



## Antibacterial Activity of *Bauhinia acuminata* L. Seed Protein Extract with Low Hemolytic Activity Against Human Erythrocytes

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### ABSTRACT

Crude extract showing strong antibacterial activity against various pathogenic bacteria both gram positive and gram negative was isolated from the seed kernels of *Bauhinia acuminata* L. The Minimum Inhibitory Concentration (MIC) values against the most sensitive strain, *Bacillus subtilis* (ATCC 7058) and the least sensitive strain, *Pseudomonas aeruginosa* (ATCC 27853) were 8.34 and 66.72  $\mu\text{g}/\text{mL}$  respectively. The concentration of crude extract which gave 50% hemolysis compared to Triton X-100 treatment ( $\text{HC}_{50}$ ) value was 715.3  $\mu\text{g}/\text{mL}$ . At the MICs range, the crude extract did not show significant hemolytic activity ( $<5.0\%$ ). Diminishment of antibacterial activity by the crude extract after treatment with Proteinase K and Pronase supports the responsible compound of peptides (proteins) in the crude extract. The scanning electron microscopy (SEM) experiments indicated that the bacteriolytic properties of this crude extract might be related to their disruptive action on the cell membrane, characterized by a number of bubble-like formations or even cell lysis.

**Keywords:** antibacterial activity, antimicrobial peptides, *Bauhinia acuminata* L., leguminous plant.

### 1. INTRODUCTION

Bacteria may cause numerous diseases ranging from basic diseases found in daily life such as diarrhea, acne and tooth decay to very harmful diseases like tuberculosis and anthrax. Traditional antibiotics which have been previously used successfully for controlling bacterial pathogens are now less effective. This situation is due to the increasing antibiotic

resistance which is currently shown by several bacteria [1]. The search for new antibacterial compounds which have different mechanisms of action from those in current use is an alternative way for solving this problem.

Many types of molecules with antibacterial activity have been isolated from plants [2, 3]. Among them, peptides with antimicrobial

activity have recently been reported. They are recognized as important as components of the innate defense system of bacteria, fungi, insects, animals and plants. Most of these defense peptides normally have multitasked activities. Some peptides can selectively inhibit gram positive or negative bacteria although antimicrobial peptides with gram positive and gram negative bacteria growth inhibiting ability have been reported [4]. In addition, some peptides can inhibit other types of microorganisms including fungi and virus. Seeds of leguminous plants have been reported to produce a number of peptides and proteins with antimicrobial activities [5]. These seed extracts including their peptides and proteins have been interested by our group for number of years.

In this report, the antibacterial activity of crude extract from *Bauhinia acuminata* L. seed kernels was evaluated, including the effect of this extract on human red blood cells.

## 2. MATERIALS AND METHODS

### 2.1 Microorganisms and Plant Seeds

Six bacteria were collected including the Gram-positive species: *Staphylococcus epidermidis* (clinical isolate), *Staphylococcus aureus* (ATCC 25923) and *Bacillus subtilis* (ATCC 7058) and Gram-negative species: *Pseudomonas aeruginosa* (ATCC 27853), *Shigella flexneri* (DMST 4423, Department of Medical Sciences Type Culture Collection, Thailand) and *Escherichia coli* O157:H7. Strains of *B. subtilis* (ATCC 7058), *S. flexneri* (DMST 4423), *P. aeruginosa* (ATCC 27853) were kindly provided by Dr. Rungruedee Thiwthong, Mahasarakham University and others were obtained from Assoc. Prof. Dr. Sompong Thammasirirak, Khon Kaen University, Thailand. *S. epidermidis* (clinical isolate) strain was isolated from a patient of Srinagarind Hospital, Khon Kaen University by Assoc. Prof. Dr. Sompong Thammasirirak

and the strain was collected at the Department of Microbiology, Faculty of Science, Khon Kaen University, Thailand. They were grown in Luria-Bertani (LB, 10 g/L NaCl, 5 g/L yeast extract and 10 g/L tryptone) medium. The seeds of *Bauhinia acuminata* L. were collected from Mahasarakham Province of Thailand in September 2008 and identified by Mr. Pasakorn Bunchalee, Department of Biology, Faculty of Science, Mahasarakham University, where a voucher specimen (P. Bunchalee 12/01/09) was deposited.

