

## Inhibitory Effects of Curcumin on the Expression of NorA Efflux Pump and Reduce Antibiotic Resistance in *Staphylococcus aureus*

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One of the important pathogen with increasing resistance rate in hospitalized patients is *Staphylococcus aureus*. The useful results for antimicrobial activity of curcumin made it a proper candidate to enhance the inhibitory effect of some certain antibiotics like ciprofloxacin. The aim of this study was to investigate the inhibitory effects of curcumin on the expression of NorA efflux pump and decrease ciprofloxacin resistance in *Staphylococcus aureus*. One hundred *S. aureus* isolates were acquired from different clinical specimens at the milad hospital (Tehran, Iran). Susceptibility test to ciprofloxacin was done by Kirby-Bauer disk diffusion test and microdilution method, conforming to the CLSI guidelines. Activity of the efflux pump was recognized using CCCP as a chemical efflux pump inhibitor. MIC of curcumin was evaluated with Broth Microdilution method. Bacterial culture was performed near the curcumin and RNA extraction was done. cDNA was synthesized and NorA gene expression was examined by Real-time PCR. The expression of NorA was significantly decreased in this isolated when it was treated with curcumin before RNA extraction compared with absent of curcumin. Our results showed that curcumin can increase ciprofloxacin susceptibility through inhibition of the NorA efflux pump. Combination of curcumin with ciprofloxacin can reduce the antibiotic resistance.

**Keywords:** *Staphylococcus aureus*, curcumin, Antibiotic Resistance, Efflux Pump Inhibitor, NorA.

*Staphylococcus aureus* is the mostly gram-positive isolated bacteria in all of the infections. *S. aureus* has a wide range of infectious diseases, from skin infections to more crucial invasive infections such as abscess formation, suppuration, endocarditis, pneumonia, meningitis, fatal septicemia and bacteremia<sup>1, 2</sup>. The growth of *S. aureus* infections has been associated with hospitalization and immunocompromised

situations<sup>3</sup>. The clinical emphasis of *S. aureus* virulence factors are toxins, enzymes and surface proteins that terminate to rapid development of drug resistance<sup>1</sup>.

On the other hand the wide spread use of antibiotics, developed drug resistance rapidly<sup>2, 3</sup>.

Antibiotic resistance has become an important subject for the therapy of *S. aureus* infections. Resistance can be achieved via drug inactivation, antibiotic target modification or drug export by efflux pumps. *S. aureus* encodes various multidrug resistance efflux pumps<sup>4</sup>. To date more than 10 efflux pumps have been explained for *S. aureus*. Most of them belong to the major

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superfamily (MFS), including NorA, NorB, NorC, MdeA and SdrM (chromosomally encoded) and QacA/B pumps (plasmid-encoded) <sup>5</sup>.

NorA is responsible to export a many type of drugs and chemical substance, such as ethidium bromide, fluoroquinolones, benzalkonium chloride, cetrimide, acriflavine and tetraphenylphosphonium bromide <sup>4</sup>.

NorA was overexpressed in more than half of bloodstream isolates of *S. aureus* <sup>6</sup>. This bacteria is less susceptible to quinolones due to over expression of NorA efflux pump <sup>5</sup>.

NorA has more affinity to hydrophilic fluoroquinolones (ciprofloxacin, norfloxacin, enoxacin) than hydrophobic compounds (sparfloxacin, trovafloxacin, levofloxacin) <sup>7</sup>. The pumps activity can be inhibited by proton gradient (such as carbonyl cyanide m-chlorophenyl hydrazone (CCCP). Inhibition of NorA activity could improve fluoroquinolones acting <sup>7</sup>.

compound antibiotics with efflux pump inhibitors (EPI) probability terminate susceptibility again to antibiotics that before cannot be used. Combination therapy might synergistically enhancement the susceptibility of the bacteria <sup>6</sup>.

In this study, we explain for the first time the potentiating effect of curcumin against expression of NorA gene in *S. aureus*.

Curcumin is a potent natural food-grade from the root of the rhizome *Curcuma longa* with antimicrobial compound <sup>8</sup>. curcumin has multitude biological activities including immunosuppressive activities, antitumor, anti-inflammatory, antioxidant and antimicrobial effects <sup>9</sup>.

