Synergistic antibacterial activity of Curcumin with antibiotics against *Staphylococcus aureus*

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Abstract: This study evaluated the synergistic antibacterial activity of Curcumin with 8 different antibiotic groups. Two reference, one clinical and ten environmental strains of *Staphylococcus aureus* (*S. aureus*) were tested. Disc diffusion assay with 25µg/mL Curcumin demonstrated synergism in combination with a majority of tested antibiotics against *S. aureus.* However, checkerboard micro dilution assay only showed synergism, fractional inhibitory concentration index (FICI) <0.5 in three antibiotics i.e. Gentamicin, Amikacin, and Ciprofloxacin. Other antibiotics showed indifferent interactions but no antagonism was observed. In time-kill curve, appreciable reduction of bacterial cells was also observed in combination therapy (Curcumin + antibiotics) compared to monotherapy (Curcumin or antibiotic(s) alone). The antibiotics with higher synergistic interaction with Curcumin are arranged in a decreasing order: Amikacin > Gentamicin > Ciprofloxacin.

Keywords: Curcumin; antibacterial; *Staphylococcus aureus*; synergism; antibiotics.

INTRODUCTION

Turmeric (*Curcuma longa*) is a perennial plant native to tropical South Asia. Its tuberous rhizome (root-like structures) has been used traditionally to flavor food, dye cloths and treat a variety of human ailments (Goel *et al*., 2008; Gupta *et al*., 2011). Polyphenolic curcuminoids give turmeric its characteristic yellow color and Curcumin, the principle curcuminoid found in turmeric, is well-known for its multiple pharmacological and biological properties (Gupta *et al*., 2011; Zhou *et al*., 2011; Shen and Ji, 2012). Recently, its potential in anti-cancer therapy has been highlighted and actively explored (Teiten *et al*., 2010). Curcumin also possesses potent anti-bacterial activity against a wide range of bacteria (Kim *et al*., 2012; Na *et al*., 2011; De *et al*., 2009). Interestingly, its synergistic activities with various anti-microbial drugs have also been reported (Sharma *et al*., 2009, 2010).

Enhancement of antibacterial activity of Curcumin against *S. aureus* has been reported using disc diffusion method (Moghaddam *et al*., 2009) and more recently by broth micro dilution method (Mun *et al*., 2013). However, assays using these methods have never been performed in parallel against an identical group of antibiotics and bacterial strains. Such variations in experimental design can significantly alter antibacterial activities. Here, we studied the combination effect of Curcumin with eight different groups of antibiotics (amino glycosides, β-lactams, quinolones, glycopeptides, macrolides, lincosamides, tetracyclines and fusidic acid) against two reference strains, one clinical and ten environmental isolates of *S. aureus* using modified disc diffusion assay and validated by checkerboard micro dilution assay.

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MATERIALS AND METHODS

Bacterial isolates

Ten strains of *S. aureus* were isolated from the environment using BD BBL Chromagar plate *S. aureus* (BBL214982, USA) and serologically confirmed by *S. aureus* Plus Kit (30950102ZL33, Remel Inc, 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) from Merck, Germany.

Reagents and chemicals

Curcumin (CB0346) was from Bio Basic Canada and DMF (D4551) was from Sigma-Aldrich. Antibiotic discs (Amikacin 30µg, Gentamicin 10µg, Ampicillin10µg, Penicillin 10µg, Ciprofloxacin 5µg, Vancomycin 30µg, Erythromycin 10µg, Clindamycin 2µg, Tetracycline 30µg, and Fusidic acid 10µg) were from Oxoid, UK. Antibiotics (Ciprofloxacin, Gentamicin, Vancomycin, Amikacin, Ampicillin, Clindamycin, Erythromycin, Tetracycline, Penicillin and Fusidic acid) were obtained from Pharmacy, AMDI, USM, Penang. Müeller Hinton broth (MHB) was from Hi Media Laboratories, India and LB Agar (244520) was from BD.

