Tropical Journal of Pharmaceutical Research February 2018; 17 (2): 307-317
ISSN: 1596-5996 (print); 1596-9827 (electronic)
© Pharmacotherapy Group, Faculty of Pharmacy, University of Benin, Benin City, 300001 Nigeria.

Available online at http://www.tjpr.org
http://dx.doi.org/10.4314/tjpr.v17i2.16

Original Research Article

Siamese crocodile plasma synergizes with ceftazidime against ceftazidime-resistant *Enterobacter cloacae*

Nitaya Rojtinnakorn[1], Yosapong Temsiripong[2], Thippawan Pimchan[3], Griangsak Eumkeb[1]*
[1]School of Preclinic, , [2]Sriracha Moda Farm, Sriracha District, Chonburi 20110, Thailand[3]School of Biology, Institute of Science, Suranaree University of Technology, Nakhon Ratchasima, 3000

For correspondence: **Email: griang@sut.ac.th; **Tel:** +66 44 224260; **Fax:** +66 44 224650*

Sent for review: 27 April 2017 *Revised accepted: 17 January 2018*

Abstract

Purpose: *To evaluate whether Siamese crocodile plasma exhibits antibacterial properties and if it synergizes with ceftazidime against ceftazidime-resistant Enterobacter cloacae (CREnC).*
Methods: *Protein fractions were from crocodile plasma and tested them on CREnC strains. Multiplex polymerase chain reaction (PCR) screening test was performed for extended-spectrum β-lactamase (ESBL) phenotype and AmpC gene. The effects of the antibacterial agents were analyzed using a bacterial suspension standard curve, minimum inhibitory concentration (MIC), Checkerboard assays, viability curves, membrane permeability assays, enzyme assays, and transmission electron microscopy.*
Results: *CREnC strains expressed ESBL-AmpC gene combinations. The MICs of resuspended protein 1 (P1), protein 5 (P5), ceftazidime, cefotaxime, and benzylpenicillin against all tested CREnC and E. coli strains were in the range of > 1024 μg/mL, indicating resistance. However, P1 and P5 exhibited a synergistic effect against test CREnC and E. coli strains when used in combination with ceftazidime and cefotaxime, with fraction inhibitory concentration indices of < 0.062 and 0.28, respectively. A kill curve demonstrated that the combination treatments had synergistic activity and inhibited β-lactamase.*
Conclusion: *The synergistic activity of P1 and P5 in combination with ceftazidime is achieved in multiple ways, including increased cytoplasmic and outer membrane permeability, β-lactamase inhibition, and peptidoglycan damage. Therefore, the combination therapy of Siamese crocodile plasma and ceftazidime may be a novel therapeutic approach for treating recalcitrant E. cloacae infection.*

Keywords: *Crocodylus siamensis, ceftazidime-resistant Enterobacter cloacae, synergistic activity, β-lactamase activity*

INTRODUCTION

A widespread increase in antimicrobial resistance of the Enterobacteriaceae family has created a significant challenge for treatment. In particular, *Enterobacter cloacae* is a causative pathogen for a variety of infections in several organ systems, including circulatory, gastrointestinal, respiratory, urinary, central nervous, musculoskeletal, and integumentary system [1]. From 2011 to 2013, the multidrug-resistant (MDR) rates (%) of *E. cloacae* identified from ICU and non-ICU infections in North America and Europe were 12.2 (ICU) and 10.0 (non-ICU), and 18.2 (ICU) and 15.5 (non-ICU), respectively [2]. Resistance to extended-spectrum cephalosporins occurs in approximately 31 % of *Enterobacter* spp identified in ICUs in the United States [3], and effective antibiotics to treat bacterial infections driven by producers of extended-spectrum β-lactamases (ESBLs), β-lactamases of the AmpC type, or co-expressing ESBLs are sorely lacking [4]. Accordingly, development of novel antibacterials is in significant demand and represent a critical unmet need.

Animals provide a unique source for novel antibacterial agents. For example, crocodiles suffer terrible fighting injuries without experiencing infections, even in pathogen-ridden water, indicating that crocodiles likely have an extremely potent immune system. Merchant and colleagues reported that the antibacterial spectrum of American alligator (*Alligator mississippiensis*) serum for both Gram-negative and positive species was more comprehensive than human serum [5]. In addition to the American alligator, the plasma of the Siamese crocodile (*Crocodylus siamensis*) has a broad antibacterial spectrum for *Escherichia coli*, *Klebsiella pneumoniae*, *Salmonella typhi*, *Staphylococcus epidermidis*, *Staphylococcus aureus*, *Pseudomonas aeruginosa*, and *Vibrio cholera* [6]. However, no studies have investigated the effects of the antibacterial action of Siamese crocodile (*C. siamensis*) plasma, or its synergistic action with ceftazidime against ceftazidime-resistant *E. cloacae* (CREnC).

Hence, we used transmission electron microscopy (TEM), membrane permeability assays (outer membrane and cytoplasmic membrane), and β-lactamase inhibition assays to evaluate how Siamese crocodile plasma fractions, alone and in combination with ceftazidime, affect CREnC viability and drug resistance.

EXPERIMENTAL

Bacterial strains and antibiotics

Clinical isolates of CREnC DMST 21394, DMST 21549, DMST 19719, and *E. coli* DMST 20662, 29237, 29239 were obtained from the Ministry of Public Health in Thailand. We obtained *E. coli* ATCC 25922 from the American Type Culture Collection (ATCC), USA, and used it as a reference strain. An 18 h culture was prepared at 37 °C using Cation-adjusted Mueller-Hinton broth (CAMHB), purchased from Oxoid (Basingstoke, United Kingdom). Bovine serum albumin (BSA), ceftazidime (CTZ), cefotaxime (CFT), benzylpenicillin (BZP), nisin, and polymixin B (PMX) were purchased from Sigma-Aldrich (Dorset, England).

Separation of protein from crocodile plasma

All animal experimental protocols were approved under the U.K. Animals (Scientific Procedures) Act, 1986 and the Animal Care and Use Committee of Suranaree University of Technology (approved serial number: 30/2553). Blood of 40 healthy Siamese crocodiles aged between 1 - 3 years (Sriracha Moda Farm, ChonBuri, Thailand) was drawn from a dorsal vein and subsequently kept in an EDTA tube at 4 °C. Then, 40 mL of blood was centrifuged at 4000 *x g* for 10 min. The resulting crude plasma was fractionated using ion exchange chromatography. Crude plasma was diluted in Tris-HCl buffer (Tris-HCl, 25 mM, pH 8.1) and placed in a Q-Sepharose fast flow column (size = 25 mL), then eluted with a linear NaCl gradient in Tris-HCl buffer (varying concentrations of NaCl at pH 8.1 included 0.1, 0.2, 0.3, 0.4 and 0.5 M for fractions 1, 2, 3, 4, and 5, respectively). To obtain only protein fractions, salt was eliminated and dialyzed overnight against diethyl-aminoethyl buffer (DEAE) using a dialysis membrane (Spectra/Por 1) (Spectrum, Houston, TX, USA, cut off 250 kDa) [7].