### 2.2 Extraction of *Bauhinia acuminata* Seed Kernels

Since we are interested in peptides with antibacterial activity, the extraction method used was that reported as successful for antimicrobial peptide extraction. The extraction was performed as described by Franco et al. [6] with some modifications. Briefly, the seed kernels were ground in liquid nitrogen to a fine powder. Extraction was performed by extracting 1 g of powdered seed kernel with 3 mL of extraction solution (0.01 M HCl containing 0.15 M NaCl) and stirred at 4°C for 2 h. The extracted solution after filtering through cheese cloth was heated at 85°C for 10 min and then centrifuged at 6,000 rpm and 4°C for 30 min.

### 2.3 Protein Determination

Protein concentrations were determined as described by Bradford method [7]. Briefly, an aliquot of appropriate dilution of sample (10-50 µL) was mixed with 200 µL of commercial protein determination reagent (Bio-Rad), and made up to 1 mL with distilled water. After mixing and stand in room temperature for 5 min, the absorbance was read at 595 nm, and the protein content was calculated with a bovine serum albumin (BSA) as standard.

#### 2.4 Antibacterial Activity and Minimum Inhibitory Concentration (MIC) Assay

Disc diffusion assay was performed as described by Lo Cantore et al. [8] with some modifications as follows. Overnight cultures of the reference strains bacteria were grown in LB medium. Fifty microliters of overnight culture was added to 5 mL of a new broth and incubated at 37°C with shaking at 120 rpm until reaching the mid-exponential phase. Then 1 mL of appropriate dilution of this culture was added to 5 mL warm melted LB agar. After mixing, the overlay gel was poured over sterile 15 mm × 100 mm glass petri dishes containing 10 mL LB agar as underlay gel. The sterile discs (6 mm in diameter) were then placed on plates. The crude extract and a negative control (0.01 M HCl containing 0.15 M NaCl) were applied onto each different disc. Ten microliters of Kanamycin (100 mg/mL) were also loaded onto a disc and used as antibacterial positive control. The plates were incubated for 12-18 h at 37°C. After incubation, the diameters of the inhibition zone surrounding the disc were measured. The experiments were carried out in triplicate.

The MIC assay was performed as follows. The tested bacteria were first grown to mid-exponential phase and diluted with the double strength LB broth. The 0.45 µm filtered *B. acuminata* L. crude extract was prepared with 2-fold serial dilutions by double strength LB broth in a 96-well microtiter plate. After that 50 µL of bacterial suspension as previously prepared was mixed with 50 µL of the 2-fold diluted serial dilution of crude extract in a microtiter plate well with three replicates for each test sample. The MIC was determined after incubation for 18 h at 37°C [9]. Kanamycin was used as antibacterial positive control. The MIC value was defined as the lowest concentration of the crude extract which gave no visible growth on the plate.

#### 2.5 Treatment of Crude Extract by Proteinase K and Pronase

In order to figure out whether antibacterial activity is the cause of peptides (or proteins) in the crude extract, the crude extract was treated with two proteolytic enzymes, Proteinase K and Pronase. The proteinase K (or Pronase) was added into 200 µL of crude extract (3.55 mg/mL protein) in which the protein amount ratio of protein substrate and proteolytic enzymes equaled to 1:8. The treatment reaction was performed at 37°C for 20 h [10]. After treatment, the supernatant was obtained by centrifugation at 10,000 rpm for 5 min and used for antibacterial activity assay. Aliquots of proteinase K and pronase treated mixtures were subjected to a 15% Tris-tricine sodium dodecyl sulfate-polyacrylamide gel electrophoresis (Tris-tricine SDS-PAGE) and stained with coomassie brilliant blue R-250. At least 2 independent experiments have been performed.

