

Impact of bovine and human serum albumin on Curcumin *in vitro* activity against *Staphylococcus aureus*

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Abstract: This study evaluated the impact of pH (7.4 and 6.5), bovine serum albumin (BSA), and human serum albumin (HSA) on Curcumin activity against 2 reference, 1 clinical, and 10 environmental strains of *Staphylococcus aureus* (*S. aureus*). Minimal inhibitory concentrations (MICs) of Curcumin against *S. aureus* were statistically indifferent ($p > 0.05$) at pH 7.4 and pH 6.5. Activity of Curcumin against *S. aureus* was reduced by two folds in the presence of 1.25-5% BSA/HSA.

Keywords: Curcumin, antibacterial activity, *Staphylococcus aureus*, plasma protein binding, serum albumin.

INTRODUCTION

Turmeric (*Curcuma longa*) is a perennial plant native to tropical South Asia. Its tuberous rhizomes (root-like structures) have been used since ancient times to flavor food, dye cloths, and treat a variety of human ailments. Polyphenolic Curcuminoids give turmeric its characteristic yellow color. Curcumin, the principal Curcuminoid found in turmeric, is attributed for diverse pharmacological properties of turmeric (Zhou *et al.*, 2011; Shen and Ji, 2012; Gupta *et al.*, 2011). Curcumin also possesses broad spectrum activity against Gram-positive and Gram-negative bacteria (Kim *et al.*, 2012; Na *et al.*, 2011; De *et al.*, 2009).

Curcumin binds strongly to bovine serum albumin (BSA) and human serum albumin (HSA) (Sahoo *et al.*, 2007; Barik *et al.*, 2003; Yallapu *et al.*, 2011; Mohammadi *et al.*, 2009). However, the effect of protein binding on Curcumin's pharmacological activities has not been studied. The influence of protein binding on the activity of antimicrobial agents is somewhat controversial. It is however believed that pharmacologic effects of an antimicrobial agent are associated to its unbound concentration at the site of infection (Mittra, 2007; Wolfgang, 2008; Kim *et al.*, 2011). Most *in vitro* antibiotic susceptibility tests are performed with standard microbiological media (Mueller-Hinton broth), without supplementation of serum albumin. However, studies that consider the effect of serum albumin, on efficacies of antimicrobial agents *in vitro* may better forecast *in vivo* drug efficacy.

In preliminary studies, we have found Curcumin most active against Gram-positive bacteria, in particularly *Staphylococcus aureus* (*S. aureus*). In this study, we aim to investigate the effect of BSA and HSA on the *in vitro* activity of Curcumin against 2 reference, 1 clinical, and

10 environmental strains of *S. aureus*.

MATERIAL AND METHODS

Bacterial strains

Ten strains of *S. aureus* were isolated from the environment using BD BBL Chromagar plate *S. aureus* (BBL214982) and serologically confirmed by *S. aureus* Plus Kit (30950102ZL33) from RemelInc, USA. One clinically isolated *S. aureus* strain was obtained from Microbiology Diagnostic Laboratory, Advanced Medical and Dental Institute (AMDI), USM, Penang. Reference *S. aureus* ATCC 25923 (MSSA) and ATCC 43300 (MRSA) were purchased from ATCC. All strains were maintained as glycerol stocks at -80°C and cultured on Mueller Hinton Agar (MHA).

Reagents and chemicals

Curcumin (CB0346) and BSA (AD0023) were from Bio Basic Canada. HSA was from CSL Limited, Australia. Dimethylformamide (DMF) (D4551) was from Sigma-Aldrich. Antibiotic discs Oxacillin (1 μg disc) and Cefoxitin (30 μg disc) were from Oxoid. Antibiotics (Ciprofloxacin, Gentamicin, Vancomycin, Amikacin, Ampicillin, Clindamycin, Erythromycin, Tetracycline, Penicillin, and Fusidic acid) were obtained from Pharmacy, Advanced Medical and Dental Institute, USM, Penang. Curcumin stocks were prepared in neat DMF at 10mg/mL. Mueller Hinton broth was from Hi Media Laboratories. LB Agar (244520) was from BD.