The protein fractions were monitored spectrophotometrically and pooled to give 5 fractions. We used ion exchange chromatography to further separate the pooled fractions. DEAE-Toyopearl 650 M (65 μm particle size) anion exchange resin was equilibrated using Tris-Chul buffer and elution from the column was performed in Tris-HCl buffer using a linear gradient of NaCl and infiltration of the column with trifluoroacetic acid (0.1 %). The molecular weights (MW) of proteins contained in the separated protein 1 (P1) to separated protein 5 (P5) fractions were evaluated using SDS-PAGE. Separated fractions were lyophilized and stored at - 70 °C. Protein concentrations of resuspended P1 and P5 were measured using a Coomassie assay [8], and standard curves were generated using BSA.

Screening test for ESBL phenotype and AmpC gene detection

The presence of ESBL, AmpC, and Metallo-β-lactamase (MBL) in test *E. cloacae* strains was phenotypically determined as reported previously [9]. Briefly, 5 x 10^5 CFU/mL of the test bacteria were challenged with a serial two-fold dilution of ceftazidime and cefotaxime with and without their respective inhibitors (4 µg/mL of clavulanic acid [ESBL inhibitor], 200 µg/mL of cloxacillin [AmpC inhibitor], and 0.5 mM of EDTA [MBL inhibitor]). The results were interpreted within 6 h of incubation at 37 °C by calculating the ratio of the MIC value of the well without β-lactamase inhibitors versus the MIC of the well with β-lactamase inhibitors. An eightfold or higher MIC ratio denoted a positive result for the presence of β-lactamase. In addition, we performed multiplex PCR for screening and confirmation of genes for AmpC (DHA and EBC) and ESBL (CTX-M-3, CTX-M-14, SHV, SHV-5, and TEM) [10].

Bacterial suspension standard curve

We performed bacterial suspension standard curves as previously described, with a few modifications [11]. The modifications were CAMHB and Mueller Hinton agar was used as a culture medium instead of Iso-sensitest broth and Iso-sensitest agar, respectively [12].

Susceptibility test

We determined the minimum inhibitory concentrations (MICs) for resuspended crocodile plasma fractions and antibiotics with a broth microdilution assay in a 96-well microtiter plate as previously described [12]. The susceptibility panel was prepared by dispensing 100 µL of 2048 µg/mL resuspended P1 or P5 (with sterile water) into the first column wells, and 80 µL of CAMHB (pH 5.9) with 20 µL of 1 x 10^6 CFU/mL into the test wells to get a 1024 µg/mL suspension. Then, serial twofold dilutions of P1 or P5 suspension were carried out by aliquoting 100 µL of suspension in the first column wells into the second column; subsequent columns contained 20 µL of 5 x 10^6 CFU/mL diluted bacteria plus 80 µL of CAMHB. After 20 h at 37 °C, the lowest concentration that did not induce visible growth was noted as the MIC.

Checkerboard assay

The interaction between resuspended P1 and P5, and ceftazidime, cefotaxime, or benzylpenicillin against CREnC was determined using checkerboard assays, as described in Eumkeb *et al.* [13]. We determined the interactions between agents by calculating the fractional inhibitory concentration index (FICI) for each combination. We then determined each individual treatment fractional inhibitory concentration (FIC) by determining which concentration (when used in combination) completely inhibited bacterial growth. FICI was calculated using Equation 3 below:

FICP1 = MICP1C/MICP1A …………… (1)

FICC = MICCC/MICCA ……………… (2)

FICI = FICP1 + FICC ………………… (3)

Where FICP1 is the FIC of P1, MICP1C represents the P1 MIC in combinatorial treatments, MICP1A is the P1 MIC, FICC is the ceftazidime FIC, MICCC is the ceftazidime MIC in the combination, and MICCA is the MIC of ceftazidime alone. A FICI value of a combination ≤ 0.5 was considered synergistic; FICI > 0.5 - < 1.0 was considered partially synergistic; FICI equal to 1.0 was considered additive; FICI > 1.0 - ≤ 4.0 was considered indifferent; and FICI > 4.0 was considered antagonistic [14].

Kill curves

Kill curves were performed according to a previous report with minor modifications [13]. Inocula of 5x10^5 CFU/mL of CREnC 21394 were exposed to individual antibacterial agents, used at half-MIC concentrations (512 µg/mL). In combination experiments, concentrations of the individual compounds were used at the MIC required for synergism (32 µg/mL). Cultures containing either 0.9 % Sodium chloride (NaCl), this was the vehicle treatment condition, or BSA (512 µg/mL) were used as controls.

Transmission electron microscopy (TEM)

We used a previously reported method to evaluate bacterial structure and morphology by TEM following treatment with test compounds [15]. We chose to use half-MIC for each individual agent (P1 at 512 µg/mL, P5 at 512 µg/mL, or ceftazidime at 512 µg/mL), and used concentrations below the FIC for each combination (P1 at 16 µg/mL plus ceftazidime at 16 µg/mL and P5 at 16 µg/mL plus ceftazidime at 16 µg/mL). This ensured that most of the bacteria were damaged, but not killed. Briefly, adjusted 4 h log phase cultures (5 x 10^5 CFU/mL) were exposed to antibacterial compounds and resuspended fractions.

The cells were pelleted, fixed, dehydrated, infiltrated, and embedded. Sectioned samples were counterstained and examined with a Tecnai G2 electron microscope. Using micrographs, we evaluated the effects of the agents on cell size by quantifying cell area (nm^2) (cell width x cell length). Experiments were repeated three times, and data is reported as the mean ± SEM.

Outer membrane (OM) permeability assay

OM permeability of CREnC 21394 in response to individual and combination treatments of P1 (512 µg/mL), P5 (512 µg/mL), ceftazidime (512 µg/mL), and ceftazidime (16 µg/mL) plus 16 µg/mL of either P1 or P5, was determined using a nitrocefin assay as described in [16]. Briefly, inocula of log phase cultures were treated with resuspended P1, P5, and ceftazidime at the same concentrations used for TEM. Nitrocefin (20 µg/mL) was used as the β-lactamase substrate. We measured the assay in a plate reader using a wavelength of 500 nm over 30 min at 37 °C. Wells with 7 µg/mL polymixin B (positive control) and without antibacterial agents (negative control) were also included.

