Malays. Appl. Biol. (2018) 47(6): 145–152

ANTIMICROBIAL ACTIVITY OF PARTIALLY PURIFIED PEPTIDES ISOLATED FROM THE SKIN SECRETIONS OF BORNEAN FROGS IN THE FAMILY OF RANIDAE

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Accepted 20 December 2018, Published online 31 December 2018

The emergence of drug resistant bacteria has now become a major public health problem worldwide (Cohen, 2000; Kumarasamy *et al*., 2010; Sengupta *et al*., 2013). WHO report (2017) on global surveillance of antimicrobial resistance revealed a widespread development of resistance in both gram positive and gram negative bacteria which had threatened millions of people worldwide. A rapid increase in the number of drug-resistant bacteria and the incidence nosocomial infections pose a challenge to conventional therapies using existing antibiotics, leading to the need in finding alternative microbicides to control these infections (Lakshmaiah *et al*., 2015). Thus, a discovery of new and effective treatments that can replace current available antibiotics has become a critical area of research globally.

Anurans inhabit a wide variety of habitat types from barren deserts to deep freshwater lakes and may spend most of their life underground or high in the cloud of a forest canopy. They are vulnerable towards injuries, predators, parasitisation, micro-organism's infections and wounds (Clarke, 1997). The frog skin plays key roles in the everyday survival of the amphibians and contributes to their ability to exploit a wide range of habitats and ecological conditions. Upon stress or injuries, frogs secrete secretion from the glandular gland as their first line host defense system against penetrating infectious microorganisms (Schadich, 2009, Hancock and Diamond, 2000). Glandular gland also serves as a toxic gland containing antimicrobial peptides (AMPs) and can be found concentrated at the head and neck of the frogs (Rollin-Smith *et al*., 2002). Most AMPs are cationic in nature and share a net positive charge at neutral pH with the high content of hydrophobic residues and an amphipathic character (Galdiero *et al*., 2013; Power & Hancock, 2003). These characteristics allow the frog skin peptides to kill bacteria through cell lysis by binding to negatively charged components of the bacterial membrane (Schadich *et al*., 2013). The AMPs attract attention due to their effectiveness in killing both gram-negative and gram-positive bacteria, without any of the undesirable effects of antibiotic resistance (Conlon and Sonnevand, 2011; Galdiero *et al*., 2015; Schadich, 2009). Thus, amphibian's skin secretions have become the target for the screening and subsequent development of AMPs.

Bornean frogs are the endogenous frogs that inhabit Borneo, an island divided between Indonesia, Brunei and East Malaysia (Sabah and Sarawak). Approximately, more than 150 species of frogs occur in Borneo (Inger and Stuebing, 2005) and are widely distributed throughout the island. Their habitats range from peat swamps, terrane, waterfalls, streams, high altitudes to the forest floors of the tropical rainforest. There are seven families of Bornean frogs present in the island which are Bombinatoridae, Bufonidae, Ceralobatrachidae, Dicroglossidae, Microhylidae, Ranidae and Rhacophoridae.

The current study focuses on the individuals belonging to the family of Ranidae, which is often referred to as 'true frog' under the suborder Neobatrachia. It has been documented that the skin secretions from frogs in the Ranidae family contained brevinin, esulentin and ranateurin that are

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able to inhibit the growth of various bacteria strains (Conlon *et al*., 2011; Kumar *et al*., 2015; Wang *et al*., 2012(a); Wang *et al*., 2012 (b)). A study on a species of Bornean frog from the family of Ranidae (Conlon *et al*., 2008) revealed that the skin secretions of *Hylarana picturata* contained AMPs belonging to the brevinin-1, brevinin-2 and temporin families. The study also revealed that skin secretion of frogs from another Ranidae family, *Odorrana hosii* contained brevinin-1, brevinin-2, esculentin-1, esculentin-2 and nigrocin-2 (Conlon *et al*., 2008). Apart from the works done by Conlon and coworkers, research on the AMPs from the skin secretions of Bornean frogs remain scarce, thus warrants increased efforts to sample these peptides and to study their potential against bacterial species associated with nosocomial infections.