Various studies about curcumin have shown the broad-spectrum antimicrobial activity for this compound including antiviral, antifungal, antibacterial and antimalarial activities.

In addition to the extended antimicrobial activity, curcumin has safety property even at high doses (12 g/day) in human, so it was used as a structural specimen to design the new drugs with improve and enhancement antimicrobial activities through the synthesis of different derivatives related to curcumin <sup>10</sup>.

Objective of this study was to investigate the inhibitory effects of curcumin on the expression of NorA efflux pump and decrease ciprofloxacin resistance in *Staphylococcus aureus*.

## MATERIAL AND METHOD

### Bacterial strains and growth conditions

One hundred *S. aureus* isolates were acquired from different clinical samples at the milad hospital (Tehran, Iran); this samples including nasal, sputum, tracheal, bladder and discharge of inpatient and outpatient with ages ranging from infants to aging.

Samples taken and right away transported to the microbiology laboratory of the Mofid hospital, Tehran, Iran. The *Staphylococcus aureus* identification was done according to standard procedures <sup>1</sup>.

Bacteria were stored at -80°C as 20% glycerol stocks and subcultured on nutrient agar plates at 37°C before testing <sup>11</sup>.

*Staphylococcus aureus* (ATCC 25923) was used as a control strain.

### Antimicrobial Susceptibility Testing

Disk diffusion method was used on Muller-Hinton agar (Merck, Germany) to determine resistance or susceptibility to ciprofloxacin, conforming to clinical laboratory standards institute (CLSI, 2015) <sup>10</sup>. Ciprofloxacin disc (CIP: 5µg) from mast company, Merseyside, UK was used. Plates incubation at 37°C for 24 hours. Results have been record and bacterial sensitivity was obtained through measure the diameter of the inhibition zones according to CLSI 2015. The reference strain was American Type Culture Collection strain (ATCC 25923) <sup>11</sup>.

### Minimum Inhibitory Concentration (MIC)

We were re-examined all of the strains by disk diffusion method, through broth microdilution method based on CLSI 2015.

Ciprofloxacin powder was melted in distilled water (16 mg powder in 3ml distilled water). The concentrations for this antibiotics were 5120 µg/mL, during working this solution is diluted 10<sup>10</sup>.

All of the 96-wells of microplate contained 100 µL Müller-Hinton broth. Then we added 100 µL of the antibiotic to the first row of microplate and carried out serial dilution in the form of a column. After making the 0.5 McFarland suspensions, it was diluted 1:20 to attained 5 × 10<sup>6</sup> CFU/mL. 10 µL of this suspension was added into the all of wells. The final concentration was nearly 5 × 10<sup>5</sup> CFU/mL. *Staphylococcus aureus* (ATCC

29213) was used as a control strain <sup>11</sup>.

#### Treatment of the Efflux Pump Inhibitor

To confirm the presence of active efflux pump system, carbonyl cyanide m-chlorophenylhydrazone (CCCP) (from Sigma Aldrich) as an efflux pump inhibitor was added to each of M-H agar plates including 0.5 to 128 µg/mL ciprofloxacin. The terminal concentration of CCCP in the M-H agar was 25 µg/mL <sup>11</sup>. Then again, MIC for ciprofloxacin was determined for second time. For controlling we used a plate containing CCCP without antibiotics. Decrease at least 4 folds of ciprofloxacin MIC after the addition of CCCP, was considered positive result for the existence of active efflux pump in samples <sup>12</sup>. *Staphylococcus aureus* (ATCC 29213) was used as a control strain <sup>11</sup>.

#### Antimicrobial Activity of curcumin

Antimicrobial activity of curcumin was tested against 31 isolated of *S. aureus* that efflux pump involved in their resistance. Curcumin was accounted the amount of material requirements according to the following formula:

$$\text{Weight(mg)} = \{ \text{The total volume(ml)} \beta \text{Density}(\mu\text{g/mL}) \} \div \text{potency}(\mu\text{g/mg})$$

Curcumin (from Sigma Aldrich) is dimethyl sulfoxide 2% (DMSO) soluble and that potency is 650 µg/mg. The Density of 5120 µg/ml was used in this study. MIC method for curcumin is the same as ciprofloxacin except that we did macrodilution for curcumin (instead of microdilution) because we need the subminimal Inhibitory concentration for extraction of RNA and the mass in microdilution is very low. MIC values ranging between <2 and 256 µg ml.