MIC determination

The susceptibility of bacteria towards Curcumin was determined using CLSI-recommended broth micro dilution assay according to M07-A9 guideline (Clinical and Laboratory Standards Institute, 2012). To investigate the effect of pH on Curcumin activity, the media pH was adjusted to pH 7.4 and 6.5 with 1N HCl. To study the effect of BSA and HSA, various concentrations (1.25, 2.5,

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5, and 10%) were added to MHB and pH was adjusted to 7.4.

Overnight bacterial cultures were diluted 10-fold in fresh medium and incubated at 37°C until they reached exponential growth phase. Serial two-fold dilutions of Curcumin in test medium were prepared in a 96-wells microtiter plate (95µL per well). The inoculum (5µL) containing 2×10^7 CFU/mL of test strain was added to wells of microtiter plate. Each plate contained negative (test medium without bacteria + 1% DMF, test medium without bacteria + Curcumin in 1% DMF) and positive (test medium with bacteria + 1% DMF) control wells. The plates were incubated at $36^\circ\text{C} \pm 1^\circ\text{C}$ for 24 hours. Bacterial growth was evaluated by measuring the turbidity at 600nm using µ Quant ELISA Reader (Bio-Tek Instruments, USA). To assay viable bacteria, samples were diluted in phosphate buffered saline (PBS) and plated on LB. Colonies were counted the following day and dose response curves were generated.

STATISTICAL ANALYSIS

All experiments were performed independently in duplicate on three separated occasions. All values are expressed as the mean $\pm$ standard error of mean (SEM). Statistical comparisons were performed using a Student's *t*-test by IBM SPSS Statistics Version 21 software. A *p*-value of less than 0.05 was considered statistically significant.

RESULTS

Antibiotic susceptibility of *S. aureus* strains

The antibiotic susceptibility of 13 *S. aureus* strains were determined according to Standard CLSI Antimicrobial Susceptibility Testing Guideline (M100-S22, CLSI) and listed in table 1. Clinical and environmental strains 1, 3, 4, 5, 7, and 8 were resistant to Ampicillin and Penicillin but susceptible to other antibiotics. Strain 8 was also resistant to Tetracycline. Environmental strain 2 was susceptible to all antibiotics except Tetracycline. ATCC 25923 and environmental strains 6, 9, and 10 showed susceptibility towards all antibiotics. ATCC 43300 (MRSA) was sensitive to Ciprofloxacin, Amikacin, Vancomycin, and Tetracycline but resistant to other antibiotics. Except for ATCC 43300, all other *S. aureus* strains were susceptible to Oxacillin (zone of inhibition 20-25mm- median 22.5mm) and Cefoxitin (zone of inhibition 25-33mm- median 27mm) as determined by the disc diffusion assay.

Effect of pH on antibacterial activity of Curcumin against *S. aureus*

The results of pH effect on Curcumin activity are listed in table 2. Curcumin appeared to be more active against *S. aureus* at pH6.5 compared to pH7.4. The MIC₅₀ of Curcumin against *S. aureus* ranged between 48-88µg/mL (median 62µg/mL) at pH7.4, compared to 38-64µg/mL

(median 46µg/mL) at pH6.5, however the MIC₅₀ difference between two pH points was not statistically significant ($p > 0.05$). The MIC₇₀ of Curcumin against *S. aureus* ranged between 70-168µg/mL (median 84µg/mL) at pH7.4, compared to 50-138µg/mL (median 84µg/mL) at pH6.5. The median MIC₇₀ of Curcumin at two pH points was identical ($p > 0.05$). The MIC₉₀ of Curcumin against *S. aureus* ranged between 120-200µg/mL (median 200µg/mL) at pH7.4, compared to 150-200µg/mL (median 200µg/mL) at pH6.5. The median MIC₉₀ of Curcumin at two pH points was identical ($p > 0.05$). These data showed that Curcumin activity against *S. aureus* was indifferent at pH7.4 and 6.5. All subsequent experiments were thus carried out at pH 7.4, which is a physiologically relevant pH.

