Altered antibacterial activity of Curcumin in the presence of serum albumin, plasma and whole blood

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Abstract: Antibacterial effect is one of the major therapeutic activities of plant-derived Curcumin. This work evaluated the effect of serum albumin, human plasma, and whole blood on the *in vitro* activity of Curcumin against eight clinical bacterial isolates by standard broth micro dilution and plate-counting methods. Toxicological effects of Curcumin towards human red blood cells (RBCs) and peripheral blood mononuclear cells (PBMCs) were also investigated. Curcumin exhibited weak activity against gram-negative bacteria, except *Escherichia coli* and *Shigella flexneri* were susceptible and was most active against gram-positive bacteria: *Staphylococcus aureus*, *Streptococcus pyogenes* and *Enterococcus faecalis*. The antibacterial activity was impaired in the presence of bovine serum albumin (BSA), human plasma and whole blood. Curcumin was not toxic to PBMCs and RBCs at 200μg/mL. Furthermore, Curcumin showed synergistic activity in combination with antibiotics: Ciprofloxacin, Gentamicin, Vancomycin and Amikacin against *Staphylococcus aureus*. This study demonstrated that the interaction of Curcumin with plasma proteins diminishes its *in vitro* antibacterial activity. Curcumin derivatives with reduced affinity for plasma protein may improve the bioavailability and antibacterial activities.

Keywords: Curcumin, antibacterial, physiological conditions, antibiotics, synergism.

INTRODUCTION

Turmeric (*Curcuma longa*) is a perennial plant that grows in tropical regions of South Asia. Its leaves, roots (rhizomes) and bulbs are used to add color and flavor to food. It has also been used in the Indian and Chinese traditional medicine to treat a variety of ailments. The major bioactive compound in turmeric is a hydrophobic polyphenol called 'Curcumin' (Zhou *et al*., 2011). Curcumin has been studied for its anti-inflammatory, anti-oxidative, anti-carcinogenic, anti-viral, anti-fungal and anti-parasitic activities (Gupta *et al*., 2011; Marathe *et al*., 2011). Curcumin was also investigated for its anti-bacterial activity against Gram-positive and Gram-negative bacteria (Kumar *et al*., 2001; Tajbakhsh *et al*., 2008; De *et al*., 2009; Wang *et al*., 2009; Bhawana *et al*., 2011; Na *et al*., 2011; Kim *et al*., 2012). These reports suggested that Curcumin possessed potent antibacterial activity against various bacterial strains. Recently, its synergistic effect with antibiotics against Methicilin-resistant *Staphylococcus aureus* (MRSA) was also reported (Mun *et al*., 2013).

Despite the assessment of antibacterial activity of Curcumin in broth media is well-established, no report has yet studied the activity in biological fluids such as plasma and whole blood. As Curcumin interacts closely with blood or serum proteins and erythrocytes (Leung and Kee, 2009; Kudva *et al*., 2011; Yallapu *et al*., 2011), the antibacterial activity can be complicated by this *in vivo* interactions. For instance, human serum albumin acts as an effective protein stabilizer to Curcumin (Leung and Kee, 2009). When the stabilized Curcumin exposed to bacteria, the antibacterial effect can either increase due to the lower degradation rate of albumin-bound Curcumin, or decrease due to the lower concentrations of freely bound Curcumin. Interestingly, the biological activities of Curcumin were also associated with the degradation products (Shen *et al*., 2012). Notably, there are many other proteins present in the blood (fibrinogen, globulin and enzymes) other than serum albumin, which can interact with Curcumin to affect its pharmacological activities. Therefore, it is not surprising to observe a sudden elevation or elimination of antibacterial effect of Curcumin in the presence of biological fluids.

In the present investigation, we report the antibacterial activity of Curcumin under various biologically relevant conditions using a plate-counting method. We have investigated the effect of pH, serum albumin, plasma and whole blood on the activity of Curcumin. Furthermore, we have tested Curcumin for its toxicological effects and explored its synergistic activity in combination with different antibiotics. This study provides an important preliminary data for Curcumin to be used in pre-clinical and clinical trials in the future.

MATERIAL AND METHODS

Reagents, chemicals and culture media

Curcumin (CB0346) and Bovine Serum Albumin (BSA) (AD0023) were from Bio Basic Canada. Dimethylformamide (DMF) (D4551) was from Sigma-

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Aldrich. Antibiotic discs Amoxicillin/Clavulanic Acid (AMC30µg), Penicillin (P10µg), Clindamycin (DA2µg), Oxicillin (OX1µg) and Gentamicin (CN10µg) 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 (M391) and Todd-Hewitt (M313) broth media were from HiMedia Laboratories. Luria-Bertani (LB) Agar (244520) and Brain Heart Infusion (BHI) Agar (221569) were from BD.