The plates were incubated at 37 °C during 24 h.

At last the lowest concentration of the antibiotics that did not have certain bacterial growth as MIC was considered <sup>11</sup>. Our data revealed that majority of the bacteria were affected by curcumin.

#### RNA Extraction

Genomic RNA was extracted from pure cultures of ciprofloxacin resistance *S. aureus* that they grow in the subminimal inhibitory concentration of curcumin (one lower dilution of the MIC) using RNX-PLUS Kit (Cat. No. RN7713C/EX6101) according to the manufacturer, also the RNA of these samples were extracted in

terms of lack curcumin. The purified RNA was used for creating cDNA.

#### Synthesis of cDNA

cDNA was synthesized using a reverse transcriptase reaction by DNaseI, RNase-free Kit (cat. No: PR891627) according to the manufacturer. Synthesis of cDNA was performed for both groups of RNA (extracted in presence and absence of curcumin). cDNA was used for Real-time PCR. The concentration of cDNA measured by nanodrop.

#### For synthesis of cDNA

We mixed 10 µl RNA, 10 µl 10X reaction buffer with MgCl<sub>2</sub> and 5 µl DNaseI, RNase-free. Then incubate at 37 °C for 30 minutes. After it we added 10 µl 50mM EDTA and incubate at 65 °C for 10 min. RNA hydrolyzes during heating with divalent cations in the absence of a chelating agent. Then used the prepared RNA as a template for reverse transcriptase. We added 10 µl of this RNA to 2 µl random hexamere and 8 µl distilled water. Then did PCR. The program of PCR was 45 °C for 59 minutes, and after that 95 °C for 5 minutes.

#### Analysis of NorA gene expression by Real-time PCR

To determine the effect of curcumin extract on norA gene expression, a real-time PCR assay was performed. The cDNA amplifications were performed using a system with Power SYBR Green PCR Master Mix (YTA) (Both groups of cDNAs). The primer pairs that we were exploit in this study is described in below table 1. *gmk* is a *S. aureus* house keeping gene and it was used as an internal control <sup>2</sup>.

The total volume of materials that used in reactions was 20 µl including 1 µl cDNA as a template 10 µl Power SYBR® Green PCR Master Mix (Applied Biosystems) and 0.5 µl of each F and R primers and 8 µl distilled water.

The qPCR cycling for NorA was performed at 94 °C for 10 min, followed by 40 cycles at 94 °C for 12s and 37s at 57 °C and finally a melting stage (72 °C for 20s) to determine the unspecific PCR product or possible primer dimers. Couple of a negative control were contained in all qPCR runs, and *gmk* gene was used as an internal control. The relative expression of norA efflux pump gene was analyzed using  $\Delta\Delta C_t$  method <sup>13</sup>.

$$\Delta C_t = C_t \text{ house keeping} - C_t \text{ NorA}$$

$$\Delta\Delta C\tau = \Delta C\tau \text{ with curcumin} - \Delta C\tau \text{ without curcumin}$$

$$2_{-(\Delta\Delta C\tau)}$$

**Statistical analysis**

Data from antimicrobial susceptibility tests were analyzed based on the latest published version of CLSI (2015). The relationship between curcumin and reduction of antibiotic resistance in isolates was analyzed by SPSS software version 21 and Pearson Chi-Square test. For all statistical tests, a *P* value of <0.05 was considered meaningful.

**RESULTS**

**Antimicrobial Susceptibility Testing**

Among 100 isolated of *S. aureus*, that tested by disc diffusion method, 31 of them were ciprofloxacin resistance (and intermediate) (31%). Source of samples and susceptibility or resistance of them against ciprofloxacin was shown in tabel2.

Situation, sex and age range of patients who were included in this study and ciprofloxacin susceptibility. were shown in Table 3, 4 and 5 respectively.