Cytoplasmic membrane (CM) permeability assay

We evaluated CM permeability with slight modifications of previous methods [16]. Briefly, we mixed suspended cells (50 µL, OD = 0.3) in wells containing 50 µL of ortho-Nitrophenyl-β-galactoside (ONPG), and the half-MICs of resuspended P1 (512 µg/mL), P5 (512 µg/mL), and ceftazidime (512 µg/mL) alone, or the half-MICs of each combination (16 µg/mL P1 plus 16 µg/mL ceftazidime and 16 µg/mL P5 plus 16 µg/mL ceftazidime) that were established as synergistic FICIs (final concentration of ONPG = 100 µg/mL). Plates were equilibrated to temperature, and ONPG consumption and intracytoplasmic cleavage were measured in a plate reader at 37 °C by monitoring absorption at 420 nm for 120 min. Nisin-containing wells (0.5 µg/mL, positive control) and untreated wells (negative control) were also included.

β-lactamase inhibition assay

We evaluated the ability of resuspended P1 and P5 to inhibit the β lactamase type IV (purified enzyme from *E. cloacae*; Sigma; Poole, England) by modifying previous methods [15]. We used 100 µg/mL benzylpenicillin, (a β-lactamase type IV substrate); this concentration was chosen as it is 50 – 60 % hydrolyzed within 5 min. Resuspended P1, P5, and ceftazidime were preincubated at 37 °C with β-lactamase type IV in a buffer containing sodium phosphate (50 mM, pH 7.0) for 5 min, followed by addition of benzylpenicillin. We analyzed 5 timepoints from 0 – 20 min, in increments of 5 min, and a solution of methanol and acetic acid (100:1) was used to stop the reaction. The uninhibited benzylpenicillin was quantified using reverse-phase HPLC. Ten microliters of each sample were analyzed using a 10 mM ammonium acetate (pH 4.5 acetic acid):acetonitrile (75:25) mobile phase, at a flow rate of 1 mL/min, a 200 nm UV detector, and an Ascentis C18 column at 35 °C. The area under the curve was used to quantify the results. BSA-treated (512 µg/mL; negative control) and 0.9 % NaCl (the vehicle control) samples were also included.

Statistical analysis

CM and OM permeability and enzyme assays were carried out in triplicate. Average cell area was measured with TEM analysis. The data as shown as mean ± standard error of the mean (SEM). Statistical differences were evaluated using one-way ANOVA, and $p < 0.01$ by Tukey's HSD post hoc test were established as significant [15].

RESULTS

Siamese crocodile plasma-derived proteins

Molecular weights from SDS-PAGE analysis of resuspended P1 and P5 fractions are depicted in Figure 1. The results showed that both resuspended P1 and P5 displayed two protein bands at 67 and 75 kDa.

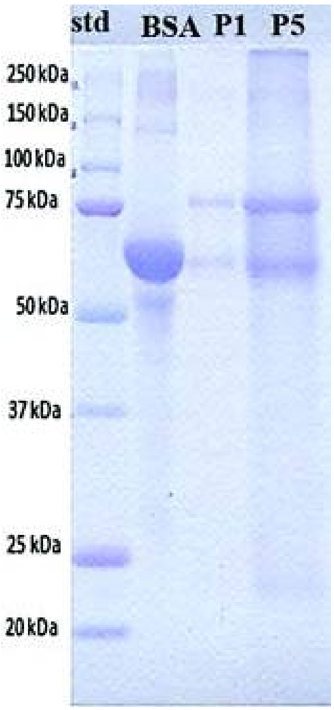

Figure 1: Molecular weight of proteins contained in re-suspended P1 and P5

ESBL phenotype and AmpC gene detection

To establish the resistance profile for the CREnC used in this study, we performed a β-lactamase phenotypic assay, and found that all test CREnC strains expressed both AmpC β-lactamases and ESBLs. These included the resistant EBC AmpC gene and the TEM ESBL gene, indicating that these strains are cephalosporin-resistant. Together, these findings reflect those reported previously by Eumkeb, Chukrathok, and Kao *et al.* who detected these resistance genes (ESBL-AmpC combinations) in CREnC strains and multiple drug resistant *E. cloacae* isolates [10, 15].

MIC and checkerboard assay data

The MIC results of resuspended P1, P5, CTZ, CFT, and BZP against CREnC strains were 1024, 1024, > 1024, > 1024, and > 1024 µg/mL, respectively (Table 1). Additionally, test *E. coli* strains were inhibited by the MICs of CTZ, CFT, BZP, P1, and P5 at 512, 512, > 1024, 512, and 512 µg/mL, respectively. In contrast, P2, P3, and P4 had MICs > 2048 µg/mL against all CREnC strains (data not shown), indicating that the active compound(s) is likely in P1 and P5.

According to the Clinical Laboratory Standards Institute, all tested CREnC strains were highly resistant to ceftazidime, cefotaxime, and benzylpenicillin, whereas the reference strain was susceptible to these antibiotics [17]. These findings suggest that the resistance genes, the ESBL-AmpC combination expressed by CREnC strains leads to cephalosporin resistance. Checkerboard data for crocodile plasma fractions (P1 and P5) with both ceftazidime and cefotaxime against CREnC and *E. coli* strains had synergistic activity at FICIs < 0.062 and 0.28, respectively. Likewise, benzylpenicillin plus either P1 or P5 displayed partially synergism at a FICI < 1.0 against all of the tested strains (Table 2) [14].

Table 1: MICs of ceftazidime, cefotaxime, benzylpenicillin, P1, and P5 against ceftazidime-resistant *E. cloacae* (CREnC) and *E. coli* strains. Data represent the medians, generated from three triplicate experiments

Strain	MICs (µg/mL)				
	CTZ	***CFT***	***BZP***	***P1***	***P5***
CREnC DMST 21394	>1024^{R}	>1024^{R}	>1024^{R}	1024ND	1024ND
CREnC DMST 21549	>1024^{R}	>1024^{R}	>1024^{R}	1024ND	1024ND
CREnC DMST 19719	>1024^{R}	>1024^{R}	>1024^{R}	1024ND	1024ND
E. coli DMST 20662	512^{R}	512^{R}	>1024^{R}	512ND	512ND
E. coli DMS 29237	512^{R}	512^{R}	>1024^{R}	512ND	512ND
E. coli DMS 29239	512^{R}	512^{R}	>1024^{R}	512ND	512ND
E. coli ATCC 25922*	0.25^{S}	0.25^{S}	1.0^{S}	512ND	512ND