Disc diffusion assay

Assay was performed on MHA plates against two ATCC reference strains and one clinical isolate. For combination experiments, Curcumin stocks were prepared in absolute ethanol at 2.5mg/mL (at sub-inhibitory concentration of 12.5µg/mL and 25µg/mL) was added into the 40^{o}C agar before pouring plates. 1x10^{7}CFU/mL of test strain in

phosphate buffered saline (PBS) were spread onto the plates followed by placement of antibiotic discs. After incubation at 37°C for 24 hrs, zones of inhibition were measured and percentages increase in zone area was calculated as $(b^2-a^2)/a^2x100$ where a and b are the inhibition zones of antibiotic alone and antibiotic plus Curcumin, respectively (Moghaddam *et al*., 2009). Assays were performed in triplicate and the standard deviations calculated.

Checkerboard micro dilution assay

MICs were determined using broth micro dilution method according to M07-A9 guideline (Clinical and Laboratory Standards Institute, 2012). Overnight bacterial cultures were diluted 10-fold in fresh medium and incubated at 37°C until they reached exponential growth phase. Two-fold serial dilutions of an antibiotic were tested in combination with sub-inhibitory concentration of Curcumin (25µg/ml in DMF) in a 96-wells microtiter plate (95µL per well). The inoculum (5µL) containing 1 x 10^7CFU/mL of test strain was added to wells of microtiter plate. The plates were incubated at 36°C±1°C for 24 hours. Bacterial growth was measured at 600 nm using µ Quant ELISA Reader (Bio-Tek Instruments, USA). To assay viable bacteria, samples were diluted in PBS and plated on LB agar at 0, 3, 6, 12 and 24 hours. Plates were incubated at 37°C and colonies were counted the following day and dose-response curves were generated. The experiments were repeated at-least three times in duplicates for each strain. The combination effects were evaluated by the sum of fractional inhibitory concentration indices (FICIs) of two compounds (Curcumin and antibiotic) in combination (FIC_a $+FIC_b=FIC_I$). FIC_a was calculated as the MIC of compound a in combination, divided by the MIC of compound a alone whereas FIC_b was calculated as the MIC of compound b in combination, divided by the MIC of compound b alone. The types of effects were classified as follows: FICI ≤0.5 =synergistic; FICI 0.6-0.9 =additive; FICI 1.0-3.9=indifferent; and FICI>4.0= antagonistic.

STATISTICAL ANALYSIS

All experiments were performed independently in duplicate on at-least three separated occasions. All values are expressed as the mean ± standard error of mean (SEM). Statistical comparisons on time-kill assay and combination effect by discs method were performed using a Student's *t*-test by IBM SPSS Statistics 21 software. A P-value of less than 0.05 was considered statistically significant.

RESULTS

Synergism by disc diffusion assay

In vitro antibacterial activity of standard antibiotics alone and in combination with Curcumin was tested against two ATCC strains and one clinical strain. Sub-inhibitory concentration of Curcumin, 12.5µg/mL and 25µg/mL were used in this assay. According to CLSI guidelines, ATCC 25923 was susceptible to all tested antibiotics whereas ATCC 43300 was resistant to aminoglycosides (Gentamicin), β-lactams (Ampicilin and Penicillin), macrolides (Erythromycin), and lincosamides (Clindamycin). The clinical strain found resistant to β-lactams, but susceptible to other antibiotics.

Table 1 shows the combination effects against all tested strains. In the presence of 12.5µg/mL Curcumin, antibacterial activities of all antibiotics against ATCC 25923 were enhanced ranging from 1.6% to 18.8%. Curcumin at 25µg/mL further enhanced the activities from 4.7% to 35.8%. Highest combination activity was seen for Tetracycline at both concentrations of Curcumin, but the difference was not statistically significant (P>0.05). In contrast, antibacterial activity in combination with 25µg/mL Curcumin was significantly higher than with 12.5µg/mL Curcumin in Gentamicin, Penicillin, Ciprofloxacin, Clindamycin and Fusidic acid. For ATCC 43300, no combination effect was observed in Ampicillin, Penicillin, Erythromycin and Clindamycin. Highest combination activity was seen for Gentamicin, 35.7% and 64.3% at 12.5µg/mL and 25µg/mL Curcumin, respectively.

The synergistic activity of Curcumin was prominent against the clinical strain. In the presence of 12.5µg/mL Curcumin, 7.3% to 10.7% of enhanced activity was observed for tested antibiotics. No combination effect was seen for Ampicillin, Ciprofloxacin, Erythromycin and Clindamycin. Curcumin at 25µg/mL enhanced the anti-bacterial effect of antibiotics from 12.1% to 49.4%. The highest synergistic effect was seen for Vancomycin followed by Ciprofloxacin and Tetracycline.