Bacterial isolates

Eight clinical bacterial isolates (*Klebsiella pneumonia*, *Esherichia coli*, *Staphylococcus aureus*, *Salmonella typhi*, *Shigella flexneri*, *Pseudomonas aeruginosa*, *Enterococcus faecalis* and *Streptococcus pyogenes*) were obtained from Microbiology Diagnostic Laboratory, Advanced Medical and Dental Institute, USM, Penang. All isolates except *P. aeruginosa* were susceptible to Amoxicillin/Clavulanic Acid (30µg). *S. typhi* and *E. faecalis* were sensitive to Penicillin (10µg) but resistant to other antibiotics. All isolates except *S. aureus* were resistant to Clindamycin (2µg) and Oxicillin (1µg) whereas all the isolates were sensitive to Gentamicin (10µg).

Anti-bacterial assays

The susceptibility of bacteria towards Curcumin was determined by Clinical and Laboratory Standards Institute (CLSI)-recommended broth microdilution assay according to M07-A9 guideline (Clinical and Laboratory Standards Institute, 2012). Antibacterial activity of Curcumin was assessed in plain Mueller-Hinton broth (MHB), Todd-Hewitt broth (THB) for *S. pyogenes* and *E. faecalis*, and in the presence of 3.5% BSA or 25% non-heat-inactivated human plasma. To investigate the effect of pH on Curcumin activity, the media pH was adjusted to pH7.4 and 6.5 with 1N hydrochloric acid (HCl). 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 $2x10^7$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±1°C for 24h (48h for *S. pyogenes* and *E. faecalis*). Bacterial growth was evaluated by measuring the turbidity at 600nm using µ Quant ELISA Reader (Bio-Tek Instruments, USA). Some experiments were carried out in 99% whole blood, 99% leucocyte-rich plasma and 99% leucocyte-depleted plasma. To assay viable bacteria, samples were diluted in phosphate buffered saline (PBS) and plated on LB Agar (BHI agar for *E. faecalis* and *S. pyogenes*). Colonies were counted the following day and dose response curves were generated as previously described (Deslouches *et al*., 2005).

Preparation of whole blood, leucocyte-rich plasma, red blood cells (RBCs) and peripheral blood mononuclear cells (PBMCs)

Venous blood was collected in heparin-containing tubes (BD). To separate leucocyte-rich plasma from RBCs, the blood was allowed to stand at room temperature for 2h. The leucocyte-rich plasma was harvested and stored. The settled RBCs were washed twice with PBS by centrifugation at 250xg for 10min at room temperature. Washed RBCs were suspended in PBS at a hematocrit of 10% and used for hemolysis assay. PBMCs were prepared by using standard Lymphoprep density centrifugation in Leucosep tubes according to the manufacturer's instructions (Greiner Bio-One, Kremsmünster, Austria). The PBMCs were washed twice with PBS by centrifugation at 250xg for 10min at room temperature. Washed PBMCs were resuspended to a final concentration of $1x10^6$cells/mL in autologous plasma and used for toxicity assay (Deslouches *et al*., 2005).

Biofilm assay

The cultures, plate preparation and incubation conditions were prepared as described above. At the end of the incubation periods, cultures from each well were decanted and planktonic cells were removed by washing three times with PBS. Cells in biofilm were fixed with methanol for 15min, air dried and stained with 1% crystal violet. Biofilm formation was quantified by measuring the absorbance at 595nm using µ Quant ELISA Reader (Bio-Tek Instruments, USA) as previously described (Pattiyathanee *et al*., 2009).

Hemolysis assay

Diluted RBCs (10% in PBS) or whole blood was treated with two-fold serially diluted Curcumin (200 to 12.5µg/mL) and incubated at 36±1°C for 48h while shaking. DMF to a final concentration of 1% was used as a negative control. Samples (70µL) were taken at 0, 1, 3, 6, 12, 24 and 48h and centrifuged at 15,000xg for 8min. 25µL of cell-free supernatant was diluted with 225µL deionized water. Hemolysis was determined by measuring the optical density (OD) of diluted supernatant at 570nm using a µ Quant ELISA Reader (Bio-Tek Instruments, USA) as previously described (Deng *et al*., 2006; Yallapu *et al*., 2011). Positive control (100% hemolysis) was prepared by lysing 70µL untreated whole blood with 630µL RBC lysis buffer (8.3g/L ammonium chloride in 0.01M Tris-HCl, pH7.5).