#### 2.6 Scanning Electron Microscopy (SEM)

Scanning electron microscopy was performed as described by Yenugu et al. [11] and Agizzio et al. [12] with slight modification. In brief, the *S. aureus* and *P. aeruginosa* were grown in LB broth to a mid-exponential phase and centrifuged at 8,000 rpm for 5 min. An aliquot of bacteria solutions, in 10 mM phosphate buffer, pH 7.2, were mixed with crude extract from *B. acuminata* L. with final concentration of 1×10<sup>6</sup> cfu/mL and 1.2 µg/mL, respectively, in final volume of 500 µL. After incubation at 37°C for 1-3 h, the bacterial cells were washed and resuspended in 10 mM phosphate buffer, pH 7.4 and fixed overnight at 4°C with 2.5% (v/v) glutaraldehyde. Subsequently, the bacterial cells were rinsed three times with the above buffer, post-fixed for 2 h at room temperature with 1.0% osmium tetroxide diluted in the same buffer and rinsed with distilled water. After this

procedure, the bacterial cells were dehydrated through a grade series of acetone at 20, 40, 60, 80 and 100%. The cells were re-suspended with 100% acetone and mounted on cover slips to dry at room temperature. The samples were coated with gold using a sputter coater. Samples were examined using a scanning electron microscope.

### 2.7 Hemolytic Activity

Hemolytic activity was evaluated as described previously by Andrä et al. [13] with a slight modification. Human erythrocyte suspensions were washed with phosphate buffer saline (PBS: 1.5 mM  $\text{KH}_2\text{PO}_4$ , 2.7 mM KCl, 8.1 mM  $\text{Na}_2\text{HPO}_4$ , 135 mM NaCl, pH 7.4) and then centrifuged at  $6,000 \times g$  for 10 min. After washing four times with PBS (or until the supernatant was colorless), the human erythrocytes were Re-suspended and diluted to 10 times of the original volume with PBS, referred as stock erythrocyte suspension. Then, 150  $\mu\text{L}$  of *B. acuminata* L. crude extract (0-577.0  $\mu\text{g}/\text{mL}$ ) in PBS was incubated with 150  $\mu\text{L}$  of stock human erythrocyte suspension (final human erythrocytes concentration, 4% v/v) for 60 min at 37°C. After the incubation period, the reaction mixtures were centrifuged at  $1,000 \times g$  for 10 min to remove intact erythrocytes. The 10-fold dilution of the supernatant of released hemoglobin was measured at 540 nm using a microplate reader. The triplicated experiments were done. Hemolytic activity was expressed as a percentage hemolysis, which was calculated using the following equation:

$$\% \text{ Hemolysis} = \frac{A_{\text{sample}} - A_{\text{buffer}}}{A_{\text{max}} - A_{\text{buffer}}} \times 100$$

Where ' $A_{\text{sample}}$ ' is  $A_{540}$  of red blood cells with peptide solution in PBS, ' $A_{\text{buffer}}$ ' is  $A_{540}$  of red blood cells in PBS, and ' $A_{\text{max}}$ ' is  $A_{540}$  of

red blood cells with 1% (v/v) Triton X-100 in PBS. No hemolysis (0%) and full hemolysis (100%) were observed in the presence of PBS and 1% (v/v) Triton X-100, respectively.

## 3. RESULTS AND DISCUSSION

### 3.1 Protein Content of Crude Extract from *B. acuminata* L. Seed Kernels

Seeds of *B. acuminata*, dry seeds 30.0 g, were extracted with 90 mL of extraction solution. After freeze drying and redissolving in a small volume, it was found that the protein concentration was 4.27 mg/mL.