**Table 1.** Primers used in this study

Gene	Primer	Primer sequence (52_32)	Product size (bp)	Reference
<i>norA</i>	norA-F	GACATTTACCAAGCCATCAA	102	14
	norA-R	TGCCATAAATCCACCAATCC		
<i>gmk</i> (internal control)	gmk-F	TCAGGACCATCTGGAGTAGGTAAAG	108	14
	gmk-R	TTCACGCATTTGACGTGTTG		

**Table 2.** Source of sample & \* CIP Susceptibility Cross tabulation

		CIP			Total
		S	R	I	
sample	urine	46	11	10	67
	blood	5	2	0	7
	wound	9	1	3	13
	nasal	2	0	0	2
	sputum	2	1	0	3
	trachel	4	2	0	6
	acit	0	1	0	1
	dischargh	1	0	0	1
Total	69	18	13	100	

**Table 3.** Situation & CIP Cross tabulation

		CIP			Total
		S	R	I	
Situation	Out patients	42	8	12	62
	in patients	27	10	1	38
Total		69	18	13	100

Between ciprofloxacin resistant isolates, 13 of them had the activated efflux pump according to CCCP results. The effect of pump Inhibitor on the treatment of efflux pump shown in the following table 6.

**Bacterial Growth Inhibition by curcumin**

In the present survey, we investigated the antibacterial activity of curcumin against ciprofloxacin resistance *S. aureus*. The bacteria were exposed to various dilutions of curcumin and it showed antibacterial activity against *S. aureus* in a dose-dependent manner (table 7).

**Inhibitory Effect of curcumin on efflux pump Gene expression**

**Table 4.** sex & CIP Crosstabulation

		CIP			Total
		S	R	I	
sex	male	29	9	6	44
	famale	40	9	7	56
Total		69	18	13	100

Real-time PCR analysis was performed to examine the effect of curcumin on expression of norA gene in ciprofloxacin resistant *S. aureus*. The expression of NorA was significantly decreased in this isolated (P< 0.05) when it was treated with curcumin extract compared with absent of

**Table 5.** age & CIP Crosstabulation

		Count			Total
		S	R	I	
age	1-5	9	1	2	12
	5-10	0	0	0	0
	10-15	0	0	1	1
	16-20	6	0	1	7
	21-25	8	0	1	9
	26-30	8	1	1	10
	31-35	10	3	1	14
	36-40	3	0	1	4
	41-45	3	3	0	6
	46-50	6	1	1	8
	51-55	3	1	1	5
	56-60	4	2	1	7
	61-65	3	1	0	4
	66-70	2	1	1	4
	71-75	2	1	1	4
	76-80	0	3	0	3
	81-85	1	0	0	1
	86-90	1	0	0	1
total	69	18	13	100	

**Table 7.** Effects of Curcumin on the ciprofloxacin resistance *S. aureus* Isolates that they have activated efflux pmp

Isolate No	Curcumin MIC	SubMIC (for extraction)
160	32	16
113	32	16
133	32	16
164	32	16
22	32	16
128	R	R
68	R	R
85	16	8
161	32	16
54	16	8
104	32	16
43	2	1
90	32	16

curcumin. The Pfafi method was used for analyse of results.

According the results, in more than 82% of sampls, curcumine redused the rate of expression of NorA gene. As well as in more than half of strains NorA expression reduction was more then 10 times in the present of curcumin compare the lack of curcumin. In 18% of samples, decrease was more than 100 times, and this is a great result. Only in 2 of the samples, we have the increase in exprestion of NorA, while in one them increase is very minor and it is negligible.

**Table 6.** Effects of CCCP on the Ciprofloxacin MIC in *S. aureus* Isolates that they have activated efflux pump

Isolate No	Cipro	Cipro+cccip
160	32	4
113	64	8
133	16	< 2
164	64	4
22	32	2
128	32	< 2
68	128	4
85	16	< 2
161	64	4
54	64	2
104	32	2
43	16	< 2
90	128	8

**Table 8.** Results impact of curcumin effects on NorA gene expression by real time PCR

Isolate No	Reduction rate	Increase rate
160	3 times	_____
113	7 times	_____
133	More than 100 times	_____
164	11 times	_____
22	More than 100 times	_____
85	_____	10 times
161	18 times	_____
54	5 times	_____
104	_____	1.4 times
43	12 times	_____
90	11 times	_____

## DISCUSSION

Emergence and extension of antibiotic resistance among bacteria have led to the essential endeavor on the discovery of new antibacterial materials and modulators of antibiotic resistance. There are various mechanisms of antibiotic resistance in *S. aureus*. One of the most principal of them is the efflux pumps, which pull out antibiotics and reduce the intracellular concentration of the antibiotic<sup>13</sup>.