Toxicity assay for peripheral blood mononuclear cells (PBMCs)

PBMCs ($1x10^6$ cells/mL in autologous plasma) were treated with two-fold serially diluted (200 to 12.5µg/mL)

Curcumin. Control tubes received DMF to a final concentration of 1%. Tubes were incubated at 36±1°C/ 5% CO_2 for 48h. Cells were sampled at 1, 3, 6, 12, 24 and 48h and subjected to Trypan blue dye exclusion assay. Cells were counted using an improved neubauerhemo cytometer to determine the cell viability as previously described (Deslouches *et al*., 2005; Hollborn *et al*., 2013; Liu *et al*., 2013).

Selective toxicity of Curcumin in human whole blood

Serial two-fold dilutions of Curcumin in whole blood were prepared in tissue culture tubes (2mL per tube). Control tubes received 1% DMF. The inocula (10µL) containing $1x10^8$cfu/mL mid-log-phase bacteria (*S. aureus*, *E. faecalis* and *S. pyogenes*) were added to each tube. The tubes were incubated at 36±1°C for 48h while shaking. Cultures were sampled at 24 and 48h and assessed for bacterial viability, hemolysis and PBMC toxicity as mentioned above (Deslouches *et al*., 2005; Liu *et al*., 2013).

Antibacterial activity of Curcumin in combination with antibiotics

Activity of Curcumin (at sub-MIC concentration of 25µg/mL) in combination with antibiotics (Ciprofloxacin, Gentamicin, Vancomycin, Amikacin, Ampicillin, Clindamycin, Erythromycin, Tetracycline, Penicillin and Fusidic Acid) was determined using broth microdilution assay as described above. The fractional inhibitory concentration (FIC) was calculated as follows:

FIC of compound a (FIC_a) =MIC of compound a in combination/MIC of compound a alone

FIC of compound b (FIC_b) =MIC of compound b in combination/MIC of compound b alone

The sum of fractional inhibitory concentration (FICs) indices of two compounds in the combination was calculated as $FIC_a + FIC_b$ =FICs. The types of effects were classified as follows: FIC ≤0.5=synergistic; FIC 0.5-1=additive; FIC 1-4=indifferent; and FIC>4= antagonistic as previously described (Sharma *et al*., 2010; Mun *et al*., 2013).

STATISTICAL ANALYSIS

Experiments were repeated for three times and in triplicate each time. Data are presented as ± standard error of mean. The significance of data was analyzed using paired *t*-test using SPSS version 20.0 as previously described (Rahayu *et al*., 2013). A *p*-value of less than 0.05 was considered statistically significant.

RESULTS

Effect of pH, BSA, and human plasma on antibacterial activity of Curcumin against Gram-negative bacteria

Since IC_{50} values (>100µg/mL) of Curcumin were not obtained for the Gram-negative bacteria (table 1), the results were discussed in the form of growth inhibition compared to untreated control at 100µg/mL of Curcumin (Table 2).

At pH7.4, 49% growth inhibition of *E. coli* was observed at 100µg/mL Curcumin in MHB (Table 2). Growth inhibition of *E. coli* was reduced to 40% and 33% in MHB/BSA and MHB/plasma, respectively. At pH6.5, 46% growth inhibition of *E. coli* was observed at 100µg/mL Curcumin in MHB. Growth inhibition of *E. coli* was reduced to 26% and 0% in MHB/BSA and MHB/plasma, respectively. This data showed that pH had no effect on Curcumin activity against *E. coli* in MHB. Compared to pH7.4, Curcumin activity in MHB/BSA and MHB/plasma was significantly reduced at pH6.5.

At pH7.4, 45% growth inhibition of *S. typhi* was observed at 100µg/mL Curcumin in MHB. Growth inhibition of *S. typhi* was reduced to 0% and 23% in MHB/BSA and MHB/plasma respectively. At pH6.5, 48% growth inhibition of *S. typhi* was observed at 100µg/mL Curcumin in MHB. Growth inhibition of *S. typhi* was reduced to 10% and 0% in MHB/BSA and MHB/plasma respectively. This data showed that pH had insignificant effect on Curcumin activity against *S. typhi* in MHB. Compared to pH7.4, Curcumin activity in MHB/BSA increased slightly at pH6.5.