In this study, the presence of AMPs in the skin secretion of several species in the Ranidae family was investigated and partially purified and characterized. Our study focused on activities of skin antimicrobial peptides of Bornean Ranidae frogs against two pathogens associated with nosocomial infections, *Staphylococcus aureus* (ATCC 25923) and *Escherichia coli* (ATCC 25922) and yeast *Candida albicans*. Natural peptide mixture of skin peptides of six different Ranidae frog species including: *Pulchrana glandulosa, Pulchrana signata, Pulchrana baramica, Chalcorana raniceps, Odorrana hosii* and *Meristogenys jerboa* were collected and assayed for microbial growth inhibition. Adult Bornean frogs of the selected species of both sexes (n=5 specimens per species) were collected at few sites in Miri and Kuching. The captured frogs were placed in a glass beaker and anaesthetized using absorbent cotton immersed in 1 ml anhydrous diethyl ether for 10 mins (Lai *et al*., 2010). The skin secretions from the dorsal part of the frogs were then washed with sterile water containing 1 mM EDTA. The secretions collected were immediately centrifuged at 3220 rev/min for 30 min at 4°C and freeze dried.

Concentration and partial purifications of crude extracts were conducted using Sep Pak C-18 (Minn *et al*., 1998). Sep Pak cartridge was first activated with 12 ml of acetonitrile (2ml per cartridge), followed by 0.1% of trifluoroacetic acid in 99.9% water. After being loaded with 5 ml of the supernatant at a flow rate of 4ml/min, the cartridge was washed with 0.1% trifluoroacetic acid in 99.9% water and the bound peptide were eluted with 12 ml of 70% acetonitrile containing 0.1% trifluoroacetic acid (2 ml per cartridge). The final elution was collected in a glass beaker and aliquoted into 1.5 ml microcentrifuge tubes. Samples were stored at -80°C freezer.

Then, 20µg/µl of peptide samples were separated on a 30% polyacrylamide gel at 200V for 60 min (Schägger, 2006). The gel was subsequently fixed using 5% glutaraldehyde solution for 2 hours and protein bands were stained using the silver staining (Gromova and Celis, 2006).

The partially purified peptides were initially screened for antimicrobial activity by disk diffusion assay. Initial dose screening assay showed no inhibition zone at the peptide concentration of lower than 250 µg/ml. Therefore, based on this finding, 500 and 250 µg/ml of peptides were impregnated into sterile disk and placed onto Mueller Hinton agar inoculated with 10^6 CFU/ml of reference strains of *Staphylococcus aureus* (ATCC 25923), *Escherichia coli* (ATCC 25922) and *Candida albicans* for 18 hours at 37°C. After an overnight incubation, the diameter of the inhibition zone was measured.

Further antimicrobial testing was performed by determining the Minimum Inhibitory Concentrations (MIC) of the partially purified peptides from the frog skin secretions according to previously published assay (Schadich, 2009, Schadich 2013). Serial dilutions of the peptides (500, 250, 125, 62.5, 31.2, 15.6, 7.8 and 3.9µg/ml) were incubated in 50 µl Luria Bertani broth containing 50 µl of 10^6 CFU/ml of reference strains of *S. aureus* (ATCC 25923) *E. coli* (ATCC 25922) and *C. albicans* for 18 hours at 37°C. After an overnight incubation, the plate was analysed by reading the absorbance at 620 nm using the ELISA plate reader (Zynth). The MIC of peptide samples were recorded as the lowest concentration where no viability was observed in the wells of 96 microtiter plate after 18 hours.

