Jurnal Teknologi

Full Paper

PRELIMINARY STUDY OF ANTIMICROBIAL ACTIVITY OF THE SKIN SECRETIONS OF MALAYSIAN FROGS

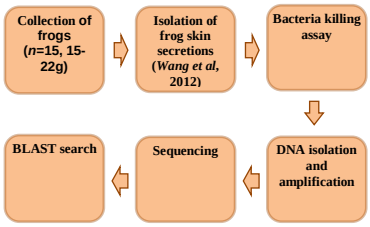
Zainon A. Rahman, Mohamad Faiz Foong Abdullah, Wan Mohamad Syahmin Wan Azmi, Aziyah Abdul-Aziz*

Faculty of Applied Sciences, Universiti Teknologi MARA Malaysia, 40450 Shah Alam, Selangor D.E., Malaysia

Article history
Received
29 July 2015
Received in revised form
10 November 2015
Accepted
14 January 2016

*Corresponding author
aziyah960@salam.uitm.edu.my

Graphical abstract



Abstract

Bioactive compounds that exhibit antimicrobial properties in frogs are parts of the animal defense against microbial infections. The lyophilized frog's skin secretions containing varies bioactive compounds were subjected to screen for their antimicrobial activity. This study was conducted as part of an effort on the search of antimicrobial peptides (AMPs) profiles of Malaysian frogs. The results indicate the collected frog skin secretion has antimicrobial effect against Gram-negative and Gram-positive bacteria. BLAST and standard phylogenetics were used to establish a preliminary identity of the frog samples.

Keywords: Bioactive compounds, antimicrobial activity, frog skin secretions

1.0 INTRODUCTION

Antimicrobial agents are agents or drugs that can kill or inhibit the growth of microorganisms. They include anti-bacterial, anti-fungal, anti-viral and anti-parasitic agents. A vast number of pathogenic microorganisms are now resistant to most of the current treatments. The evolving of microorganisms' resistance towards many antibiotics is growing and has become an important issue as a major world's health problems. It has increased in morbidity, mortality and health-care costs [1]. Prevailing misuse of antibiotics has provided the opportunity for pathogenic microorganisms to change its genetic constituents and achieve resistant mechanism. One way to make it less severe is to search for novel bioactive compounds. Some of the bioactive compounds with useful activities have been reported from the skin secretion of frogs.

A frog's skin is primarily has two types of glands which are mucus and granular glands. Both glands are located mostly at the dorsal part of the frog's skin. The range of bioactive compounds produced by frogs in the skin glands is outstandingly high, even within single species [2]. Examples of bioactive compounds are antimicrobial peptides (AMPs), analgesic peptides, algesic peptides, protease inhibitors, lectins, antioxidant peptides and neurotoxins [3]. The algesic peptides such as bradykinin and tachykinin may participate in fright behaviour during violation and aid the animal to escape from injury. In addition, the analgesic peptides may reduce sufferings. Protease inhibitors and lectins can inhibit parasite contagions and synergize with frog AMPs and take part in antimicrobial protection like AMPs do [4]. Studies have shown that the skin secretion of frogs and other amphibians are known to have large spectrum and useful antibacterial and antifungal activities. It has also been stated that a compound effective against *Staphylococos aureus* (Gram positive), *Escherichia coli* (Gram negative) and against virus that are barely affected by antibiotics was discovered from a frog species of genus Rana [5]. Other than antimicrobial activities, these bioactive compounds particularly AMPs from frog skin secretions also show other attractive biological activities such as antiprotozoa and anti-cancer cells [6]. Skin secretions of a number of frogs have also shown to contain high amount of various types of biologically active constituents that consists of thyrotropic

hormons and the myotropic peptides caerulin, xenopsin and levitide [7].