Time kill curves of Curcumin

S. aureus strains were exposed to 25, 50, 100, and 200µg/mL Curcumin for 0, 3, 6, 12 and 24 hours. Samples were harvested after each time point and plated to determine the viable cells. Against ATCC 25923 (MSSA), 25µg/mL Curcumin showed bacteriostatic activity within 6 hours (fig. 1A). However, when incubation was extended to 12 and 24 hours, little reduction in CFU count ($p < 0.05$) was observed compared to control (fig. 1A). At 50, 100, and 200µg/mL, Curcumin showed bactericidal activity at 12 (one-log reduction, $p < 0.001$) and 24 hours (2-log reduction, $p < 0.01$) respectively, after which regrowth occurred (fig. 1A).

Compared to ATCC 25923, the reduction in viable counts was lower for the ATCC 43300 (MRSA) when exposed to different Curcumin concentrations (fig. 1B). ATCC 43300 did not multiply for the first 12 hours in the presence of 100 and 200µg/mL Curcumin (fig. 1B). Compared to control, significant reduction of bacteria was observed at 12- ($p < 0.01$) and 24-hour ($p < 0.01$) at 100 and 200µg/mL Curcumin.

Clinical and environmental *S. aureus* isolate's growth was retarded in the presence of 25µg/mL (1.8×10^6 CFU/mL) and 50µg/mL Curcumin (1.48×10^6 CFU/mL) in the first 6 hours as compared to control (7.1×10^6 CFU/mL). At later time points, however, bacterial growth was arrested only at 100 and 200µg/mL Curcumin (fig. 1C). Little bactericidal activity was noted in presence of 100µg/mL (6.63×10^5 CFU/mL, $p < 0.01$) and 200µg/mL (5.03×10^5 CFU/mL, $p < 0.01$) Curcumin at 24 hours post exposure compared to 0 hours (1.1×10^6 CFU/mL). These data suggest that Curcumin exhibits potent antibacterial activity, mainly bacteriostatic against methicillin-sensitive and methicillin-resistant strains.

Effect of serum albumin on Curcumin activity

S. aureus strains were exposed to 100µg/mL Curcumin in presence of 0, 1.25, 2.5, 5 and 10% BSA or HSA in MHB at pH7.4 for 24 hours. As shown in fig. 2A, percent bacterial growth inhibition was significantly ($p < 0.05$)

Table 1: MIC ($\mu\text{g/mL}$) of various antibiotics against 13 *S. aureus* strains

<i>S. aureus</i>	Clin	Amp	Pen	Ery	Gent	Cip	Amik	Fus	Van	Tet
ATCC 25923	0.125 S	0.06 S	0.06 S	0.25 S	0.156 S	0.125 S	0.5 S	1 S	1.25 S	2 I
ATCC 43300	1.5 R	1.25 R	1.25 R	2.5 R	1.25 R	0.3 S	0.75 S	1.5 R	1 S	1 S
Clinical. Isolate	0.156 S	1.0 R	1.25 R	0.25 S	0.25 S	0.375 S	1 S	0.75 S	1 S	2 I
Env 1	0.125 S	1.25 R	0.5 R	0.325 S	0.25 S	0.375 S	1 S	0.5 S	1.5 S	2 I
Env 2	0.125 S	0.06 S	0.06 S	0.25 S	0.25 S	0.25 S	1 S	0.5 S	1 S	2.5 R
Env 3	0.25 S	0.25 R	0.25 R	0.36 S	0.5 S	0.125 S	1 S	1 S	1.25 S	2 I
Env 4	0.25 S	1.0 R	0.5 R	0.25 S	0.06 S	0.25 S	0.5 S	0.5 S	1 S	1 S
Env 5	0.125 S	1.25 R	0.5 R	0.25 S	0.06 S	0.25 S	0.5 S	0.5 S	1 S	1 S
Env 6	0.25 S	0.06 S	0.1 S	0.25 S	0.125 S	0.25 S	0.5 S	0.25 S	1 S	1 S
Env 7	0.25 S	1.0 R	1.0 R	0.25 S	0.25 S	0.25 S	0.75 S	0.5 S	1 S	2 I
Env 8	0.125 S	0.25 R	0.5 R	0.25 S	0.5 S	0.313 S	1 S	0.5 S	1 S	2.5 R
Env 9	0.156 S	0.1 S	0.06 S	0.125 S	0.125 S	0.25 S	0.5 S	0.5 S	0.5 S	1 S
Env 10	0.125 S	0.06 S	0.1 S	0.25 S	0.25 S	0.25 S	1 S	0.5 S	1 S	1.5 I