Haemolytic effect of the antimicrobials frogs' peptides was investigated using human red blood cells (RBC). The RBC were centrifuged at 1800 rcf for 10 min and resuspended in phosphate buffer saline (PBS) to give $5x10^7$ cells count. Skin secretions were prepared in the concentrations of 100, 50, 25, 12.5, 6.25, 3.125 and 1.56 µg/ml and incubated with the RBC at 37°C for 1 hour. RBC were centrifuged at 12000 g for 30 sec and the absorbance of supernatant was measured at 450 nm. Total lysis of erythrocyte suspension was obtained by incubating the cells with 1 % v/v Tween 20. Ampicilin was used as the positive control against *E. coli* and *S. aureus* while Amphotericin-B was used as the positive control against *C. albicans.*

The observed bands from all samples are found concentrated towards the bottom of the gel corresponding to the peptides of lower molecular mass (1-3.5 kDa) (Figure 1). This observation is agreeable to the published reports on similar studies where most AMPs have low molecular mass ranging from 1 to 5 kDa (Conlon 2007, Conlon *et al*., 2008; Conlon & Mechkarska, 2014; He *et al.,* 2013; Memarpoor-Yazdi *et al.,* 2013). Among the peptides isolated from the Bornean frogs are Brevinin-2LSa (2815.5Da), Esculentin-1LSa (4637.6Da), Palustrin-

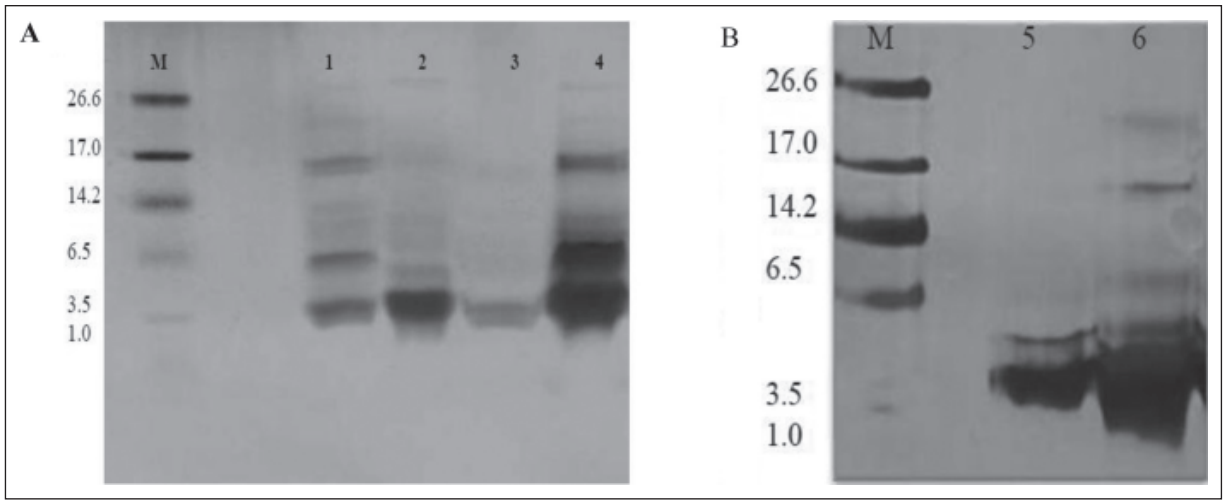

Fig. 1. Silver stained Tricine SDS-Page gel of partially purified secretions from Bornean Ranidae. (A) Lane M represents protein marker (Sigma Aldrich) ranging from 26.6 to 1.0 kDa. Lane 1: *C. raniceps*, lane 2: *P. signata,* lane 3: *P. glandulosa* and lane 4: *P. baramica*. (B) Lane M represents marker, lane 5: *O. hosii*, lane 6: *M. jerboa*.

2LSa (3020.5Da), Temporin-LSa (1361.8Da), Brevinin-2SGa (3126.7Da) and Temporin-SGa (1673Da) (Conlon and Mechkarska; 2014). Further purification steps will largely resolve the molecular weights of the corresponding peptides from the studied Bornean frogs.