Frogs belong to family Ranidae, genus *Rana* consists of more than 250 species which are broadly dispersed except for the Polar Regions. Malaysian frogs are divided into six families including Bufonidae, Dicroglossidae, Megophryidae, Ranidae, Microhylidae and Rhacophoridae. These families have 106 species of frogs in Peninsular Malaysia. Besides that, East Malaysia, Sabah and Sarawak have their own frog population. It has been reported that there are five species of frogs from the genus *Hyalarana* that are known to be in Sarawak such as *Hylarana baramica, Hylarana glandulosa, Hylarana signata, Hylarana picturata and Hylarana luctuosa* [8]. In addition, a new species of genus *Kalophrymus* was found at Gunung Mulu National Park in Sabah [9]. Because of their potential roles in fighting pathogenic microorganisms, many new and more animal–friendly methods and approaches have been used to venture this sustainable source of antimicrobials [7]. Skin secretions can now be accessed by electrical or chemical stimulations, without having to kill the animal. The advancement in molecular biology techniques have also allowed for the development of cDNA libraries and cloning of the bioactive compounds in peculiar AMPs gene sequences.

It has been documented that bioactive compounds secreted from frog's skins are known to have only a low possibility to promote resistance against microorganisms as compared to current antibiotics which has become a major problem worldwide [10]. Thus, all these characteristics show the importance of bioactive compounds extracted from frogs making them as an attractive subject in the search to find new antimicrobial characteristics. In Malaysia, such bioactive compound remains unexplored due to inadequate knowledge of their occurrence. Malaysia is known to be rich in flora and fauna, thus frogs from Malaysia may exhibit a source of bioactive compounds that can be paddled for therapeutic applications.

In this current study, the collected frogs were first exposed to diethyl ether, a process that would induce the stress level in the frogs before the frogs were fully anesthetized. The stressed frogs would release mucous secreted at the dorsal part of the frog's skin which was collected and further studied for its antimicrobial properties. The frog would then identify via the nucleotide base identification system using the 16S RNA sequencing method.

2.0 EXPERIMENTAL

2.1 Collection of Frog Skin Secretions

Adult frogs ranging between 15-22 grams were collected from Pelangai Forest, Negeri Sembilan. The collection of the frog's skin secretions was performed according to the recommendation by Wang 2013 [11] but with some modifications as approved by the Animal Research and Ethics of Universiti Teknologi MARA (UiTM) Shah Alam, Malaysia. The collected frogs were anaesthetized in a covered container using absorbent cotton immersed in 1ml anhydrous diethyl ether for 2 mins. The exudated secretions from the dorsal part of the frogs were then washed with sterile 0.1 M NaCl solution contained 1% of protease inhibitor cocktail (Sigma). The solution collected was immediately centrifuged at 5,000g for 20 mins and the supernatant was lyophilized.

2.2 Bacterial Killing Assay

The bacterial killing assay was performed according to the method proposed by Mangoni with some modifications [12]. *E. coli* ATCC 11229 inoculated in Luria Bertani (LB) broth was first grown overnight at 37°C in a rotating shaker at 150 rpm. The culture were then diluted (1:100) in fresh LB broth and further incubated at similar conditions until the growth is equivalent to McFarland 0.5 where as by OD_{625} is between 0.08 – 0.13 [13]. This cell suspension is equivalent to approximately 1 X 10^7 cells/ml. Meanwhile, a flask containing fresh LB broth with 1 ml of filter-sterile frog's skin secretion at the concentration of 1mg/ml was prepared. A volume of 10 µl of the broth were taken, diluted in 100 µl sterile distilled water and plated on LB plate to ensure that the broth containing the frog's skin secretion was free from contamination.

A volume of 1 ml of the prepared *E. coli* culture was then inoculated into the LB broth with the frog's secretion and mix thoroughly by swirling the flask. Immediately, 1 ml sample was taken and diluted in sterile distilled water before plated on duplicated LB plate and incubated overnight at 37°C to determine the viable cell count. This sample was marked as 0 min. Samples were then taken at intervals of every 10 mins for two hours. Each sample was also diluted and plated on LB to determine the viable cell count. The same procedure was also performed on *E. coli* culture inoculated in LB broth only without the frog's secretion which served as a control.

