat Shandiz SA, Tajdoost S. 2018. Anti-cancer and anti-oxidant properties of ethanolic leaf extract of *Thymus vulgaris* and its bio-functionalized silver nanoparticles. *3 Biotech* 8: 177. <https://doi.org/10.1007/s13205-018-1199-x>
- Huet AA, Raygada JL, Mendiratta K, Seo SM, Kaatz GW. 2008. Multidrug efflux pump overexpression in *Staphylococcus aureus* after single and multiple in vitro exposures to biocides and dyes. *Microbiology* 154(10): 3144-3153.
- Jansen AM, Cheffer JJC, Svendsen AB. 1987. Antimicrobial activity of essential oils: a 1976-1986 literature review. Aspects of test methods. *Planta Med* 40: 395-398.
- Katz OT, Peled N, Yagupsky P. 2004. Evaluation of the current NCCLS guidelines for screening and confirming extended-spectrum beta-lactamase production in isolates of *E. coli* and *Klebsiella* species from bacteremic patients. *Eur J Clin Microbiol Infect Dis* 23: 813-817.
- Kosmidis C, Schindler BD, Jacinto PL, Patel D, Bains K, Seo SM, Kaatz GW. 2012. Expression of multidrug resistance efflux pump genes in clinical and environmental isolates of *Staphylococcus aureus*. *Int J Antimicrob Agents* 40: 204-209.
- Kurlenda J, Grinholc M. 2012. Alternative therapies in *Staphylococcus aureus* diseases. *Cta Biochim Pol* 59(2): 171-184.
- Le Loir Y, Baron F, Gautier M. 2003. *Staphylococcus aureus* and food poisoning. *Genet Mol Res* 2(1): 63-76.
- Li XZ, Nikaido H. 2009. Efflux-mediated drug resistance in bacteria: an update. *Drugs* 69: 1555-1623.
- Matsuda K, Toyoda H, Kakutani K, Hamada M, Ouchi S. 1990. Indole as an Antibacterial Substance against *Pseudomonas solanacearum*, *Agric Biol Chem* 54(11): 3039-3040.
- Marchese A, Orhan IE, Daglia M, Barbieri R, Di Lorenzo A, Nabavi SF, *et al.* 2016. Antibacterial and antifungal activities of thymol: A brief review of the literature. *Food Chem* 210: 402-414
- Mohadjerani M, Naqinezhad A, Aghasizadeh Sharbaf M. 2016. *Platyclusus orientalis* Extracts with Antioxidant Activity from North of Iran. *J Genet Resour* 2 (2): 60-66.
- Moustafa MFM, Alamri SA, Taha TH, Alrumman SA, 2013. In vitro antifungal activity of Argemoneochroleuca sweet latex against some pathogenic fungi. *Afr J Biotechnol* 12(10): 1132-1137.
- Patil A, Jadhov V, 2014. GC-MS analysis of bioactive components from methanol leaf extract of *Toddaliaasiatica* (L.). *Int J Pharm Sci Rev Res* 29(1): 18-20.
- Pourmand MR, Yousefi M, Salami SA, Amini M. 2014. Evaluation of expression of NorA efflux pump in ciprofloxacin-resistant *Staphylococcus aureus* against hexahydroquinoline derivative by real-time PCR. *Acta Med Iran* 52(6): 424-429.
- Saiful AJ, Mastura M, Zarizal S, Mazurah MI, Shuhaimi M, Ali AM. 2008. Efflux genes and active efflux activity detection in Malaysian clinical isolates of methicillin-resistant *Staphylococcus aureus* (MRSA). *J Basic Microbiol* 48(4): 245-251.
- Savjani JK, Gajjar AK, Savjani KT. 2009. Mechanisms of resistance: useful tool to design antibacterial agents for drug-resistant bacteria. *Mini-Rev Med Chem* 9(2): 194-205.
- Saxena G, McCutcheon AR, Farmer S, Towers GHN, Hancock REW. 1994. Antimicrobial constituents of *Rhus glabra*. *J Ethnopharmacol* 42: 95-99.
- Sienkiewicz M, Lysakowska M, Denys P, Kowalczyk E. The antimicrobial activity of thyme essential oil against multidrug resistant clinical bacterial strains. *Microb Drug Resist* 18(2): 137-148.
- Tintino SR, Oliveira-Tintino CD, Campina FF, Silva RL, Costa Mdo S, Menezes IR, *et al.* 2016. Evaluation of the tannic acid inhibitory effect against the NorA efflux pump of *Staphylococcus aureus*. *Microb Pathog* 97: 9-13.
- Truong-Bolduc QC, Dunman PM, Strahilevitz J. Projan SJ, Hooper DC. 2005. MgrA is a multiple regulator of two new efflux pumps

- in *Staphylococcus aureus*. *J Bacteriol* 187: 2395-2405.
- Tukmechi A, Ownagh A., Mohebbat A. 2010. In vitro antibacterial activities of ethanol extract of Iranian propolis (EEIP) against fish pathogenic bacteria (*Aeromonas Hydroohila*, *Yersinia Ruckeri* & *Streptococcus Inae*). *Braz J Microbiol* 41: 1086-1092.
- Wayne PA. 2011. Clinical and laboratory standards Institute. In: Performance standards for antimicrobial susceptibility testing. *21th Inform Suppl* 31(1): M100-S21.
- Yoshida H, Bogaki M, Nakamura S, Ubukata K, Konno M. 1990. Nucleotide sequence and characterization of the *Staphylococcus aureus* *norA* gene, which confers resistance to quinolones. *J Bacteriol* 172: 6942-6949.