Disc diffusion assay (Table 1) indicates the presence of antimicrobial activities of the partially purified peptides against *E. coli, S. aureus and C. albicans* (Figure 2, 3 and 4). Based on the diameter of the inhibition zones formed, the highest activity was shown by the peptides (500 µg/ml) from *M. jerboa* and *P. signata* with 14.33 mm and 13.67 mm, respectively, against the gram negative *E. coli*. Peptides from *P. baramica*, *C. raniceps*, *P. glandulosa* and *O. hosii* demonstrated relatively comparable potency against *E. coli* with the formed inhibition zone diameters between 7-9 mm. Peptides from *P. baramica* and *O. hosii* (500 µg/ml) had higher antimicrobial effect on the gram positive *S. aureus* with the inhibition zones diameter of approximately 14.67 mm and 14.00 mm, respectively. As for the antimicrobial effect against *C. albicans*, most of the tested peptides showed comparable potency against the fungi with the highest diameter exhibited by the peptides from *P. baramica,* while *P. glandulosa* did not exhibit any inhibition effect on *C. albicans* at 500 µg/ml. Determinations of the minimum inhibitory concentrations (MIC) of the frogs' peptides further elucidate the antimicrobial characteristics of these peptides.

Figures 2 to 4 show increased antimicrobial potency against *E. coli, S. aureus* and *C. albicans* with increasing concentrations of the partially purified peptides from the frogs' skin. *P. glandulosa* and *P. baramica* showed the highest antimicrobial activities against *E. coli* with the MIC of 31.3 µg/ml. The MIC of *P. glandulosa, P. signata, C. raniceps* and *P. baramica* against *S. aureus* were found to be more than 500, 250 and 125 µg/ml, respectively (Table 1). *P. baramica* showed the highest antimicrobial activity against *S. aureus* as compared to other tested species with the MIC of 125 µg/ml. Previous structure-activity relationship studies suggested that the antimicrobial activity of AMPs is significantly dependent on their α-helical structure, which is affected by charge, size, helicity and hydrophobicity (Jiang *et al.,* 2008; Tossi *et al*., 2000). Thus, further investigations on the secondary structures of the purified peptides will enhance the knowledge and information regarding the Bornean frogs' AMPs.

The toxicity assay shows low haemolytic activity (less than 50% cell lysis) at the concentration below 12.5 µg/ml for all skin peptides (Figure 5). However, at the AMPs concentration of 100 µg/ml, partially purified peptides from *C. raniceps, P. baramica* and *P. glandulosa* caused more than 50% blood cell lysis. Interestingly, only *P. signata* peptides demonstrated less toxicity against the red blood cells in which at 100 µg/ml, the partially purified secretions caused approximately 23% cell lysis. It is generally accepted that AMP isolated from frog secretions have the characteristics of being toxic to mammalian cells (Jiang *et al*., 2014). The mechanism of which the RBC lyses upon reacting with AMP was not being studied in this project. However, the current findings strongly supported that the extreme toxicity of AMP is related to its higher hydrophobicity (Conlon *et al*., 2008).

To conclude, this study shows that the skin peptides from *P. baramica*, *C. raniceps*, *P. signata P. glandulosa, O. hosii* and *M. jerboa* exhibit

Table 1. Inhibition zone of post Sep-Pak peptide secretions after incubation with *E. coli, S. aureus* and *C. albicans.* 20 µl of peptide samples were incubated at 37°C with *E. coli, S. aureus* and *C. albicans*. After 24 hours, the clear zone surrounding disk impregnated with peptide samples were measured using a ruler. Values were displayed as mean ± SD of triplicates from a single experiment, representative of three separate experiments.