This bacterial killing assay method was repeated using other bacterial cultures which were *Pseudomonas aeroginosa* ATCC 10145, *Staphylococcus aureus* ATCC 25923 and *Bacillus subtilis* ATCC 6633. Each of the experiment was performed in three repeats.

2.3 Genomic DNA Isolation and Amplification

Total DNA was extracted from samples of the frog tissue using the DNEasy Blood and Tissue kit (Qiagen Germany) according to the manufacturer's instructions. DNA amplication was performed using universal primers of the 16S rRNA gene with a forward primer 16SA-L(F) 5'-CGCCTGTTTATCAAAAACAT-

3'and a reverse primer of 16SBH(R) 5'CCGGTCTGAACTCA GATCACGT-3'[14]. PCR amplification was carried out using Go Taq PCR (Promega) as per manufacturer's instructions with the following conditions: initial denaturation for 10 mins at 96°C; followed by 35 cycles of 2 min at 96°C, 0.75 min at 41°C and 0.75 min at 72°C; and a final extension for 10 mins at 72°C. The expected amplicon is about 550bp

2.4 DNA Sequencing and Data Analysis

PCR products were visualised on a 1.2% agarose gel stained with GelRed (Biotium) and purified using a commercial kit (Qiagen). DNA sequencing was performed by Eurogente AITbiotech (Singapore) using the forward primer. The resulting sequence data were then used to interrogate the nucleotide collection at Gen bank data base using the BLAST algorithm. Phylogenetic analysis was performed using Clustal X.

3.0 EXPERIMENTAL RESULTS

3.1 Antimicrobial Assay

The antimicrobial activity of the frog's skin secretion was measured by assessing the activity of the secretion against four different bacteria. In general, the results showed a decrease in the viable cells count of all the bacterial cells tested suggesting the killing ability of the secretion as displayed in Figure 1. After 20 mins incubation period, the number of viable cells in both *Escherichia* coli ATCC 11229 and *Staphylococcus aureus* ATCC 25923 were found to decrease by about 20% as compared to the cells without secretion while a small difference of 5% decrease was observed in *Bacillus subtilis* ATCC 6633. In addition, about 35% decrease in number of viable cells was seen in *Pseudomonas aeroginosa* ATCC *10145* within the same period. After 60 mins, the number of CFU decreased by 20% in the *E. coli*, *Staphylococcus aureus* and *Bacillus subtilis* cells incubated with the frog's secretion as compared with cells without secretion. However, a higher percentage of dead cells were observed in *Pseudomonas aeroginosa* with about 60% decrease in the number of viable cells.