Antibiotic susceptibility was determined according to Standard CLSI Antimicrobial Susceptibility Testing Guideline (Version M100-S22) Env-environmental strain, Clin - Clindamycin, Amp-Ampicillin, Pen-Penicillin, Ery-Erythromycin, Gent-Gentamicin, Cip-Ciprofloxacin, Amik-Amikacin, Fus-Fusidic acid, Van-Vancomycin, Tet-Tetracycline, S-Susceptible, R-Resistant, I-Intermediate.

Table 2: MIC₅₀, MIC₇₀ and MIC₉₀ of Curcumin against *S. aureus* at pH7.4 and pH 6.5

<i>S. aureus</i>	MIC ₅₀ ($\mu\text{g/mL}$)		MIC ₇₀ ($\mu\text{g/mL}$)		MIC ₉₀ ($\mu\text{g/mL}$)	
	pH 7.4	pH 6.5	pH 7.4	pH 6.5	pH 7.4	pH 6.5
ATCC 25923	48 $\pm$ 1.67	38 $\pm$ 1.93	70 $\pm$ 4.77	50 $\pm$ 3.54	187 $\pm$ 4.45	150 $\pm$ 2.37
ATCC 43300	67 $\pm$ 2.61	50 $\pm$ 1.17	88 $\pm$ 2.62	87 $\pm$ 2.01	200 $\pm$ 4.04	>200
Clinical isolate	65 $\pm$ 3.63	63 $\pm$ 0.45	81 $\pm$ 1.51	104 $\pm$ 2.89	120 $\pm$ 3.11	>200
Env 1	62 $\pm$ 3.42	50 $\pm$ 4.92	100 $\pm$ 2.08	138 $\pm$ 1.44	>200	>200
Env 2	88 $\pm$ 1.35	56 $\pm$ 3.77	168 $\pm$ 1.33	84 $\pm$ 1.01	>200	>200
Env 3	57 $\pm$ 3.32	44 $\pm$ 1.19	79 $\pm$ 0.41	81 $\pm$ 1.39	>200	>200
Env 4	52 $\pm$ 2.92	39 $\pm$ 3.20	75 $\pm$ 0.18	103 $\pm$ 4.02	>200	>200
Env 5	52 $\pm$ 2.55	39 $\pm$ 0.46	71 $\pm$ 4.95	83 $\pm$ 2.11	>200	>200
Env 6	53 $\pm$ 4.88	64 $\pm$ 1.32	80 $\pm$ 6.00	82 $\pm$ 0.98	>200	168 $\pm$ 2.86
Env 7	57 $\pm$ 3.49	45 $\pm$ 2.92	84 $\pm$ 2.02	100 $\pm$ 0.73	>200	>200
Env 8	63 $\pm$ 0.12	63 $\pm$ 1.55	90 $\pm$ 1.65	94 $\pm$ 1.12	189 $\pm$ 5.40	>200
Env 9	69 $\pm$ 2.05	46 $\pm$ 3.17	88 $\pm$ 3.61	69 $\pm$ 5.41	>200	189 $\pm$ 2.63
Env 10	82 $\pm$ 1.08	40 $\pm$ 3.33	139 $\pm$ 3.16	56 $\pm$ 4.73	>200	180 $\pm$ 1.81

Env-environmental strain.

affected in presence of 1.25% BSA (median 43.15 $\pm$ 8.13%) compared to 0% BSA (median 79.52 $\pm$ 9.23%).