	Diameter of inhibition (in mm)					
	E.coli		*S.aureus*		*C.albicans*	
	Concentration of peptide (µg/ml)					
	500	250	500	250	500	250
P.glandulosa	7.33±0.57	6.16±0.29	10.00±0.00	8.33±0.57	0.00±0.00	0.00±0.00
P.signata	13.67±1.15	11.00±0.00	9.67±2.52	7.33±0.57	8.00±0.00	8.00±0.00
P.baramica	9.66±0.57	7.33±0.57	14.67±0.57	12.33±0.57	11.00±1.15	9.33±0.57
C.raniceps	9.00±0.00	6.16±0.29	8.00±0.00	7.16±0.29	9.33±1.52	7.17±0.29
O.hosii	7.33±0.57	6.33±0.57	14.00±1.00	11.00±0.00	9.00±0.00	6.00±0.00
M.jerboa	14.33±0.00	12.00±0.00	11.33±0.57	8.33±0.57	8.00±1.00	7.00±0.00

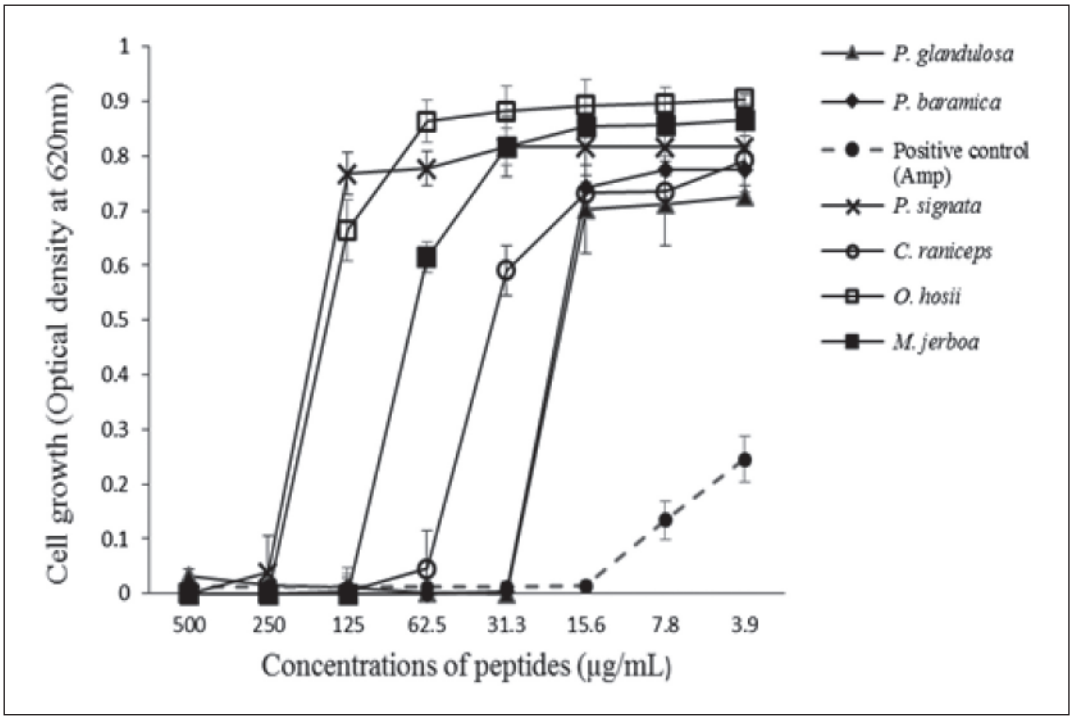

Fig. 2. Minimum inhibitory concentrations of peptides from Bornean Ranidae against the growth of *E. coli*. Partially purified peptides of *P. baramica, P. signata, P. glandulosa, O. hosii, M. jerboa* and *C. raniceps* at concentrations ranging from 500 µg/ml to 3.9 µg/ml were incubated with *E. coli* at 1×10^6 CFU/ml. After 24 hours, the bacterial growth were analysed by reading the optical density at 620 nm using ELISA plate reader. Values were displayed as mean ± SD of triplicates from a single experiment, representative of three separate experiments.