### 3.2 Minimum Inhibitory Concentration (MIC)

According to disc diffusion assay, *B. acuminata* L. kernel crude extract showed inhibition towards all tested pathogenic bacteria including *S. epidermidis* (clinical isolate), *S. aureus* (ATCC 25923) and *B. subtilis* (ATCC 7058) which are gram positive bacteria and *P. aeruginosa* (ATCC 27853), *S. flexneri* (DMST 4423) and *E. coli* O157:H7 which are gram negative bacteria (data not shown). The MIC values of the crude extract toward these strains were evaluated, with results as shown in Table 1. Sensitivity towards different bacteria by *B. acuminata* seed crude extract was *B. subtilis* (ATCC 7058) > *S. epidermidis*, *S. flexneri* (DMST 4423), *E. coli* (O157:H7) > *S. aureus* (ATCC 25923) > *P. aeruginosa* (ATCC 27853). Plant extract are generally more active on gram positive than gram negative bacteria. Mostly, plant extracts contain oils, phenolic and flavonoid compounds which response for antimicrobial activities. However, the extract of *B. acuminata* L. seeds was well active on gram negative bacteria. There might be other molecules apart from oils, phenolic and flavonoid compounds in the extract response for this ability. It has been reported that crude protein extracts of cruciferous vegetables had greater inhibitory effects against Gram-

**Table 1.** The MIC of *Baubinia acuminata* extract against selected bacteria.

Bacteria	MIC ( $\mu\text{g}/\text{mL}$ )	
	<i>B. acuminata</i> extract	Kanamycin
<b>Gram positive bacteria</b>		
<i>Bacillus subtilis</i> ATCC 7058	8.34	12.50
<i>Staphylococcus aureus</i> ATCC 25923	33.36	6.30
<i>Staphylococcus epidermidis</i>	16.68	15.70
<b>Gram negative bacteria</b>		
<i>Escherichia coli</i> O157:H7	16.68	100.00
<i>Pseudomonas aeruginosa</i> ATCC 27853	66.72	625.00
<i>Shigella flexneri</i> DMST 4423	16.68	62.50

negative bacteria than Gram-positive bacteria [14]. It is possible that the response molecule for antibacterial activity in *B. acuminata* L. crude extract might be protein or peptide. It was obvious that *B. acuminata* L. crude extract gave more nine times active than kanamycin against *P. aeruginosa*. Kanamycin have been reported to interact with the 30S subunit of prokaryotic ribosomes. It induces substantial amounts of mistranslation and indirectly inhibits translocation during protein synthesis [15]. However, many bacteria have evolved way to adapt or become resistant to currently available antibiotics. It is possible that *B. acuminata* L. extract has a different mechanism to inhibit *P. aeruginosa* with more effective than kanamycin.

*B. acuminata* crude extract showed interesting antibacterial activities which could inhibit all tested gram positive and gram negative bacteria. The bacterial inhibition activities depended on bacterial strain. A previous report [6] has shown that purified peptide from cowpea seeds had a MIC of 128  $\mu\text{g}/\text{mL}$  for *S. aureus* (ATTC 25923) and 64  $\mu\text{g}/\text{mL}$  for *E. coli* (ATTC 25922). These MIC values are about four times higher than those MIC values from our work, which implies that *B. acuminata* crude extract shows a better antibacterial ability for both bacteria. Crude protein extract of *Moringa oleifera* seeds

have been reported to possess antibacterial activities against *S. aureus* (MIC 24.0 mg/mL) and *E. coli* (28.0 mg/mL). These MIC values are much more than our results which showed that crude extract of *B. acuminata* L. seeds are more potent for bacterial inhibition [16]. Partially purified protein extract from seeds of *Robinia pseudoacacia* have been reported to have antibacterial activity with MIC values of 20  $\mu\text{g}/\text{mL}$  for *S. aureus* and 120  $\mu\text{g}/\text{mL}$  for *E. coli* [17]. From the MIC values obtained we can conclude that the ability to inhibit *S. aureus* was similar between partially purified protein extract from *R. pseudoacacia* and crude protein from *B. acuminata* L. However, crude extract of *B. acuminata* L. could inhibit *E. coli* better than partially purified protein extract from *R. pseudoacacia*.