As shown in fig. 2A, percent bacterial growth inhibition was significantly ($p < 0.05$) affected in presence of 1.25% BSA (median 43.15 $\pm$ 8.13%) compared to 0% BSA (median 79.52 $\pm$ 9.23%). The effect on percent growth inhibition was maximal in presence 2.5% BSA (median 36.727 $\pm$ 10.72%) and 5% BSA (38.852 $\pm$ 8.59%). This data suggests that Curcumin-BSA binding reached to saturation at 2.5-5% BSA. There was no significant difference of the effect of 1.25-5% BSA on percent growth inhibition. Surprisingly, Curcumin-mediated growth inhibition was least affected in presence of 10% BSA (median 60.865 $\pm$ 8.57%) compared to 1.25-5% BSA.

Similar trend was obtained for HSA as shown in fig. 2B. Percent growth inhibition was most affected in presence of 2.5% HSA, followed by 5% and 1.25% of HSA. There was no significant difference of the effect of 1.25-5% BSA on percent growth inhibition. Similar to BSA, Curcumin-mediated growth inhibition was least affected in presence of 10% HSA (median 67.23 $\pm$ 8.89%). In 10% HSA, percent growth inhibition was identical to 0% BSA (median 76.10 $\pm$ 7.53%).

DISCUSSION

Curcumin is reportedly more stable at pH6.5 (Wang et al., 1997) and unstable at neutral and alkaline pH, where it rapidly undergoes auto-oxidation (Gordon and Schneider, 2012). Curcumin has been tested for its antibacterial and antifungal activities at acidic pH (Rai et al., 2008; Khalil

et al., 2012). However, Curcumin has not been evaluated for its antimicrobial activities at two pH points (pH7.4 and 6.5) in parallel. Here, we have assessed the activity of Curcumin against 13 *S. aureus* strains at pH7.4 and pH6.5 in parallel. We demonstrated that the antibacterial effect of Curcumin was identical at two different pH.

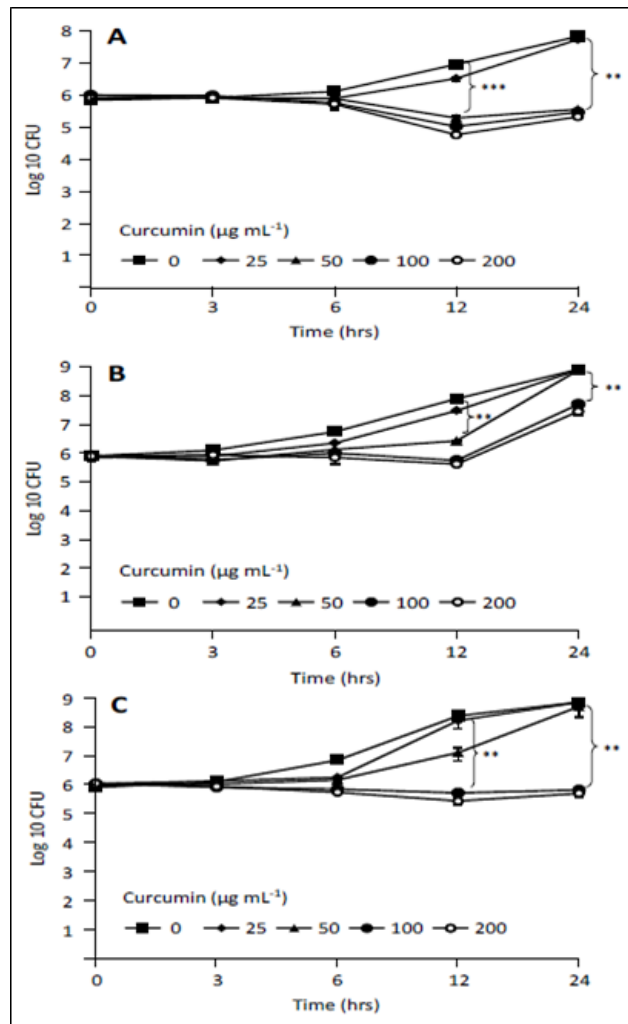


Fig. 1: Time kill curves of Curcumin against *S. aureus* strains. (A) Reference ATCC 43300 (MRSA); (B) ATCC 25923 (MSSA); (C) 1 clinical and 10 environmental strains were exposed to 25, 50, 100 and 200 µg/mL Curcumin for 0, 3, 6, 12, and 24 hours. Samples were harvested after each time point and plated to determine the viable cells. *** $p < 0.001$; ** $p < 0.01$.