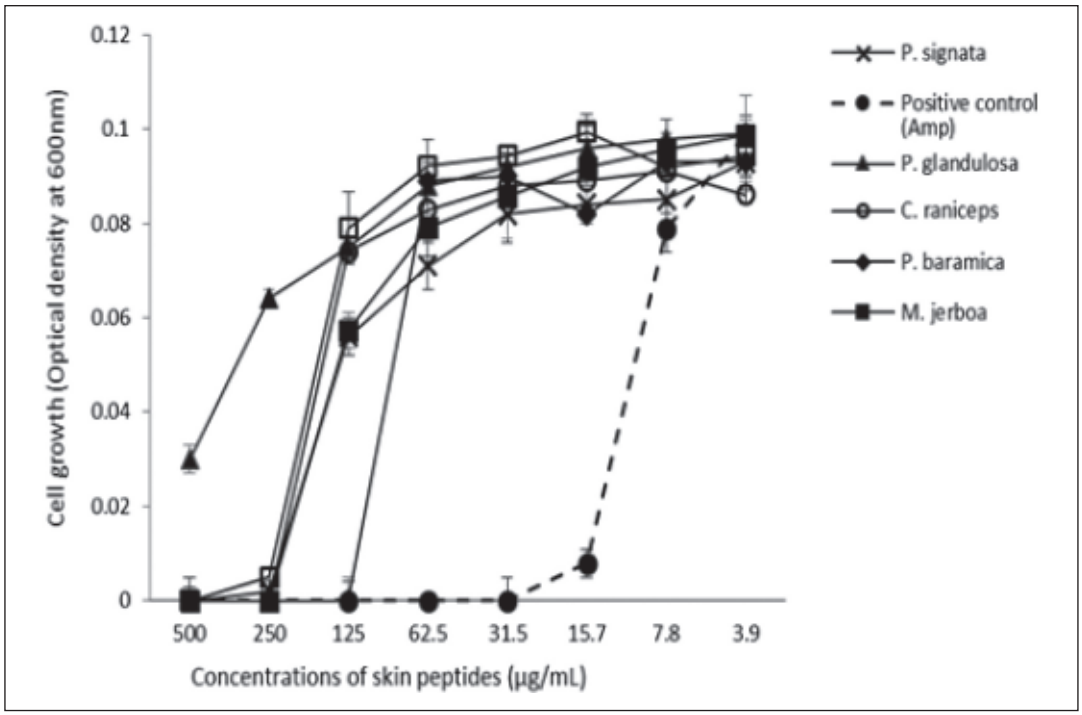

Fig. 3. Minimum inhibitory concentrations of peptides from Bornean Ranidae against the growth of *S.aureus*. Partially purified peptides of *P. baramica, P. signata, P. glandulosa, O. hosii, M. jerboa* and *C. raniceps* at concentrations ranging from 500 µg/ml to 3.9 µg/ml were incubated with *S. aureus* at 1×10^6 CFU/ml. After 24 hours, the bacterial growth were analysed by reading the optical density at 620 nm using ELISA plate reader. Values were displayed as mean ± SD of triplicates from a single experiment, representative of three separate experiments.