At pH7.4, 44% growth inhibition of *S. flexneri* was observed at 100µg/mL Curcumin in MHB. Growth inhibition of *S. flexneri* was reduced to 10% and 0% in MHB/BSA and MHB/plasma respectively. At pH6.5, 56% growth inhibition of *S. flexneri* was observed at 100µg/mL Curcumin in MHB. Growth inhibition of *S. flexneri* was reduced to 11% and 31% in MHB/BSA and MHB/plasma respectively. This data showed that Curcumin was more active against *S. flexneri* in MHB at pH6.5. Curcumin activity was similar in MHB/BSA at pH7.4 and pH6.5. Compared to pH7.4, Curcumin showed growth inhibition against *S. flexneri* in MHB/plasma at pH6.5.

Effect of pH, BSA, and human plasma on antibacterial activity of Curcumin against Gram-positive bacteria

At pH7.4, Curcumin showed higher activity against *S. aureus* in MHB/BSA (IC_{50} of 44µg/mL) or MHB/plasma (IC_{50} of 52µg/mL) than in MHB (IC_{50} of 89µg/mL) (Table 1). At pH6.5, Curcumin was more active against *S. aureus* in MHB (IC_{50} of 44µg/mL) than in MHB/BSA (IC_{50} of 81µg/mL) or MHB/plasma (IC_{50} of 65µg/mL). This data showed that Curcumin was more active against *S. aureus* in MHB at pH6.5 than pH7.4. Compared to pH6.5, Curcumin showed better activity against *S. aureus* in MHB/BSA and MHB/plasma at pH7.4.

At pH7.4, Curcumin showed higher activity against *S. pyogenes* in THB/BSA (IC_{50} of 60µg/mL) and THB/plasma (IC_{50} of 60µg/mL) than in THB (IC_{50} of 75µg/mL). At pH6.5, Curcumin showed higher activity

Table 1: Curcumin activity in basal media, basal media containing 3.5% BSA and basal media containing 25% human plasma at pH7.4 and pH6.5

	pH 7.4 (µg/mL)			pH 6.5 (µg/mL)		
	Medium	Medium + 3.5% BSA	Medium + 25% plasma	Medium	Medium + 3.5% BSA	Medium + 25% plasma
Klebsiella pneuomoniae	IC_{50}>100 IC_{90}>100	IC_{50}>100 IC_{90}>100	IC_{50}>100 IC_{90}>100	IC_{50}>100 IC_{90} >100	IC_{50}>100 IC_{90} >100	IC_{50}>100 IC_{90}>100
Escherichia coli	IC_{50}>100 IC_{90}>100	IC_{50}>100 IC_{90}>100	IC_{50}>100 IC_{90}>100	IC_{50}>100 IC_{90}>100	IC_{50}>100 IC_{90}>100	IC_{50}>100 IC_{90}>100
Pseudomonas aeruginosa	IC_{50} >100 IC_{90}>100	IC_{50}>100 IC_{90}>100	IC_{50}>100 IC_{90}>100	IC_{50}>100 IC_{90}>100	IC_{50}>100 IC_{90}>100	IC_{50}>100 IC_{90}>100
Salmonella typhi	IC_{50}>100 IC_{90}>100	IC_{50}>100 IC_{90}>100	IC_{50}>100 IC_{90}>100	IC_{50}>100 IC_{90}>100	IC_{50}>100 IC_{90}>100	IC_{50}>100 IC_{90}>100
Shigella flexneri	IC_{50}> 100 IC_{90}>100	IC_{50}>100 IC_{90}>100	IC_{50}>100 IC_{90}>100	IC_{50}= 69 IC_{90}>100	IC_{50}>100 IC_{90}>100	IC_{50}>100 IC_{90}>100
Staphylococcus aureus	IC_{50}=89 IC_{90}>100	IC_{50}=44 IC_{90}>100	IC_{50}=52 IC_{90}=98	IC_{50}=44 IC_{90}>100	IC_{50}=81 IC_{90}>100	IC_{50}=65 IC_{90}>100
Streptococcus pyogenes	IC_{50}=75 IC_{90}>100	IC_{50}=60 IC_{90}>100	IC_{50}=60 IC_{90}>100	IC_{50}=54 IC_{90}>100	IC_{50}=63 IC_{90}>100	IC_{50}=75 IC_{90}>100
Enterococcus faecalis	IC_{50}>100 IC_{90}>100	IC_{50}=82 IC_{90}>100	IC_{50}=63 IC_{90}>100	IC_{50}>100 IC_{90}>100	IC_{50}=84 IC_{90}>100	IC_{50}>100 IC_{90}>100

Muller-Hinton broth (MHB) was used as basal media for all isolates except *S. pyogenes* and *E. faecalis* in which Todd-Hewitt broth (THB) was used to enhance the growth.