Synergism by checkerboard micro dilution assay

To validate the disc diffusion assay results, checkerboard assays were performed on two reference strains (Table 2) to ensure the reproducibility and to evaluate the variation of methods if any. The combination effect of Curcumin and 10 antibiotics were categorized into 4 different classes: synergistic, additive, indifferent and antagonistic based on the FICI. In ATCC 25923, synergistic effect was observed for Ciprofloxacin (FICI 0.5) and additive activity was seen for Gentamicin (FICI 0.8). Against the ATCC 43300, synergistic activity was seen for both antibiotics i.e. Gentamicin and Ciprofloxacin with FICI of 0.4 and additive activity was observed for Amikacin (FICI 0.7) and Fusidic acid (FICI 0.8).

To explore further whether the observed synergistic and additive effects of Curcumin against two ATCC reference strains can be extended to other *S. aureus* isolates, similar experiments were performed on ten environmental and one clinical isolate of *S. aureus* (Table 3).

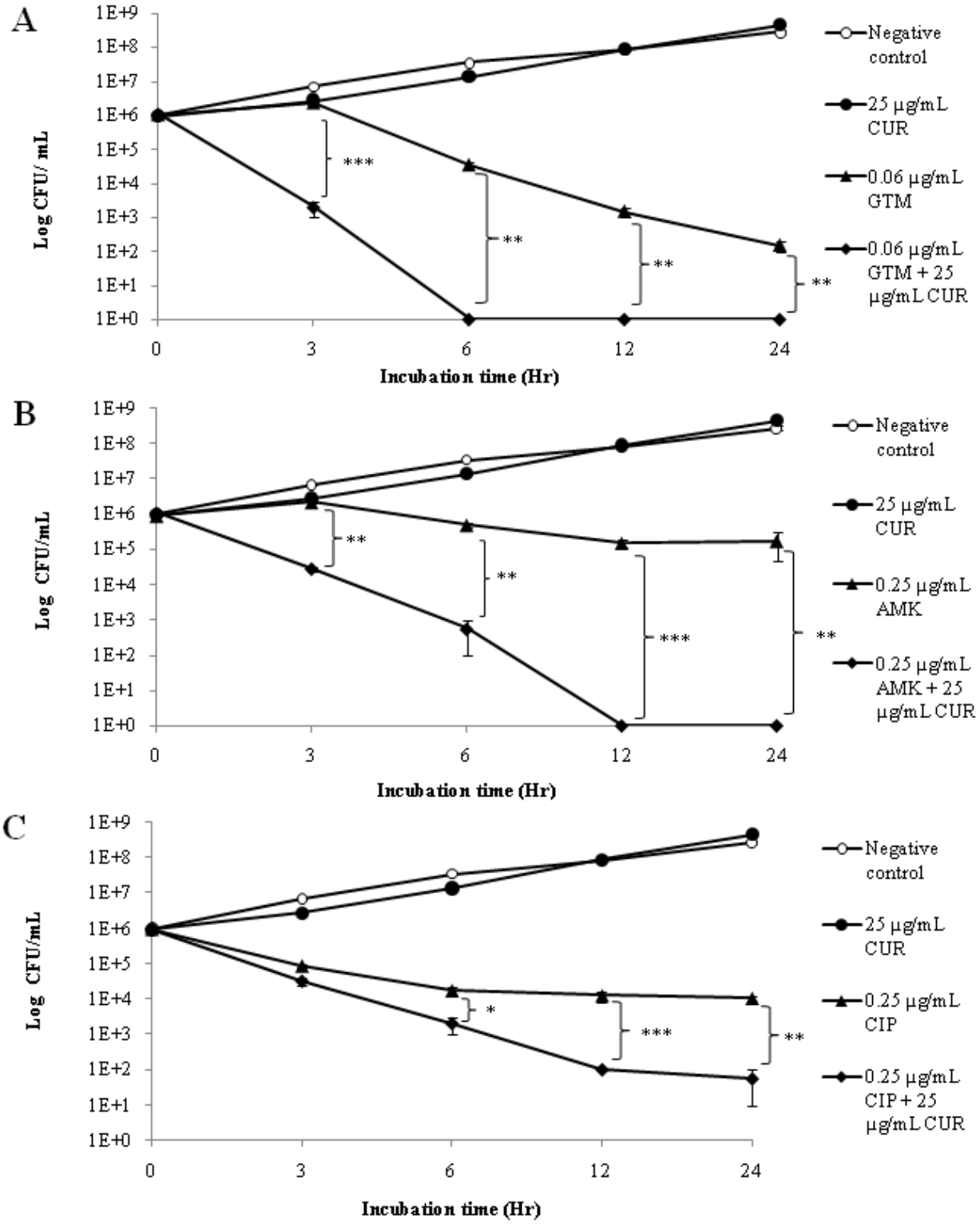

Fig. 1: Time kill curves of Curcumin (CUR) and antibiotics against ATCC 25923 (MSSA) strain. (A) Gentamicin (GTM). (B) Amikacin (AMK). (C) Ciprofloxacin (CIP). At sub-inhibitory concentration of Curcumin and antibiotics, synergism was seen within six hours post-exposure. Data are reported as the mean and SEM from three independent experiments (***P<0.001, **P< 0.01, *<0.05).

Table 1: Combination effect of 12.5µg/mL and 25µgmL Curcumin against two ATCC reference strains (MSSA 25923 and MRSA 43300) and one clinical strain by discs method

Antibiotics (µg/disc)	Increase in fold area (%)					
	MSSA		MRSA		*S. aureus*	
	ATCC 25923		ATCC 43300		Clinical strain	