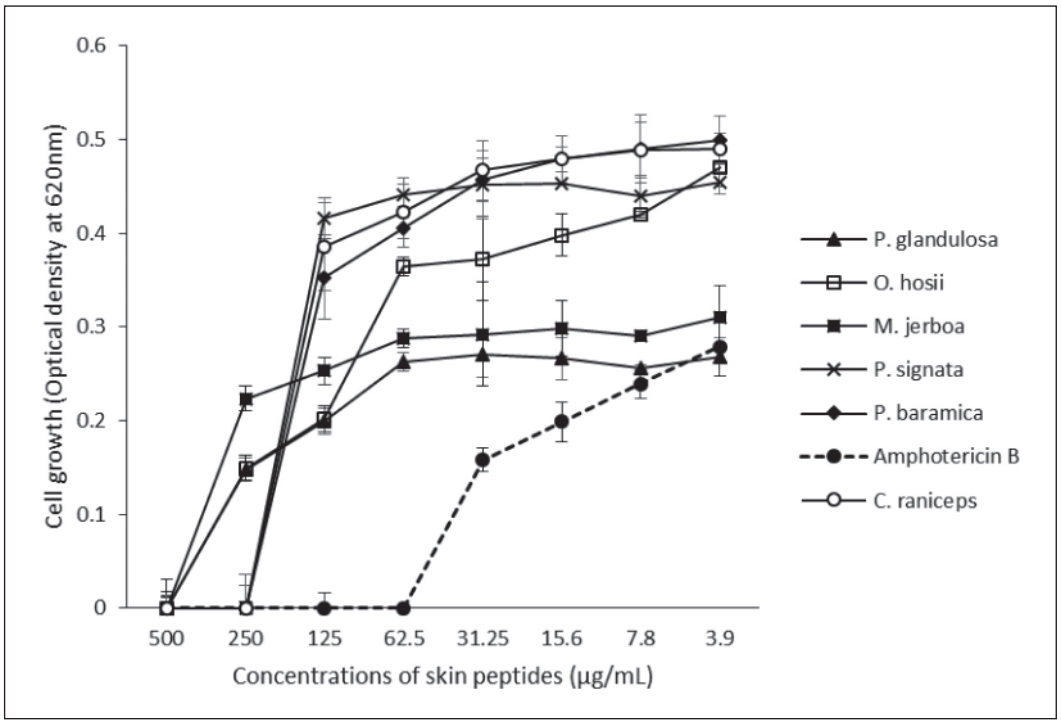

Fig. 4. Minimum inhibitory concentrations of peptides from Bornean Ranidae against the growth of *C. albicans*. Partially purified peptides of *P. baramica, P. signata, P. glandulosa, O. hosii, M. jerboa* and *C. raniceps* at concentrations ranging from 500 µg/ml to 3.9 µg/ml were incubated with *C. albicans* at 1×10^6 CFU/ml. After 24 hours, the fungal growth were analysed by taking the optical density at 620 nm using ELISA plate reader. Values were displayed as mean ± SD of triplicates from a single experiment, representative of three separate experiments.