In addition to the standard broth microdilution method, we have assessed the antibacterial activities of Curcumin at time-response fashion using a plate-counting method. We showed that Curcumin exhibits potent antibacterial activity, mainly bacteriostatic against methicillin-sensitive and methicillin-resistant strains, consistent to the previous studies (Mun *et al.*, 2013; Moghadamtousi *et al.*, 2014). In the present study, serum albumins significantly affect the antibacterial action of Curcumin against *S. aureus*

strains. Curcumin has been reported to bind to BSA and HSA (Sahoo *et al.*, 2007; Barik *et al.*, 2003; Yallapu *et al.*, 2011; Mohammadi *et al.*, 2009), which explains the reduction of antibacterial effect. Unbound drug molecules can more readily and efficiently traverse through the plasma membrane of a cell (Berginc *et al.*, 2012; Wan *et al.*, 2012).

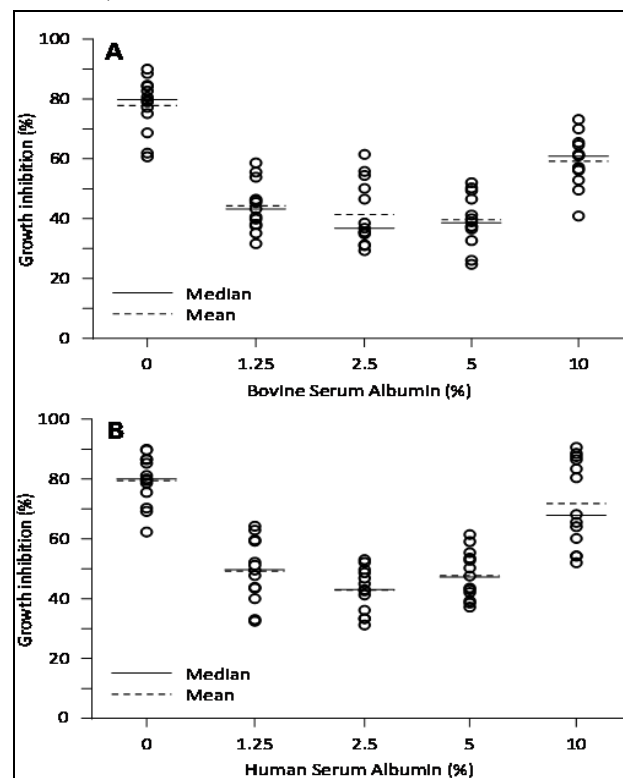


Fig. 2: Effect of BSA and HSA on Curcumin-mediated growth inhibition of *S. aureus* strains. Bacteria were exposed to 100 µg/mL Curcumin in presence of 0, 1.25, 2.5, 5 and 10% (A) BSA or (B) HSA in MHB at pH7.4 for 24 hours. Both BSA and HSA significantly affected Curcumin-mediated growth inhibition of *S. aureus* strains at 1.25-5%. Highest effect was noticed at 2.5% BSA/HSA.

CONCLUSION

The studies presented herein demonstrate that while Curcumin possesses similar activity at pH7.4 and 6.5, its bacteriostatic activity against *S. aureus* is reduced in the presence of physiologically relevant levels of BSA and HSA. However, assessment with culture media containing only serum albumin (bovine or human) is a crude approximation of the physiological state. It would be required to assess the activity of Curcumin in human plasma or whole blood with its range of proteins/components that may exert more subtle effects on the activity (experiments under progress). Further studies investigating the effect(s) of serum albumin, human plasma, and whole blood on the other biological activities of Curcumin would be desirable. Curcumin derivatives

with reduced affinity for plasma proteins may enhance Curcumin bioavailability.

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