A good MIC value against the tested bacteria indicates this extract has a high potential for widespread applications. This extract might be used for decontamination of *E. coli* or *S. flexneri* in food samples. The extract might be used for control of *P. aeruginosa* which was reported to be resistant to common antibiotic classes [18].

### 3.3 Proteinase K and Pronase Treatment

The *B. acuminata* crude extract after treatment with protein hydrolytic enzymes, proteinase K and pronase revealed a lesser



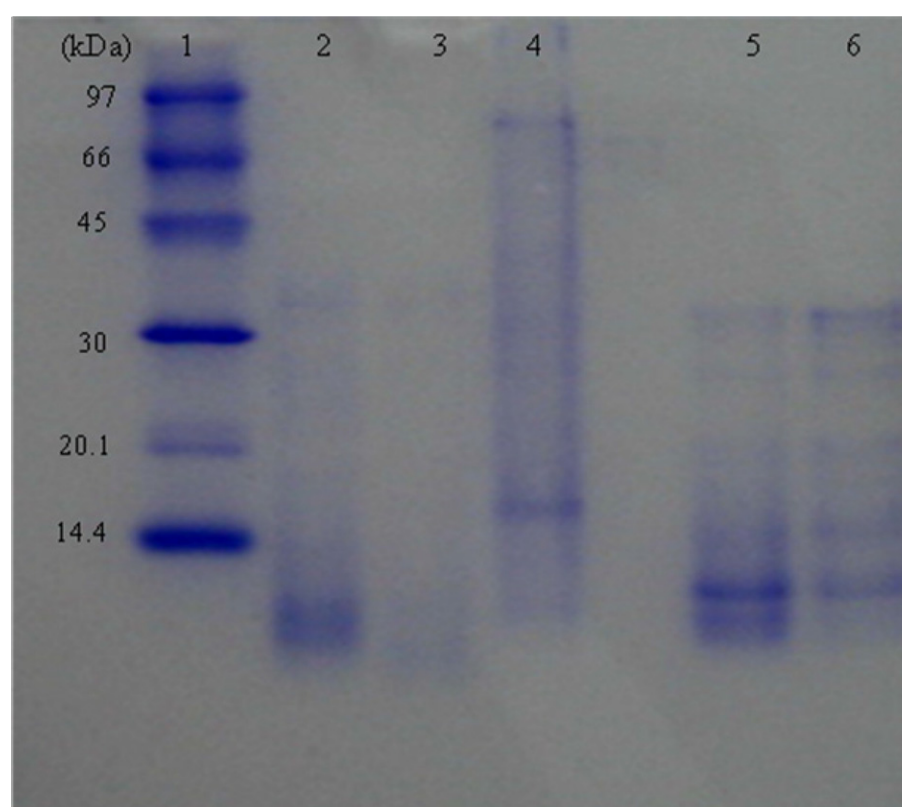
The decline of antibacterial activity in

inhibition against *S. aureus* compared to control reactions. Data are shown in Table 2. The decline of antibacterial activity in the protease treated *B. acuminata* crude extract, might be due to a partial hydrolysis of the crude peptides by an enzymatic process. Figure 1

represents the protein patterns of each reaction. The *B. acuminata* extract treated with pronase and proteinase K showed different protein patterns from untreated extract. However, some protein bands have not been digested, especially the proteins with

**Table 2.** Inhibition zone of *Baibinia acuminata* crude extract after treatment with proteinase K and pronase.

Samples	Loaded protein ( $\mu\text{g}$ )	Inhibition zone (mm)	
		C+	test
Crude extract + buffer	100	23.0	8.0 $\pm$ 0.0
Crude extract + Proteinase K	100	23.0	5.3 $\pm$ 0.6
Crude extract + Pronase	100	25.0	4.0 $\pm$ 0.0