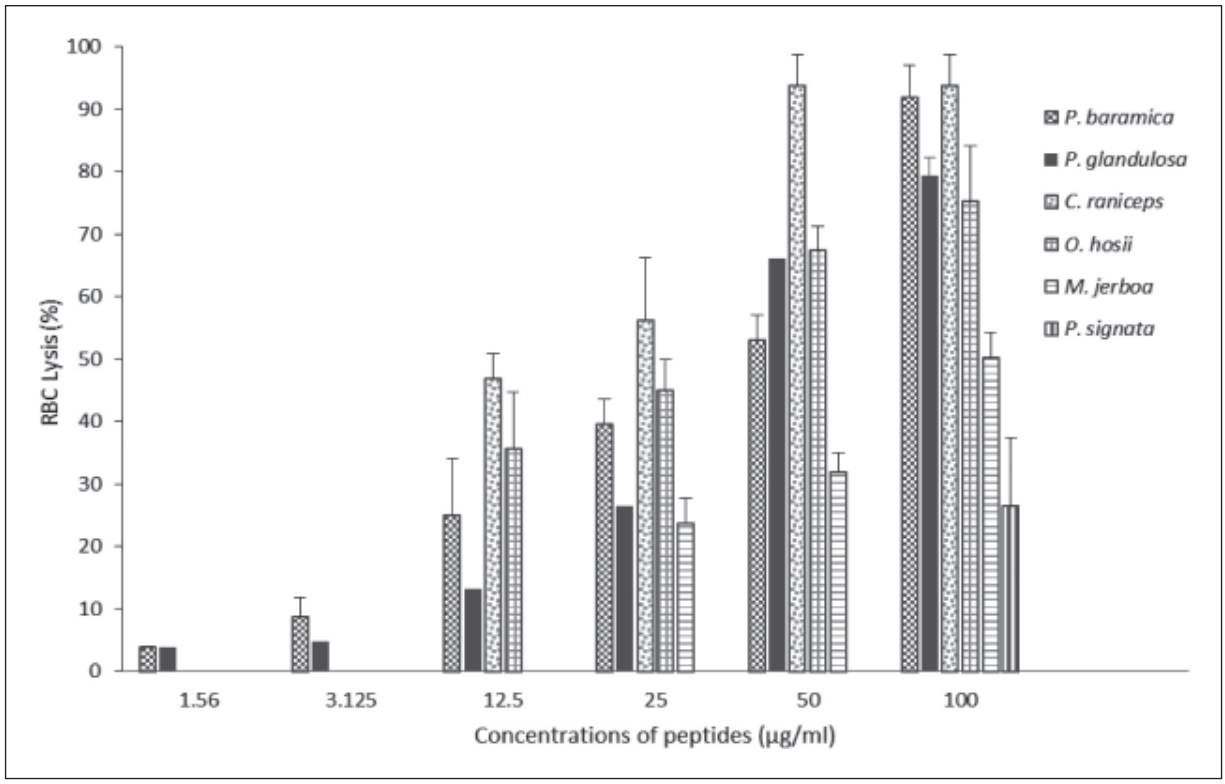

Fig. 5. Hemolytic activity of frog skin secretions on human erythrocytes. Skin secretions after Sep-Pak purification were incubated with 5×10^7 RBC at 37°C in the concentration ranges from 1.56 to 100 µg/ml. After 1 hour, the RBC lysis were analysed using ELISA plate reader. Results are displayed as mean ± SD of triplicates from a single experiment representative of three separate experiments.

Table 2. Minimum Inhibitory Concentrations of Bornean Ranidae peptides against bacterial growth. Partially purified peptides of *P. baramica, P. signata, P. glandulosa, O. hosii, M. jerboa* and *C. raniceps* at concentrations ranging from 500µg/ml to 3.9 µg/ml were incubated with *E. coli, S. aureus* and *C. albicans* at 1×10^6 CFU/ml. After 24 hours, the bacterial growth were analysed by taking the optical density at 620 nm using ELISA plate reader. Results are displayed as representative of three separate experiments

	Minimum inhibitory concentrations (µg/ml)		
	E. coli	*S. aureus*	*C. albicans*
P. glandulosa	31.3	>500	>500
P. signata	250	250	250
P. baramica	31.3	125	250
C. raniceps	62.5	250	250
O. hosii	250	250	125
M. jerboa	125	250	125

apparent antimicrobial activity against gram positive and gram negative bacteria as well as being associated with relatively strong haemolytic activity. The current preliminary data can fill the gap on the knowledge of frogs AMPs and gives deeper insight on the potential of the skin peptides of Bornean frogs against nosocomial bacterial pathogens. Further works need to be conducted to isolate and purify these peptides to homogeneity and subsequently perform the structural characterization of the purified peptides. The potency of these skin peptides against microorganisms makes them the attractive candidates for the development into therapeutically anti-infective agents. However, due to the adverse haemolytic effects of the peptides on the red blood cells, certain chemical modifications should be done prior to further development of these host defense peptides.

ACKNOWLEDGEMENTS

We would like to thank the UNIMAS Frog Research Team and research officer from Sarawak Biodiversity Centre for helping us with frog identifications and samples purifications. This work is fully supported by the FRGS/ST04(01)/1074/2013(20) grant from the Ministry of Higher Education Malaysia.

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