*Positive control was *E. coli* (ATCC 25922). ^{S} = susceptible; ^{R} = resistance; ND = no data in Clinical Laboratory Standards Institute; CTZ = ceftazidime; CFT = cefotaxime; BZP = benzylpenicillin

Table 2: FICs and FICIs of ceftazidime, cefotaxime, benzylpenicillin, P1, and P5 against ceftazidime-resistant *E. cloacae* (CREnC) and *E. coli* strains. Each experiment was performed in triplicate

Strain	**FIC$^{\Psi}$: FICI$^{\Sigma}$**					
	CTZ+P1	***CTZ+P5***	***CFT+P1***	***CFT+P5***	***BZP+P1***	***BZP+P5***
CREnC DMST 21394	32+32: <0.062^{SI}	32+32: <0.062^{SI}	32+32: <0.062^{SI}	32+32: <0.062^{SI}	512+512: <1.0^{PS}	512+512: <1.0^{PS}
CREnC DMST 21549	32+32: <0.062^{SI}	32+32: <0.062^{SI}	32+32: <0.062^{SI}	32+32: <0.062^{SI}	512+512: <1.0^{PS}	512+512: <1.0^{PS}
CREnC DMST 19719	32+32: <0.062^{SI}	32+32: <0.062^{SI}	32+32: <0.062^{SI}	32+32: <0.062^{SI}	512+512: <1.0^{PS}	512+512: <1.0^{PS}
E. coli DMST 20662	16+128: 0.28^{SI}	16+128: 0.28^{SI}	16+128: 0.28^{SI}	16+128: 0.28^{SI}	512+256: <1.0^{PS}	512+256: <1.0^{PS}
E. coli DMST 29237	16+128: 0.28^{SI}	16+128: 0.28^{SI}	16+128: 0.28^{SI}	16+128: 0.28^{SI}	512+256: <1.0^{PS}	512+256: <1.0^{PS}
E. coli DMST 29239	16+128: 0.28^{SI}	16+128: 0.28^{SI}	16+128: 0.28^{SI}	16+128: 0.28^{SI}	512+256: <1.0^{PS}	512+256: <1.0^{PS}
E. coli ATCC 25922*	0.03+128: 0.38^{SI}	0.03+128: 0.38^{SI}	0.03+64: 0.25^{SI}	0.03+64: 0.25^{SI}	0.5+256: 0.5^{SI}	0.5+256: 0.5^{SI}

* Positive control was *E. coli* (ATCC 25922). ^{SI} = synergistic; ^{PS} = partially synergistic; CTZ = ceftazidime; CFT = cefotaxime; BZP = benzylpenicillin. The FIC$^{\Psi}$: FICI$^{\Sigma}$ value of CTZ + P1 at 32+32: <0.062^{SI} in each row below this column is the MIC of ceftazidime at 32 µg/mL plus P1 at 32 µg/mL in the combination. Accordingly, the FICI$^{\Sigma}$ value of CTZ + P1 was < 0.062^{SI}, which exhibited a synergistic interaction

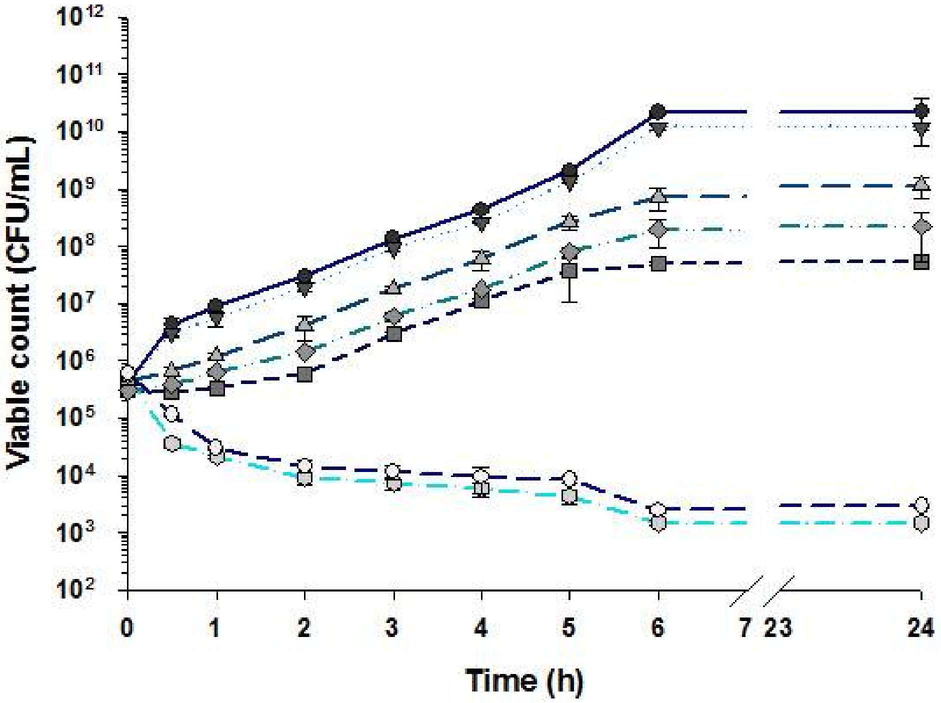

Figure 2: Viability of CREnC 21394 after treatment with: (●) = Control (0.9 % NaCl); (▼) = BSA; (▲) = P1; (◆) = P5; (■) = ceftazidime; (⬣) = P1 + ceftazidime; and (○) = P5 + ceftazidime. Compound and BSA concentrations were 512 µg/mL for individual treatments, and 32 µg/mL each for combination treatments. Data plotted are mean ± SEM (n = 3)

Kill curve data

We next evaluated the effects of individual and combination treatment with ceftazidime, P1, and P5 on CREnC 21394 viability (Figure 2). Treatment with BSA, a negative control, like the untreated control, had no effects on viability. Similarly, individual treatment with ceftazidime, P1, or P5 slightly decreased viability. However, we found that combination treatment with ceftazidime and P1 or P5 remarkably decreased cell viability by 6 h, and reduced viability (to 5 x 10^3 CFU/mL) was sustained until the end of the experiment (24 h). Our findings reflected those of the checkerboard assay, which demonstrated synergism of combination treatments, illustrated by decreased cell numbers (≥ 2log10 CFU/mL), in comparison with cells treated with only ceftazidime [18].

TEM

To determine the effects of the antimicrobial agents on cellular structure, we performed TEM of clinical isolates of CREnC 21394 treated with resuspended P1, P5, and ceftazidime alone and in combination during the log phase of growth (Figure 3).

The untreated control cells exhibited a standard appearance, with a normal peptidoglycan layer, and easily distinguished cytoplasmic membranes (Figure 3a). P1, P5, and ceftazidime individually treated cells are displayed in Figure 3b, 3c, and 3d, and exhibit small disruptions in cell envelopes. The average cross-sectional area of individually treated cells was slightly less than that of control cells, but we found no statistically significant differences ($p > 0.01$) (Figure 4).