Table 2: The percentage of growth inhibition in the presence of 100µg/mL Curcumin

	pH 7.4 (µg/mL)			pH 6.5 (µg/mL)		
	Medium	Medium + 3.5% BSA	Medium + 25% plasma	Medium	Medium + 3.5% BSA	Medium + 25% plasma
Klebsiella pneuomoniae	31	13	0	16	19	14
Escherichia coli	49	40	33	46	26	0
Pseudomonas aeruginosa	16	3	26	33	2	21
Salmonella typhi	45	0	23	48	10	0
Shigella flexneri	44	10	0	56	11	31
Staphylococcus aureus	55	87	92	69	55	65
Streptococcus pyogenes	77	86	69	77	88	78
Enterococcus faecalis	19	59	56	47	59	31

against *S. pyogenes* in THB (IC_{50} of 54µg/mL) than in THB/BSA (IC_{50} of 63µg/mL) and THB/plasma (IC_{50} of 75µg/mL). This data showed that Curcumin was more active against *S. pyogenes* in THB at pH6.5 than pH7.4. Compared to pH6.5, Curcumin showed better activity against *S. pyogenes* in THB/BSA and THB/plasma at pH7.4.

At pH7.4, Curcumin showed higher activity against *E. faecalis* in THB/BSA (IC_{50} of 82µg/mL) and THB/plasma (IC_{50} of 63µg/mL) than in THB (IC_{50}>100µg/mL). At pH6.5, Curcumin showed activity against *E. faecalis* in THB/BSA (IC_{50} of 84µg/mL) but not in THB/plasma (IC_{50}>100µg/mL) or THB (IC_{50}>100µg/mL). Compared to pH6.5, Curcumin showed better activity against *E. faecalis* in THB/BSA and THB/plasma at pH7.4.The MIC of Curcumin against *E. faecalis* has been previously reported as 293µg/mL (Gunes *et. al*., 2013).

Antibacterial activity of Curcumin in plasma, leucocyte-rich plasma, and whole blood

As *S. aureus*, *S. pyogenes* and *E. faecalis* showed relatively higher susceptibility towards Curcumin when tested in the presence of 3.5% BSA or 25% plasma, we tested Curcumin against three of the isolates in 99% plasma (leucocyte-depleted and rich) and whole blood. As shown in Fig. 1A, Curcumin activity against *S. aureus* was markedly reduced in 99% whole blood (IC_{50} of >200µg/mL), 99% leucocyte-rich plasma (IC_{50} of >200µg/mL) and 99% leucocyte-depleted plasma (IC_{50} of >200µg/mL) than 25% plasma (IC_{50} of >52µg/mL). Curcumin activity was most affected in whole blood followed by leucocyte-rich and leucocyte-depleted plasma. Curcumin activity against *E. faecalis*, and *S. pyogenes* was also reduced in whole blood, leucocyte-rich plasma, and leucocyte-depleted plasma than 25% plasma (Fig. 1B and 1C). Similar to *S. aureus*, Curcumin loss of

Table 3: Activity of Curcumin in combination with antibiotics against *S. aureus*

Antibiotics[a]	Test concentration (µg/mL)	IC_{50} (µg/mL)	FIC [$(C_A/MIC_A) + (C_B/MIC_B)$]	Activity
Ampicillin Ampicillin + Cur	0 -12.6 0 -12.6	3.91 4.69	1.0	Indifferent
Clindamycin Clindamycin + Cur	0-12.5 0-12.5	7.03 3.91	1.0	Additive
Erythromycin Erythromycin + Cur	0-0.25 0-0.25	0.09 0.11	1.0	Indifferent
Ciprofloxacin Ciprofloxacin + Cur	0-0.25 0-0.25	0.16 0.05	0.5	Synergistic
Tetracycline Tetracycline+ Cur	0-1 0-1	0.41 0.44	1.0	Indifferent
Gentamicin Gentamicin+ Cur	0-0.25 0-0.25	0.09 0.03	0.5	Synergistic
Penicillin Penicillin + Cur	0-50 0-50	7.82 6.24	0.56	Additive
Vancomycin Vancomycin+ Cur	0-1 0-1	0.55 0.38	0.5	Synergistic
Amikacin Amikacin+ Cur	0-0.5 00.5	0.49 0.19	0.25	Synergistic
Fusidic Acid Fusidic Acid + Cur	0-0.5 0-0.5	0.13 0.09	1.0	Additive

Cur-Curcumin; [a]25µg/mL sub-inhibitory concentration of Curcumin was added along with the stated antibiotic concentrations.

activity against *E. faecalis* and *S. pyogenes* was highest in whole blood followed by plasma. These data suggested that anti-bacterial activity of Curcumin was reduced in the presence of plasma proteins.