Inhibitors of bacterial resistance make a possibility for the treatment of the patient that they have antibiotic-resistant infections. Using natural inhibitors may improve re-treatment of patients that they used ineffective antibiotics in clinics and could prevent the emergence of new antibiotic resistance strains<sup>15</sup>.

Teow and *et al.* in themselves study tested the synergistic antibacterial activity of curcumin with 8 different antibiotic groups. Disc diffusion assay with Curcumin demonstrated synergism in combination with a majority of antibiotics against *S. aureus*. However, micro dilution assay only showed synergism in three antibiotics i.e. ciprofloxacin, gentamicin and amikacin. Other tested antibiotics showed indifferent interactions however no antagonism was observed<sup>16</sup>, the results of this study is similar ours study, it may be because of the area condition (both of have done in asia) and the same methods.

Mun and *et.* tested the antibacterial activity of curcumin by the broth microdilution method, checkerboard dilution test, and time-kill assay. Antimicrobial activity of curcumin was apperceived against all tested strains. In the checkerboard test, curcumin markedly reduced the MICs of the antibiotics oxacillin, ampicillin, ciprofloxacin and norfloxacin used against MRSA<sup>17</sup>, this study has done in korea, so both of this study and this study were done in same geographical conditions, and the other hand may be the similarity of results is because of same protocols.

Same as our study, Zhou and *et.* showd that curcumin and erythromycin combined treatment noticeably suppressed bacterial growth and substantially alleviated bone infection. Combination of curcumin and erythromycin direction a much stronger efficiency against MRSA induced osteomyelitis in rats than monotherapy<sup>18</sup>.

In different study Wang *et al.* used of curcumin as natural antibacterial and antifungal against various of foodborne pathogens such as *Staphylococcus aureus*, *Escherichia coli*, *Yersinia enterocolitica*, *Bacillus cereus*, *Aspergillus niger* and etc. They used microcapsule of curcumin for improve its stability and solubility. It display broad spectrum inhibitory effect against all organisms by Oxford cup methods. In this study improved that curcumin has more antibacterial activity against Gram-positive bacteria than Gram-negative bacteria. Besides that, its antifungal activity is much higher than antibacterial activity<sup>19</sup>.

Both of above studies have done in china; Because of close relationship between China and Iran, it possible that the source of the tested bacteria being the same.

Gunes *et al.* check out the effect of curcumin against standard bacterial strains in high concentrations and demonstrated the strong antibacterial activity of curcumin at high doses on animal<sup>20</sup> this study has done in turkey, it is possible the similarity of the results be due to the neighboring two countries and the same origine of bacteria strains and same resistance gene.

In Korea Mun *et al.* did a study in the our way, that the result was the same as our result. According to time-kill curves they showed that combination of curcumin and oxacillin decreased the bacterial counts under the lowest detectable limit after 24h. Also, they demonstrate that curcumin reduced the MICs of Oxacillin, ampicillin, ciprofloxacin and norfloxacin<sup>17</sup>.

According to investigations, in all studies curcumin had an antimicrobial effect and no results have been found against this subject.

In another study Hu *et al.* were detected the antimicrobial activity of curcumin against *S. mutans* and check out the inhibitory ability of the curcumin on purified sortaseA by Western-blot and real-time PCR. They improved curcumin can inhibit purified *S. mutans* sortaseA with a half-MIC and it reduce *S. mutans* biofilm formation<sup>21</sup>.

Lzui and *et.* proved Curcumin inhibited the growth of *Prevotella intermedia*, *P. gingivalis*, *Treponema denticola* and *Fusobacterium nucleatum* in a dose-dependent manner. Bacterial development was suppressed near completely at very low concentrations of curcumin<sup>22</sup>.

## CONCLUSION

This study showed that resistance through the norA efflux pump was high and curcumin reduced expression of norA gene and decrease antibiotic resistance.

Also all former investigations have shown the vast antimicrobial activity of curcumin. Curcumin has safety property even at high doses (12 g/day) in human, so it was used as a structural specimen to design the new drugs with improve and enhancement antimicrobial activities through the synthesis of different derivatives related to curcumin. So using curcumin or its derivatives as antibacterial compounds needs further investigations.

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