Figure 3 e and f show the effects of re-suspended P1 and P5 plus ceftazidime treatment on cell morphology. Most of the combination-treated cells displayed obvious morphological changes, illustrated by irregular cell shapes. We observed changes in the outer membrane, peptidoglycan layer, and cytoplasmic membrane damage that resulted in ribosomal and leakage of intracellular material. Additionally, the combination treatments decreased cell area compared to that of cells treated individually or not at all ($p < 0.01$). Furthermore, the P5 and ceftazidime combination group exhibited the smallest cell area (Figure 4).

OM permeability

To evaluate the efficacy of P1, P5, ceftazidime, and combination treatments against bacterial membrane permeability, we measured OM permeability using nitrocefin cleavage as a readout. Our results revealed that resuspended

P1, P5, and ceftazidime alone caused a significant increase in OM permeability ($p < 0.01$) (Figure 5). Moreover, treatment with ceftazidime plus P1 or P5 resulted in significantly higher OM permeability than those of these agents treated alone and controls ($p < 0.01$).

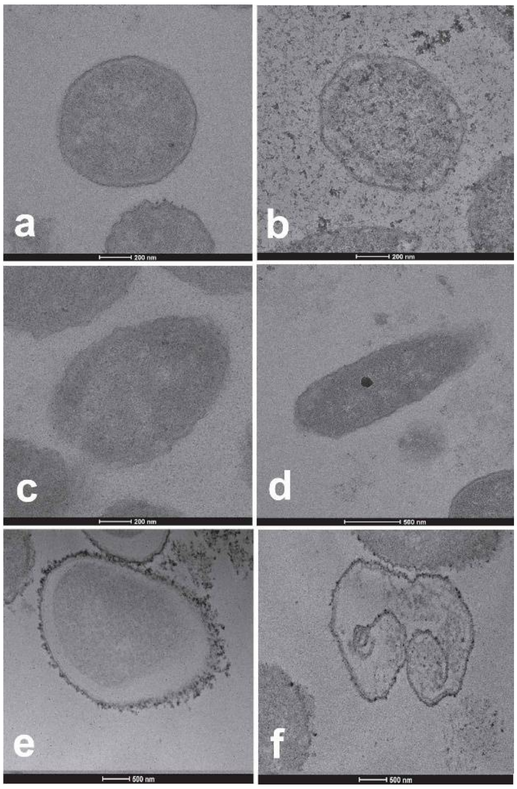

Figure 3: TEM images of CREnC 21394 after 4 h of culture in CAMHB. **a** = Control, **b** = CREnC 21394 treated with P1, **c** = CREnC 21394 treated with P5, **d** = CREnC 21394 treated with ceftazidime, **e** = CREnC 21394 treated with ceftazidime plus resuspended P1, and **f** = CREnC 21394 treated with ceftazidime plus resuspended P5 (scale bar for **a** - **c** = 200 µm; **d** - **f** = 500 µm). Compound concentration was 512 µg/mL for individual treatments, and 16 µg/mL each for combination treatment; 0.9 % NaCl was the vehicle treatment applied

CM permeability

To evaluate CM permeability in response to treatment with P1, P5, ceftazidime, or combinations of the three, we quantified ONPG cleavage by CREnC 21394 following treatment. We found that combination treatment with resuspended P1 or P5 plus ceftazidime resulted in significantly greater CM permeability, illustrated by increased OD levels compared to individual treatments and the control treatment ($p < 0.01$) (Figure 6). These results demonstrate that ceftazidime acts synergistically with P1 or P5 to increase CM permeability, suggesting that these combinations could be effective agents against CREnC.

β-lactamase inhibition

Next, we evaluated the efficacy of different treatments on β-lactamase inhibition by quantifying the ability of β-lactamase to break down benzylpenicillin. We found that the combination of P1 or P5 plus ceftazidime treatment inhibited benzylpenicillin depletion, compared to untreated controls and individually treated cells ($p < 0.01$). In addition, individual P1, P5, and ceftazidime treatment resulted in significantly higher remaining benzylpenicillin than control cells ((Figure 7, $p < 0.01$).

DISCUSSION

We compared the MWs of resuspended P1 - P5 fractions using SDS-PAGE (23 - 160 kDa), with those described in a previous report by Threenet *et al.*, who found that the protein profiles of Siamese crocodile serum presented 6 bands at MWs 225, 121, 67, 62, 45, and 25 kDa, respectively [19]. Therefore, the range of P1 - P5 MWs in our study was consistent with that previously reported.

Crocodile-derived antibacterial has shown some success to date. For example, crocosin isolated from *C. siamensis* plasma inhibits the growth of susceptible strains of *S. typhi* and *S. aureus* [20]. In addition, another study demonstrated that *Crocodylus siamensis* hepcidin (Cshepc) has antibacterial properties and inhibits growth of Gram-negative and Gram-positive bacterial species [21].

Using a checkerboard assay, we found that both resuspended P1 and P5 plus ceftazidime, cefotaxime, or benzylpenicillin exhibited very high, high, or partial synergistic activity respectively against CREnC strains.

Our findings demonstrate that the resistance of these CREnC strains, which express ESBL-AmpC combination resistance genes, was reversed by combining ceftazidime treatment with either resuspended P1 or P5, which significantly increased the efficacy of ceftazidime. Additionally, we found that resuspended P1 and P5 exhibited greater effects in combination with cetazidime and cefotaxime than benzylpenicillin. Our kill curve results confirmed that the bactericidal effects of P1 or P5 synergize with ceftazidime, demonstrated by a decrease in viability ≥ 3log10 CFU/mL [22]. Similarly, other compounds, including apigenin and naringenin also enhance ceftazidime activity against CREnC strains [15].