Effect of Curcumin on biofilm formation

Effect of Curcumin activity on *H. pylori* biofilm formation has been reported previously (Pattiyathanee *et al*., 2009). We wished to investigate whether Curcumin would inhibit biofilm formation of other bacterial species. We found that Curcumin at sub-MIC concentrations did not inhibit the biofilm formation by any of the bacterial species tested.

Hemolytic activity of Curcumin

As a measure of toxicity, we determined Curcumin-induced hemolysis of human erythrocytes in diluted RBCs (10% in PBS) and heparinized whole blood. Curcumin did not exhibit hemolytic activity even at a concentration of 200µg/mL when tested against diluted RBCs (10% in PBS). However, Curcumin exhibited mild (10%) hemolysis at 200µg/mL when tested against whole blood. The Curcumin-induced hemolytic effects were apparent at 24h and 48h incubation but not at earlier time points (Fig. 2).

Toxicity of Curcumin in PBMCs

We determined the toxicity of Curcumin against human PBMCs by incubating PBMCs with Curcumin (200 to 12.5µg/mL) for 1, 3, 6, 12, 24 and 48h and then staining the cells with trypan blue. Curcumin did not exhibit toxicity at any time point even at concentration of 200µg/mL when tested against PBMCs.

Activity of Curcumin in combination with antibiotics

We tested Curcumin (25µg/mL) in combination with various antibiotics against above mentioned bacterial isolates. Table 3 lists the results of combination activity of Curcumin against *S. aureus*. Curcumin showed synergistic activity in combination with Ciprofloxacin, Gentamicin, Vancomycin and Amikacin. Synergistic activity was highest for Amikacin with an FIC value of 0.25. Combination of Curcumin with Clindamycin, Penicillin and Fusidic Acid showed additive effects. Curcumin showed no combination effects on Ampicillin, Erythromycin and Tetracycline activity.

DISCUSSION

At physiological pH, Curcumin undergoes auto-oxidation in aqueous medium (Gordon and Schneider, 2012). Being more stable at pH6.5 (Wang *et al*., 1997), Curcumin was tested for its anti-bacterial and anti-fungal activities at acidic pH, and in the presence of ascorbic acid (Rai *et al*., 2008; Khalil *et al*., 2012). However, Curcumin's antimicrobial activity at pH7.4 (physiological pH) and pH6.5 has not been compared in parallel. Here, we tested the activity of Curcumin against eight clinical bacterial isolates in basal medium (MHB or THB), basal medium containing 3.5% BSA and basal medium containing 25% human plasma. These tests were performed in parallel at pH7.4 and pH6.5 using a standardized broth micro dilution method (Clinical and Laboratory Standards Institute, 2012).