	12.5µg/mL	25µg/mL	12.5µg/mL	25µg/mL	12.5µg/mL	25µg/mL
Amikacin (30)	5.4±1.7	13.8±1.8	21.7±3.0	45.8±6.6*	7.8±0	33.1±0***
Gentamicin (10)	7.2±0.1	9.6±3.1***	35.7±10.5	64.3±1.5***	7.3±0	30.6±0***
Ampicilin (10)	1.6±1.0	11±1.1	0	0	0	14.8±0***
Penicillin (10)	0	7.7±1.0***	0	0	10.7±3.4	27.4±3.6
Ciprofloxacin (5)	0	4.7±1.5***	0	4.8±3.0***	0	44.1±2.1***
Vancomycin (30)	9.2±2.9	14.1±5.2	23.3±6.6	32.4±3.7*	8.9±5.6	49.4±5.6
Erythromycin (10)	2.2±1.4	10.9±2.3	0	0	0	12.1±0***
Clindamycin (2)	0	10.9±2.3**	0	0	0	12.1***
Tetracycline (30)	18.8±3.6	35.8±3.1	4.7±1.5	14.1±0.1***	9.6±1.6	40.8±2.1
Fusidic acid (10)	2.4±1.5	9.9±3.1*	4.7±1.5	16.8±1.6	0	33.6±1.6***

Data are reported as the mean and SEM from three independent experiments (***P<0.001, **P< 0.01, *<0.05).

Table 2: Checkerboard assay of 10 antibiotics and 25µg/mL Curcumin against 2 ATCC reference strains (MSSA 25923 and MRSA 43300)

Antibiotics	MIC_{100} of each agent (µg/mL)							
	MSSA ATCC 25923				MRSA ATCC 43300			
	Alone	Combination	FICI*	Outcome	Alone	Combination	FICI*	Outcome
Amikacin	0.5	0.5	1.0	Indifferent	0.75	0.5	0.7	Additive
Gentamicin	0.156	0.125	0.8	Additive	1.25	0.5	0.4	Synergistic
Ampicilin	0.06	0.06	2.0	Indifferent	1.25	1.5	1.2	Indifferent
Penicillin	0.06	0.06	2.0	Indifferent	1.25	1.5	1.2	Indifferent
Ciprofloxacin	0.125	0.06	0.5	Synergistic	0.3	0.125	0.4	Synergistic
Vancomycin	1.25	1.25	1.0	Indifferent	1	1.5	1.5	Indifferent
Erythromycin	0.25	0.375	1.5	Indifferent	2.5	2.75	1.1	Indifferent
Clindamycin	0.125	0.15	1.2	Indifferent	1.5	2	1.3	Indifferent
Tetracycline	2	2	1.0	Indifferent	1	1.5	1.5	Indifferent
Fusidic acid	1	1.5	1.5	Indifferent	1.5	1.25	0.8	Additive

FICI* - the sum of fractional inhibitory concentration index MIC_{100} of Curcumin was 250µg/mL as previously determined.