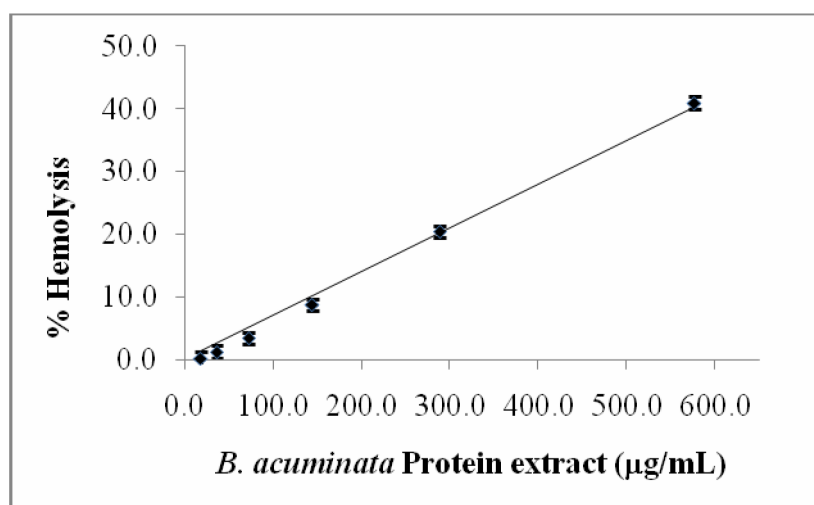
**Figure 1.** Tris-tricine SDS-PAGE of *Baibinia acuminata* crude extract treated with proteolytic enzymes. 1, molecular mass marker; 2, crude extract treated with pronase; 3, pronase; 4, crude extract without proteolytic enzyme treatment; 5, crude extract treated with proteinase K; 6, proteinase K.

low molecular weight. Therefore, these proteins might be involved in antibacterial activity. It has been reported that within one plant there may be more than one type of antimicrobial peptides [19], thus the remaining antibacterial activity of protease treated extract might come from the undigested antimicrobial peptide. There are some reports of protease inhibitors from *Bauhinia* sp., For example, *Bauhinia* serine proteinase inhibitors [20], *Bauhinia* Kunitz-type proteinase inhibitors [21-23] and *Bauhinia variegata* trypsin inhibitor (BvTI) [24]. These inhibitors might partly obstruct protease working, resulting in only some part of proteins in the extract being digested.

### 3.4 Hemolytic Activity

Several antimicrobial peptides have been reported to exhibit cytotoxic activity against eukaryotic cells. Therefore, *B. acuminata* L.

crude extract in which peptides were expected as antibacterial molecules was tested for hemolytic activity against human erythrocytes. It was found that *B. acuminata* crude extract showed hemolytic activity against human erythrocytes in a dose-dependent manner with concentration of peptide at which 50% hemolysis compared to Triton X-100 treatment ( $HC_{50}$ ) value equaled 715.3  $\mu\text{g}/\text{mL}$  (Figure 2). The well-known hemolytic peptide mellitin has been reported to cause 50% hemolysis at 7.5  $\mu\text{g}/\text{mL}$  [25]. The  $HC_{50}$  value from *B. acuminata* crude extract is far larger than that of mellitin and accompanies consideration with the MIC values against the least sensitive tested bacteria, *P. aeruginosa*, which was found to have a  $HC_{50}$  value about 10 times larger than the MIC value (66.72  $\mu\text{g}/\text{mL}$ ). These results imply that the application of this crude extract might be possible for safe use with humans.