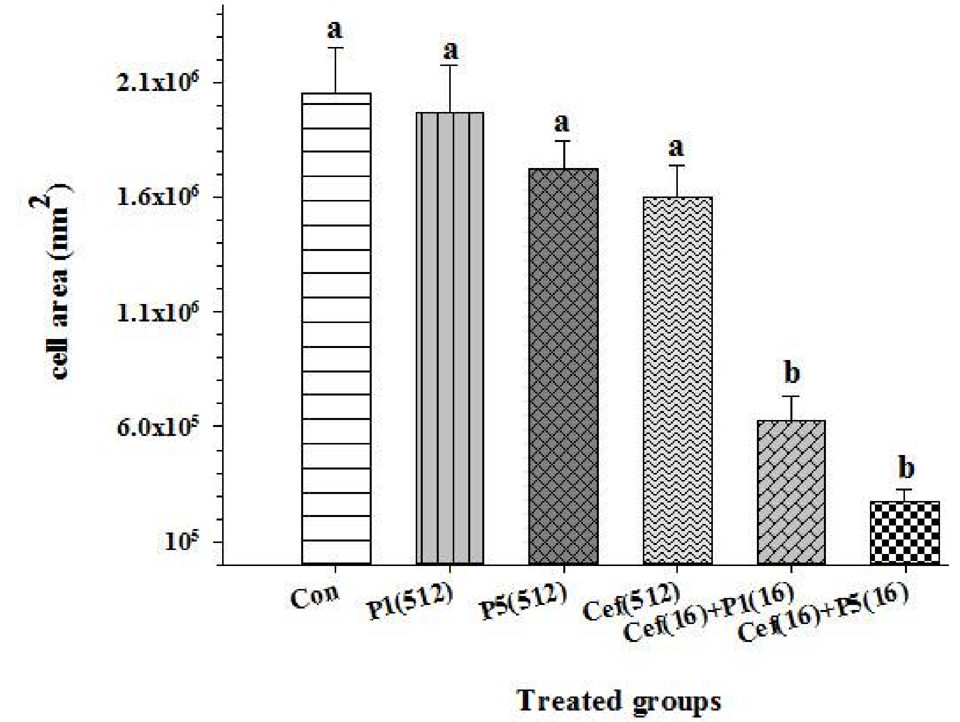

Figure 4: Quantification of cell area following treatment with: Con = control; P1 (512) = P1 (512 µg/mL); P5 (512) = P5 (512 µg/mL); Cef (512) = ceftazidime (512 µg/mL); cef (16) + P1 (16) = ceftazidime + P1 (both 16 µg/mL); and cef (16) + P5 (16) = ceftazidime + P5 (both 16 µg/mL); 0.9 % NaCl was the vehicle treatment applied. Data are mean ± SEM (n = 3). Different letters indicate groups with statistical significance compared with other groups (Tukey's HSD test, $p < 0.01$)

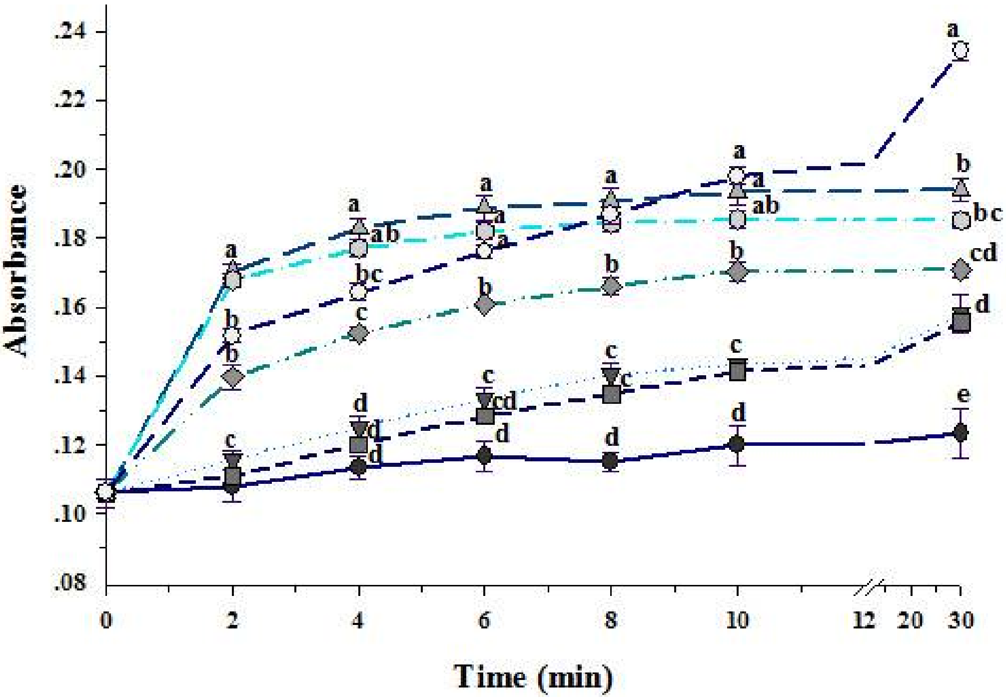

Figure 5: CREnC 21394 OM permeability measurements over time following exposure to: (●) = Control; (■) = P1; (▼) = P5; (◆) = ceftazidime; (⬢) = ceftazidime + P1; (▲) = ceftazidime + P5; and (○) = polymixin B (7 µg/mL). Compound concentration was 512 µg/mL for individual treatments and 16 µg/mL each for combination treatment; 0.9 % NaCl was used as vehicle control. The data are plotted are mean ± SEM (n = 3). Different letters indicate groups with statistical significance compared with other groups (Tukey's HSD test, $p < 0.01$)

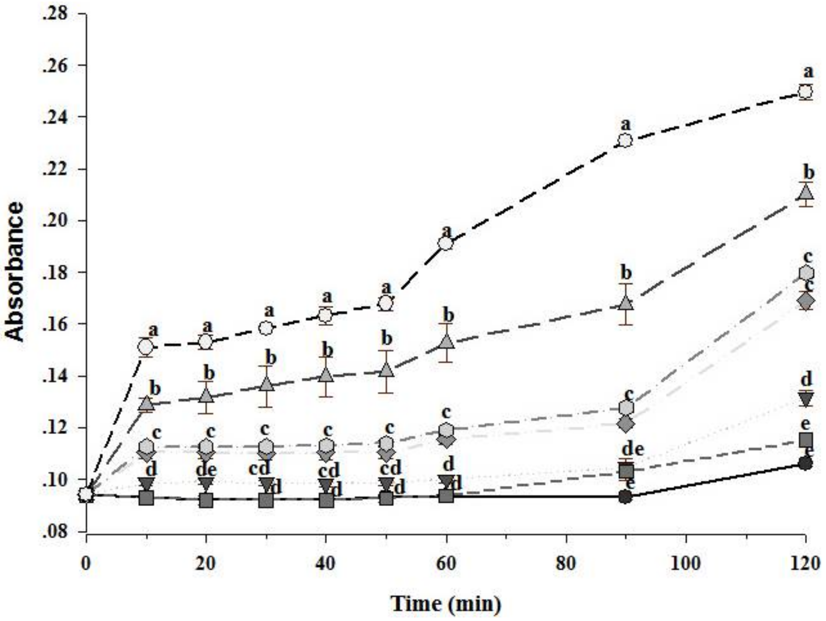

Figure 6: CREnC 21394 CM permeability measurements over time following exposure to: (●) = Control; (■) = P1; (▼) = P5; (◆) = ceftazidime; (⬢) = ceftazidime + P1; (▲) = ceftazidime + P5; and (○) = Nisin (8 µg/mL). Compounds were used at 512 µg/mL for individual treatments, and at 16 µg/mL each for combination treatments; 0.9 % NaCl was used as vehicle control. Data plotted are mean ± SEM (n = 3). Different letters indicate groups with statistical significance compared with other groups (Tukey's HSD test, $p < 0.01$)