Median of MIC_{100} of each antibiotic alone and in combination with Curcumin was determined to individually calculate the FICI. Median of FICI was then determined to classify the outcome of effect of each antibiotic. In combination with Curcumin, Amikacin (FICI 0.5), Gentamicin (FICI 0.25) and Ciprofloxacin (FICI 0.5) showed synergistic effects against these strains. In the tested antibiotics alone, MIC_{100} of Amikacin, Gentamicin, and Ciprofloxacin were 1µg/mL, 0.25µg/mL, and 0.25µg/mL, respectively. When tested in combination with Curcumin, these were reduced to 0.5µg/mL, 0.06µg/mL and 0.125µg/mL. Additive effect was observed for tetracycline (FICI 0.76). Other drugs exhibited indifferent interactions and no antagonistic interaction was found.

Time-kill assay

Synergistic activities of Gentamicin, Amikacin and Ciprofloxacin (previously shown by the disc diffusion and broth dilution assays) were examined in a multi-time point assay and the time-response curves were plotted as shown in fig. 1. Bacteria continued to multiply in the presence of 25µg/mL Curcumin just as they did in the control (no Curcumin or antibiotics) cultures. Bacteria were inhibited to various degrees in the presence of antibiotics.

When treated with Gentamycin at 0.06µg/mL, the starting inoculum of $1x10^6$ CFU/mL reduced to $2x10^2$ CFU/mL after 24h exposure. However when the bacteria were exposed to Gentamycin + Curcumin, 100% inhibition was achieved within 6 hours of exposure. At the same time point, $3.4x10^4$ bacteria were still surviving in cultures that were treated with Gentamicin alone. The Synergistic interaction was observed within 3-hour exposure where the bacteria were reduced to $0.4x10^3$ CFU/mL. The differences were statistically significant (P<0.001).

At sub-inhibitory concentration of Amikacin (0.25µg/mL), no absolute-killing of *S. aureus* was

Table 3: Interaction of 10 different antibiotics and 25µg/mL Curcumin against 10 environmental isolates and 1 clinical isolate of *Staphylococcus aureus* by checkerboard micro dilution assay

Amikacin and Curcumin			Gentamicin and Curcumin			Ampicillin and Curcumin			Penicillin and Curcumin		
MIC_{100} (µg/mL)			MIC_{100} (µg/mL)			MIC_{100} (µg/mL)			MIC_{100} (µg/mL)		
Drug	Median	Range	Drug	Median	Range	Drug	Median	Range	Drug	Median	Range
AMK	1.00	0.50-1.00	GTM	0.25	0.06-0.50	AMP	0.25	0.06-1.25	PNC	0.50	0.06-1.25
CUR	250	250-300	CUR	250	250-300	CUR	250	250-300	CUR	250	250-300
COM*	0.50	0.25-0.50	COM*	0.0625	0.03-0.25	COM*	0.25	0.125-1.50	COM*	0.25	0.06-1.25
FICI	0.50	0.25-1.00	FICI	0.25	0.12-1.00	FICI	1.26	0.50-2.50	FICI	1.00	0.60-2.08
Outcome	Synergistic		Outcome	Synergistic		Outcome	Indifferent		Outcome	Indifferent	

Ciprofloxacin and Curcumin			Vancomycin and Curcumin			Erythromycin and Curcumin		
MIC_{100} (µg/mL)			MIC_{100} (µg/mL)			MIC_{100} (µg/mL)		
Drug	Median	Range	Drug	Median	Range	Drug	Median	Range
CIP	0.25	0.125-0.375	VCM	1.00	0.50-1.50	ERT	0.25	0.125-0.36
CUR	250	250-300	CUR	250	250-300	CUR	250	250-300
COM*	0.125	0.125-0.375	COM*	1.00	0.50-1.50	COM*	0.38	0.1875-0.50
FICI	0.50	0.33-2.00	FICI	1.00	0.50-2.00	FICI	1.50	1.16-2.00
Outcome	Synergistic		Outcome	Indifferent		Outcome	Indifferent	