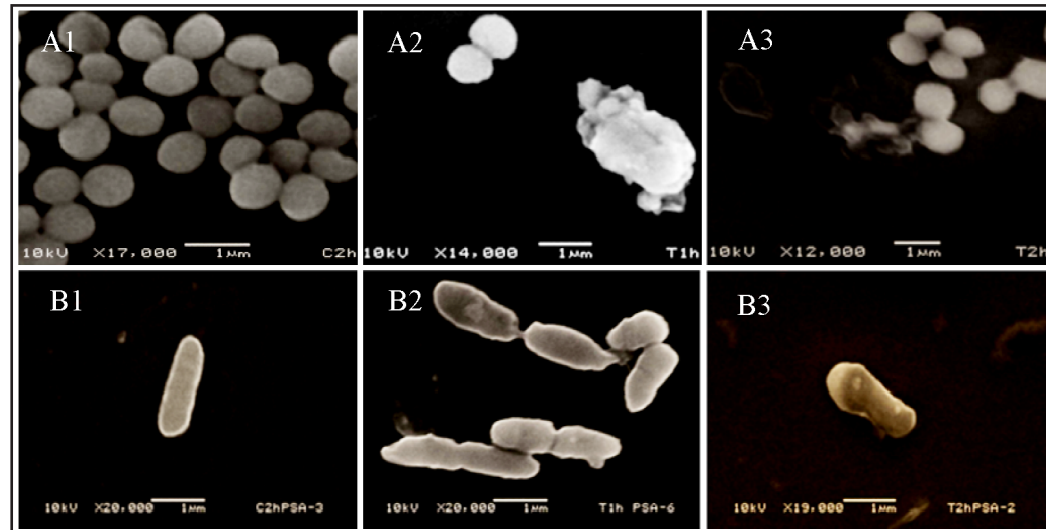


**Figure 2.** The susceptibility of freshly collected erythrocytes to hemolysis by *Bauhinia acuminata* crude kernel extract.

### 3.5 Scanning Electron Microscopy (SEM)

*S. aureus* and *P. aeruginosa* were treated with *B. acuminata* crude extract at 37°C for 1 h and 2 h. Then, scanning electron microscopy

of these bacteria was performed and compared to control (Figure 3). It was found that normal morphology of both control bacteria were observed while morphological changes with



**Figure 3.** Scanning electron micrographs of *S. aureus* (A) and *P. aeruginosa* (B) treated with *Baubinia acuminata* crude protein extract at 37°C for 1 h (A2 and B2) and 2 h (A3 and B3) and compared with controls (A1 and B1). Abnormal morphology (membrane bleb or even destruction) of both tested bacteria *S. aureus* (A2-3) and *P. aeruginosa* (B2-3) were observed while controls showed normal morphology.

membrane bleb or destruction of treated bacteria were seen.

It is believed that the membrane would be the target for antimicrobial peptides [12]. Our results demonstrate that SEM of bacteria treated with *B. acuminata* crude extract shows membrane bleb or destruction. This result supports the possibility that the antimicrobial peptide might be the response molecule for the ability to inhibit bacteria of *B. acuminata* crude extract.

This research has shown that seed crude extract of *B. acuminata* kills bacteria by destroying their membranes. However, this property is less active with human red blood cells which are eukaryote cells. The reason is that the membrane composition of prokaryotes and eukaryotes are different. Zaloff [26] described the outer membranes of bacteria as being heavily populated by lipids with negatively charged phospholipid head groups. In contrast, the outer leaflet of the membrane of eukaryotes is composed principally of

lipids with no net charge. This result from *B. acuminata* crude extract indicates that the extract is possibly safe to utilize in human or other eukaryotes to protect pathogenic bacteria attack or to treat pathogenic bacteria caused diseases.

#### 4. CONCLUSION

Crude extract of *B. acuminata* L. seeds showed strong antibacterial activity against various pathogenic bacteria both gram positive and gram negative with the MICs ranging from 8.34 to 66.72 µg/mL without showing significant hemolytic activity (<5.0% at the MICs range) against human erythrocytes with a HC<sub>50</sub> value compared to Triton X-100 treatment equal to 715.3 µg/mL. Partial loss of bacterial inhibition activity of the crude extract after treatment with proteolytic enzymes supports the hypothesis that peptides or proteins take responsibility for antibacterial activity. The SEM results indicate that the bacteriolytic properties of this crude extract



might be related to their disruptive action on the cell membrane, characterized by a number of bubble-like formations (blebs) or even cell lysis. This research implies the possible application of crude or peptides (proteins) extract of *B. acuminata* L. in foods and pharmaceuticals.

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