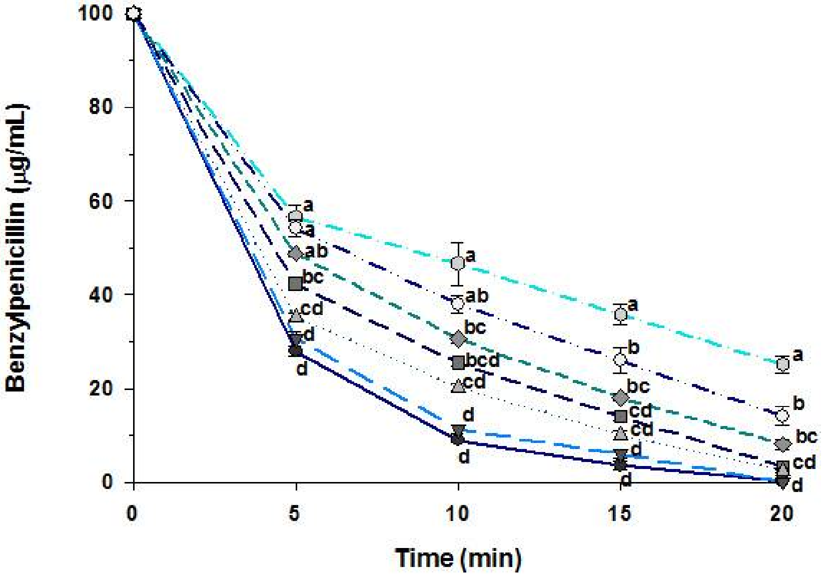

Figure 7: Inhibitory effect of resuspended P1 or P5 on β-lactamase activity. The graph illustrates remaining benzylpenicillin for each treatment group at different timepoints. (●) = control (0.9 % NaCl); (▼) = BSA (negative control); (■) = P1; (◆) = P5; (▲) = ceftazidime; (○) = ceftazidime + P1; and (⬢) = ceftazidime + P5. Compound concentration was 512 µg/mL for individual treatments and 16 µg/mL for each combination treatments; 0.9 % NaCl was the vehicle treatment applied. Data plotted are mean ± SEM (n = 3). Different letters indicate groups with statistical significance compared with other groups (Tukey's HSD test, $p < 0.01$)

Our TEM results demonstrate that cells treated with a combination of either P1 or P5 plus ceftazidime revealed clear morphological damage and significantly decreased cell area. These results are consistent with earlier findings that Siamese crocodile (*C. siamensis*) crude plasma or plasma fractions caused roughening and blebbing of the cell membrane of *S. aureus, S. typhi, E. coli*, *V. cholerae*, *P. aeruginosa,* and *S. epidermidis* [6, 20].

Combination treatment of ceftazidime with resuspended P1 or P5 significantly increased OM permeabilization of CREnC cells compared to control. This enhancement of OM permeability enhancement may be due to the presence of cationic polypeptides in the resuspended protein fractions that may interact via hydrophilic interfacing with the lipopolysaccharide core, or interact electrostatically, disrupting the polarity of the core and blocking interactions between saccharides [23]. Similarly, the novel antibacterial peptides Leucrocin I (molecular mass, approx 806.99 Da) and Leucrocin II (molecular mass, approx. 956.3 Da), isolated from white blood cells of *C. siamensis*, have been reported to combat *S. epidermidis*, *S. typhi*, and *V. cholerae* by increasing OM permeability [24].

The CM permeability findings suggest that one mechanism of action of resuspended protein fractions and ceftazidime combination treatment may be through increasing non-specific CM permeability, which results in cell death following leakage of cellular contents. This is similar to a report that found that apigenin and ceftazidime treatment promotes CREnC CM permeability [15].

β-lactamase plays a crucial role in inactivating β-lactam antibiotics by cleaving their β-lactam ring, resulting in loss of bactericidal efficacy. Additionally, a previous study reported that clavulanic acid, a β-lactamase inhibitor that has played an important role in fighting β-lactam-resistant bacteria, activity is lost through the same mechanism as β-lactam antibiotics [25]. Since P1 and P5 are structurally unlike clavulanic acid, P1 and P5 may not stimulate β-lactamase induction. Unlike resuspended P1 or P5, conventional β-lactamase inhibitors cannot reverse bacterial resistance [15].

The β-lactamase inhibition assay demonstrated that the benzylpenicillin level of the ceftazidime-treated group remaining following the assay was higher than BSA-treated and control groups. Furthermore, the highest remaining of benzylpenicillin levels were observed following P5 and ceftazidime combination treatment. These findings provide evidence that β-lactamase, an enzyme that hydrolyzes the β-lactam ring of the β-lactam antibiotics results in loss of bactericidal activity, from *E. cloacae* (penicillinase from *E. cloacae*) may act more slowly on ceftazidime than benzylpenicillin, both drugs contain a β-lactam ring, due to the fact that ceftazidime is a substrate for the enzyme.

CONCLUSION

Data from this study demonstrate that resuspended P1 or P5 from crocodile plasma, in combination with ceftazidime or cefotaxime has a synergistic effect against CREnC strains. The synergistic activity of these combinations may be due to OM and CM disruption, resulting in increased cell permeability, inhibition of β-lactamase activity, and potential damaging effects on the peptidoglycan structure. Therefore, it seems that P1 or P5 can be used in combination with ceftazidime to treat ceftazidime-resistant *E. cloacae* infection, which is currently resistant to the majority of antibiotics. Additional studies will determine whether this therapeutic approach is feasible in animals and humans.

DECLARATIONS

Acknowledgement

The authors thank Thailand Research Fund via Royal Golden Jubilee PhD Program (Grant no. PHD/0023/2554) and Sriracha Moda Farm for providing Siamese crocodile blood.

Conflict of interest

No conflict of interest is associated with this work.

Contribution of authors

We declare that this work was done by the authors named in this article and all liabilities pertaining to claims relating to the content of this article will be borne by them.