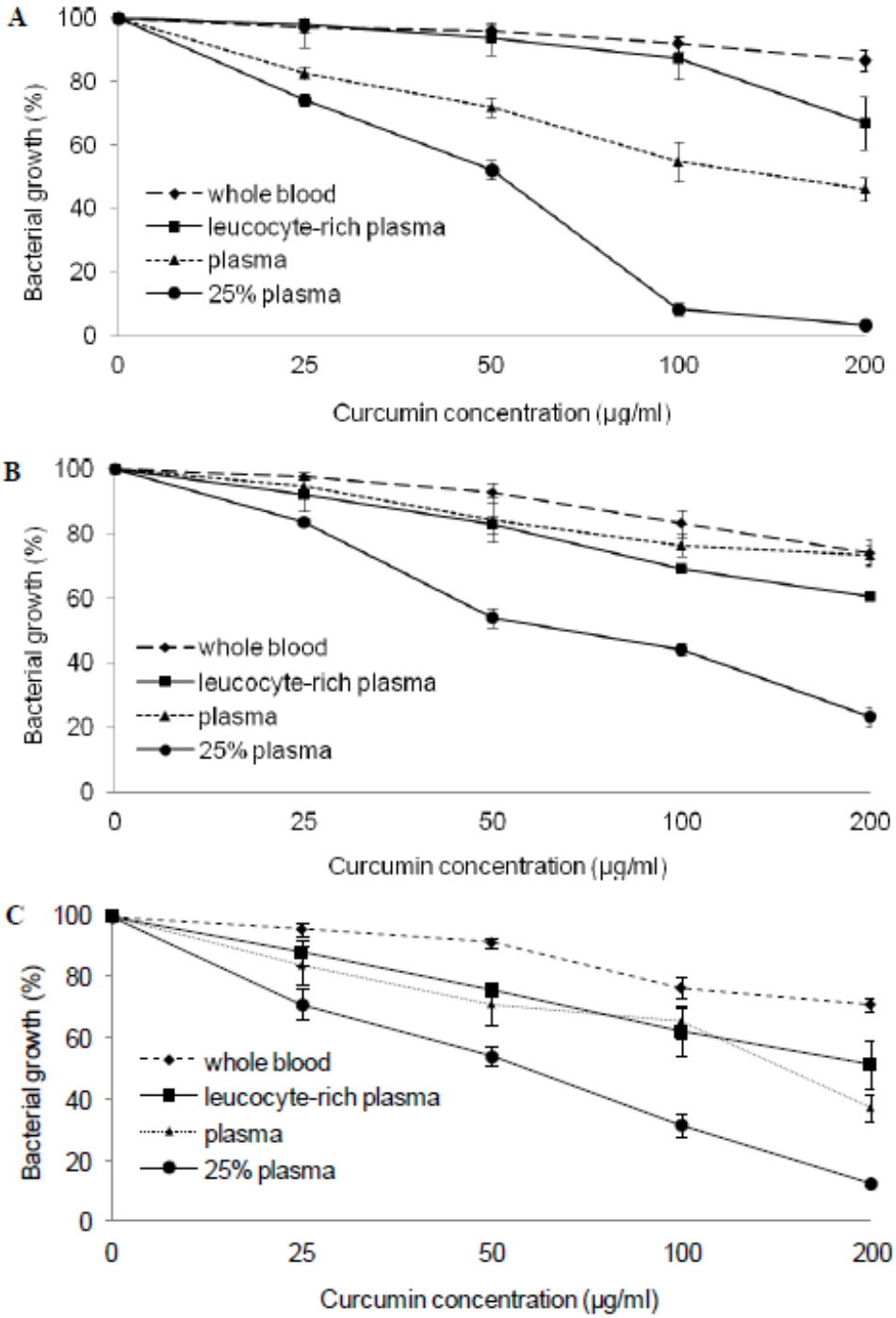

Fig. 1: Curcumin activity against *Staphylococcus aureus* (A), *Streptococcus pyogenes* (B), and *Enterococcus faecalis* (C) in whole blood, leucocyte-rich plasma, leucocyte-depleted plasma and 25% plasma.

Curcumin showed bactericidal activity against three Gram-negative bacteria: *E. coli*, *S. typhi* and *S. flexneri*, which were shown by previous studies in the laboratory broth (Marathe *et al*., 2012; Mary *et al*., 2012; Gunes *et al*., 2013; Rahayu *et al*., 2013). The MIC values of Curcumin against *E. coli* in the laboratory broth were previously reported as 163µg/mL (Gunes *et al*., 2013), 300µg/mL (Bhawana *et al*., 2011) and >375µg/mL (Tajbakhsh *et al*., 2008), respectively. The MICs varied from each report due to the different experimental settings such as varied susceptibility of bacterial strains or isolates, Curcumin preparations, anti-bacterial assays and assay conditions. This urges the development of a standardized antibacterial assay to allow comparison between studies.

Antibacterial activity of Curcumin against *S. typhi* was also supported by previous study (Mary *et al*., 2012)

which demonstrated a zone inhibition of 9mm using disc diffusion method. Limited literatures have reported antibacterial activity of Curcumin against *S. flexneri*. Various Curcumin analogues synthesized by Elavarasan *et al* were active against *S. flexneri* shown by disc diffusion method. Pretreatment of macrophages with Curcumin also attenuated the infection of clinically isolated *S. flexneri* (Marathe *et. al*., 2012), suggesting that Curcumin retained its activity intracellularly at slightly acidic environment (Casey *et al*., 2010). This study supports our observation that antibacterial effect of Curcumin against *S. flexneri* is more prominent at pH6.5 than pH7.4.

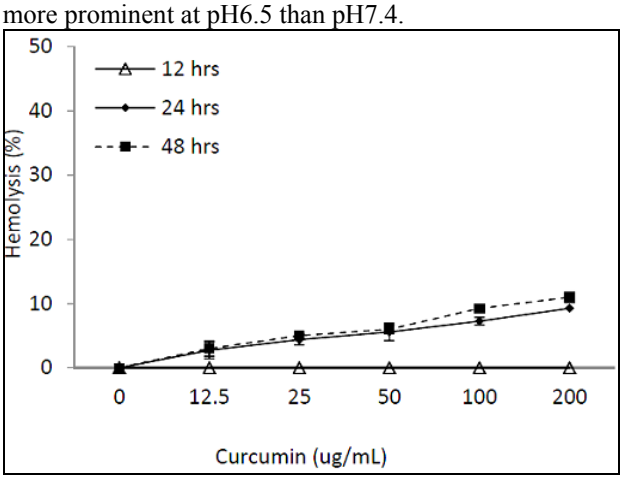

Fig. 2: Hemolytic activity of Curcumin (0-200µg/ml) on human whole blood at 12, 24 and 48 hours.