Clindamycin and Curcumin			Tetracycline and Curcumin			Fusidic acid and Curcumin		
MIC_{100} (µg/mL)			MIC_{100} (µg/mL)			MIC_{100} (µg/mL)		
Drug	Median	Range	Drug	Median	Range	Drug	Median	Range
CLD	0.16	0.125-0.25	TTC	2.00	1.00-2.50	FSD	0.50	0.25-1.00
CUR	250	250-300	CUR	250	250-300	CUR	250	250-300
COM*	0.15	0.125-0.50	COM*	1.50	0.50-2.00	COM*	0.75	0.25-1.00
FICI	1.00	0.50-2.00	FICI	0.76	0.51-1.51	FICI	1.50	0.67-3.00
Outcome	Indifferent		Outcome	Additive		Outcome	Indifferent	

COM* - combination of Curcumin with antibiotics. FICI - the sum of fractional inhibitory concentration index.

achieved but the starting inoculum ($1x10^6$CFU/mL) was reduced to $1.7x10^4$CFU/mL after 24 hours (Fig. 1B). However, *S. aureus* was completely inhibited at 12 hour in the presence of 25µg/mL Curcumin ($P<0.001$). This data suggested the enhancing activity of Curcumin on anti-bacterial effect of Amikacin. The synergistic interaction was observed after 3-hour incubation time where there were $0.6x10^2$ CFU/mL differences ($P<0.01$) of bacteria seen in Amikacin alone and in combination. At sub-inhibitory concentration of Ciprofloxacin (0.25µg/mL), no absolute-killing of *S. aureus* was achieved (fig. 1C). The starting inoculum ($1x10^6$CFU/mL) was reduced to $1.1x10^4$ CFU/mL after 24 hours. In the presence of 25µg/mL Curcumin, more prominent killing was observed. The synergistic interaction was observed after 6-hour incubation time where there were $1.55x10^4$ CFU/mL differences ($P<0.05$) of bacteria seen in Ciprofloxacin alone and in combination. At 24 hour, the bacteria were reduced to 50CFU/mL compared to ciprofloxacin alone ($1.1x10^4$ CFU/mL) ($P<0.01$).

DISCUSSION

The present data suggested that the synergism of Curcumin highly depends on the *S. aureus* strains with varied antibiotics susceptibility profiles. The mechanism of Curcumin synergistic activity is somehow associated with the mechanism of antibiotic resistance presented by bacterial strains. Majority of antibiotics exhibited indifferent interactions in combination with Curcumin but no antagonism was observed. There was an insignificant variation between both set of results (disc diffusion and broth micro dilution), mostly due to the method of result interpretation. Overall, both methods produced consistent results. For instance, results from disc diffusion method showed the statistically high combination effect ($P<0.001$) of Ciprofloxacin and Gentamicin with 25µg/mL Curcumin against MSSA, MRSA and the clinical strain (table 1), which were also interpreted as synergism in broth micro dilution assay. *Vice versa*, antibiotics that showed synergistic and additive effect in broth assay, also exhibited high combination effects in

disc diffusion assay. Comparatively, disc diffusion assay is less laborious and less prone to errors whereas broth micro dilution assay is more error-prone in the interpretation. Note worthily, the results might also vary due to the difference of experimental design as the bacteria mobility was restricted in agar method compared to liquid culture. This may significantly affect the growth pattern of bacteria thus making the direct comparison not possible.

Appreciable synergisms were observed in amino glycosides (Gentamicin and Amikacin) and quinolones (Ciprofloxacin). Interactions of Curcumin with multiple targets in bacteria including a wide variety of enzymes (Zhou *et al*., 2011; Lin, 2007) and its inhibition to FtsZ assembly (Singh and Panda, 2010; Rai *et al*., 2008) are possible explanations for these synergistic interactions Curcumin might possibly reduce the hydrolyzation or lysis of the drugs by interacting with bacterial enzymes to maximize the bactericidal effects (Zhou *et al.,* 2011). Aminoglycosides bind ribosomal target and block protein synthesis. Curcumin might enhance its antibacterial effect by assisting in this mechanism such as interacting with those ribosomal targets to facilitate the machinery blocking of protein. Quinolones interferes with bacterial enzymes, which synthesize DNA and protein. Identically, Curcumin may also enhance the inhibitory activity of quinolones by interacting to destroy the enzymes that are responsible of protein synthesis.

CONCLUSIONS

Curcumin enhances the antibacterial activities of antibiotics, markedly Gentamicin, Amikacin and Ciprofloxacin. The synergism was shown by both disc diffusion and broth micro dilution methods with the latter one yielded more sensitive results.

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