REFERENCES

1. *Sanders WEJ, Sanders CC. Enterobacter spp.: pathogens poised to flourish at the turn of the century. Clin Microbiol Rev 1997; 10: 220-241.*
2. *Lob S, Biedenbach D, Badal R, Kazmierczak K, Sahm D. Antimicrobial resistance and resistance mechanisms of Enterobacteriaceae in ICU and non-ICU wards in Europe and North America: SMART 2011–2013. J Glob Antimicrob Resist 2015; 3: 190-197.*
3. *Paterson DL. Resistance in gram-negative bacteria: enterobacteriaceae. Am J Med 2006; 119: S20-28.*
4. *Seral C, Gude MJ, Castillo FJ. Emergence of plasmid mediated AmpC beta-lactamasas: Origin, importance, detection and therapeutical options. Rev Esp Quimioter 2012; 25: 89-99.*
5. *Merchant ME, Roche C, Elsey RM, Prudhomme J. Antibacterial properties of serum from the American alligator (Alligator mississippiensis). Comp Biochem Physiol B Biochem Mol Biol 2003; 136: 505-513.*
6. *Kommanee J, Preecharram S, Daduang S, Temsiripong Y, Dhiravisit A, Yamada Y, Thammasirirak S. Antibacterial activity of plasma from crocodile (Crocodylus siamensis) against pathogenic bacteria. Ann Clin Microbiol Antimicrob 2012; 11: 22.*

7. *Selcer KW, Nespoli LM, Rainwater TR, Finger AG, Ray DA, Platt SG, Smith PN, Densmore LD, McMurry ST. Development of an enzyme-linked immunosorbent assay for vitellogenin of Morelet's crocodile (Crocodylus moreletii). Comp Biochem Physiol C Toxicol Pharmacol 2006; 143: 50-58.*
8. *Bradford MM. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal Biochem 1976; 72: 248-254.*
9. *Teethaisong Y, Eumkeb G, Chumnarnsilpa S, Autarkool N, Hobson J, Nakouti I, Hobbs G, Evans K. Phenotypic detection of AmpC β-lactamases, extended-spectrum β-lactamases and metallo-β-lactamases in Enterobacteriaceae using a resazurin microtitre assay with inhibitor-based methods. J Med Microbiol 2016; 65: 1079-1087.*
10. *Kao CC, Liu MF, Lin CF, Huang YC, Liu PY, Chang CW, Shi ZY. Antimicrobial susceptibility and multiplex PCR screening of AmpC genes from isolates of Enterobacter cloacae, Citrobacter freundii, and Serratia marcescens. J Microbiol Immunol Infect 2010; 43: 180-187.*
11. *Liu IX, Durham DG, Richards RM. Baicalin synergy with beta-lactam antibiotics against methicillin-resistant Staphylococcus aureus and other beta-lactam-resistant strains of S. aureus. J Pharm Pharmacol 2000; 52: 361-366.*
12. *Clinical Laboratory Standards Institute. Methods for Dilution Antimicrobial Susceptibility Tests for Bacteria That Grow Aerobically. In:Matthew AW, Franklin RC, William AC, Micheal ND, George ME, David WH et al., editors. Clinical and Laboratory Standards Institute document M7-A7 Vol 29. 9th ed. Pennsylvania: Clinical and Laboratory Standards Institute; 2013; pp 16-34.*
13. *Eumkeb G, Siriwong S, Thumanu K. Synergistic activity of luteolin and amoxicillin combination against amoxicillin-resistant Escherichia coli and mode of action. J Photochem Photobiol B 2012; 117: 247-253.*
14. *Marques MB, Brookings ES, Moser SA, Sonke PB, Waites KB. Comparative in vitro antimicrobial susceptibilities of nosocomial isolates of Acinetobacter baumannii and synergistic activities of nine antimicrobial combinations. Antimicrob Agents Chemother 1997; 41: 881-885.*
15. *Eumkeb G, Chukrathok S. Synergistic activity and mechanism of action of ceftazidime and apigenin combination against ceftazidime-resistant Enterobacter cloacae. Phytomedicine 2013; 20: 262-269.*
16. *Pimchan T, Maensiri D, Eumkeb G. Synergy and mechanism of action of alpha-mangostin and ceftazidime against ceftazidime-resistant Acinetobacter baumannii. Lett Appl Microbiol 2017; 65: 285-291.*
17. *Clinical and Laboratory Standards Institute. Performance Standards for Antimicrobial Susceptibility Testing; Twenty-Third Informational Supplement. CLSI document M100-S23. 23rd ed. Wayne, Pennsylvania; Clinical and Laboratory Standards Institute; 2013.*
18. *Eliopoulos GM, Moellering RC. Antimicrobial combinations. In: Antibiotic in Laboratory Medicine. 4th ed. Edited by Lorian V. Baltimore, MD; Williams and Wilkins; 1996: 330-396*
19. *Threenet E, Siruntawineti J, Chaeychomsri W. Protein profiles of siamese crocodile blood. In: 49 Kasetsart University Annual Conference, Bangkok (Thailand), 1-4 Feb 2011; 2011.*
20. *Preecharram S, Jearranaiprepame P, Daduang S, Temsiripong Y, Somdee T, Fukamizo T, Svasti J, Araki T, Thammasirirak S. Isolation and characterisation of crocosin, an antibacterial compound from crocodile (Crocodylus siamensis) plasma. Anim Sci J 2010; 81: 393-401.*
21. *Hao J, Li YW, Xie MQ, Li AX. Molecular cloning, recombinant expression and antibacterial activity analysis of hepcidin from Simensis crocodile (Crocodylus siamensis). Comp Biochem Physiol B Biochem Mol Biol 2012; 163: 309-315.*
22. *Basri DF, Xian LW, Abdul Shukor NI, Latip J. Bacteriostatic antimicrobial combination: antagonistic interaction between epsilon-viniferin and vancomycin against methicillin-resistant Staphylococcus aureus. Biomed Res Int 2014; 2014: 461756.*
23. *Junkes C, Harvey RD, Bruce KD, Dolling R, Bagheri M, Dathe M. Cyclic antimicrobial R-, W-rich peptides: the role of peptide structure and E. coli outer and inner membranes in activity and the mode of action. Eur Biophys J 2011; 40: 515-528.*
24. *Pata S, Yaraksa N, Daduang S, Temsiripong Y, Svasti J, Araki T, Thammasirirak S. Characterization of the novel antibacterial peptide Leucrocin from crocodile (Crocodylus siamensis) white blood cell extracts. Dev Comp Immunol 2011; 35: 545-553.*
25. *Tzouvelekis LS, Zissis NP, Gazouli M, Tzelepi E, Legakis NJ. In vitro comparative assessment of beta-lactamase inhibitors and their penicillin combinations against selected enterobacteria. Int J Antimicrob Agents 1997; 8: 193-197.*