Compared to Gram-negative bacteria, Curcumin showed better activity against Gram-positive bacteria and *S. aureus* was more susceptible. MICs of Curcumin against *S. aureus* have been reported ranged from 150µg/mL to 219µg/mL (Tajbakhsh *et al*., 2008; Bhawana *et al*., 2011; Gunes *et al*., 2013). When macrophages were pretreated with Curcumin to combat *S. aureus,* the infection was not attenuated, but unexpectedly aggravated (Marathe *et al*., 2012). This suggest that antibacterial effect of Curcumin is reduced at slightly acidic and protein-saturated environment (Casey *et al*., 2010). This study also supports our study that exhibited better effect against *S. aureus* at pH7.4 in the presence of BSA and plasma.

Curcumin binds with serum albumin (Pulla-Reddy *et al*., 1999; Barik *et al.,* 2003; Bourassa *et al*., 2010) while plasma protein binding affects pharmacokinetics (PK) and pharmacodynamics (PD) of therapeutic compounds (Zeitlinger *et al*., 2012). However, effect of the plasma protein binding on the antibacterial activity of Curcumin has not been investigated. In the present study, we demonstrates that the plasma proteins affect the antibacterial effects of Curcumin. Curcumin might interact with serum albumin or other proteins which limit the free-bound Curcumin (Leung and Kee, 2009; Kudva et. al., 2011; Yallapu *et al*., 2011) to exert the antibacterial effects. As non-heat-inactivated plasma was used, the immunological components present in the plasma or blood such as enzymes and globulins may metabolize the Curcumin (Wang *et al*., 1997), hence eliminating the antibacterial activities. Greater loss of Curcumin activity in whole blood and 99% leucocyte-rich plasma also suggested some role of hematocytes in the reduction of Curcumin activity. The higher viscosity of RBCs suspension might limit the interactions of bacterial isolates and Curcumin and reduce the activities. As Curcumin reacts with RBCs (Kudva *et al*., 2011; Yallapu *et al*., 2011), another possibility is the reduced bio-availability of Curcumin for antibacterial activities as a result of RBCs-bound Curcumin.

The hemolysis result is consistent with previous report (Kudva *et al*., 2011) that exhibited an approximately 8% of RBCs lysis after 6h incubation with 32µM or 11.8µg/mL Curcumin. The difference of values might be due to the assay conditions yet both studies suggested that Curcumin possesses mild hemolytic activity. Contradictorily, Curcumin and its analogues were shown to possess anti-hemolytic effect (Deng et. al., 2006) at low concentrations (15µM or 5.5µg/mL). Comparison cannot be made as the test or working concentration is at least twenty-folds higher in the present investigation.

Curcumin exhibits cytotoxic effects against various cancer cell lines, but not the normal cells in vitro including various types of immune cells such as natural killer cells, T cells and macrophages (Varalakshmi *et al*., 2008). Curcumin also selectively induced apoptosis in cutaneous T-cell lymphoma (CTCL) cell lines and PBMCs from CTCL patients, but not the PBMCs from healthy donors (Zhang *et al*., 2010), which is in agreement with our result. These suggest that the biological activity of Curcumin is specific and selective, more importantly, it is safe to be used in human trials.

Consistent with our result, synergistic effect of Curcumin with various antibiotics have also been previously reported against *S. aureus* both using broth micro dilution method (Mun *et al*., 2013) and disc diffusion method (Moghaddam *et al*., 2009). These results suggested that Curcumin may be developed into a potential candidate for combination therapy against *S. aureus* infections.

CONCLUSION

Low aqueous solubility severely limits Curcumin use as a medicine. Efforts have been made to overcome this limitation. Our work highlights the effect of plasma proteins on the antibacterial activity of Curcumin. Based on our data, it becomes clear that Curcumin's strong affinity towards plasma protein significantly limits its antibacterial efficacy. Further studies investigating the effects of plasma on the other biological properties of Curcumin would be desirable. Curcumin derivatives with reduced affinity for plasma protein may improve Curcumin bioavailability.

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