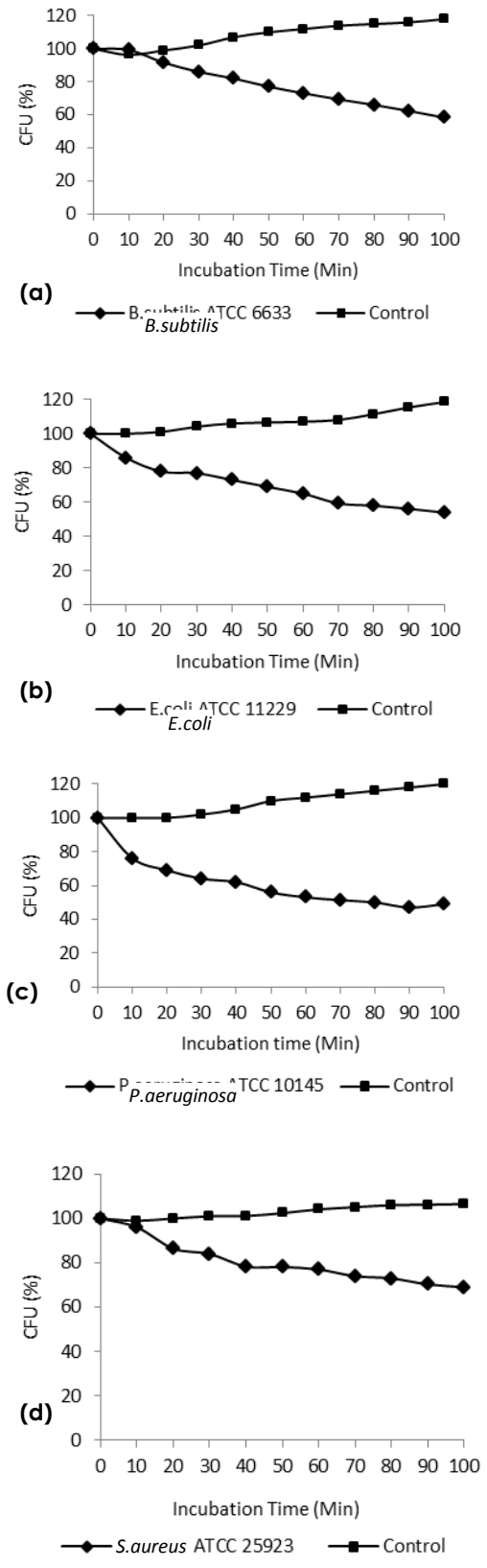


Figure 1 Antimicrobial activity of frog's skin secretion against; **a**. *Bacillus subtilis* ATCC 6633, **b**. *Escherichia coli* ATCC 11229, **c**. *Pseudomonas aeruginosa ATCC 10145*, **d**. *Staphylococcus aureus* ATCC 25923. All results are average of three replicates

Similarly, at the end of the incubation period of 100 mins, the frog's skin secretion showed the highest killing activity against *Pseudomonas aeroginosa* with about 80% decrease of the viable cells. The study showed that the frog skin secretions studied were able to kill both Gram negative (*Escherichia coli Pseudomonas aeruginosa)* and Gram positive (*Bacillus subtilis, Staphylococcus aureus*) bacteria. Similar results were reported from the skin secretions of various amphibians [1, 5, 11, 12].

The bacterial killing activity displayed by the frog's skin secretions suggests the presence of antimicrobial compounds in the secretions. Similar findings have been reported by other researchers. Amphibians are known to have rich source of broad spectrum antimicrobial compounds and these compounds are released as skin secretions when the animals are stressed, threatened or infected. One of the main functions of these compounds is to protect the amphibians against microbes in their natural habitat. In most of the studies, these antimicrobial compounds were found to be peptides. Among the variety of such peptides that has been isolated from several frog species include brevinin, esculentin and temporins [5, 6, 10, 11].

To date amphibian skin secretions have been reported to have a number of 500 antimicrobial peptides, and many new ones are being discovered from various sources. Report from many workers showed that a-helical magainins, the prototypic amphibian AMPs, have strong membrane-penetrable activity towards Gram-positive and Gram-negative bacteria, fungi, yeasts, and viruses. Nowadays there has been increasing interest on AMP temporins collected from the Rana species due to their effective and efficient antimicrobial activity against Gram-positive bacteria [15]. The medicinal potential of frog skin bioactive peptides as anti-infective agents also has potential for alternative clinical applications as anti-cancer, anti-viral, anti-diabetic, or immunomodulatory drugs [16]. Thus if the frog's skin secretions collected is purified, there might be a potential source of a new antibiotic in the future. However, more research needs to be carried out to determine the compound presents that contribute to the pathogenic bacteria killing activity.

3.2 Molecular identification

Figure 2 represents the results of the amplification of the 16sRNA gene of the frog's skin secretion. BLAST results were retrieved using about 550bp of the 5'end of the amplified 16SrRNA sequences. Table 1 showed that all the hits values were 0.0. The maximum identity was 100% for the top three hits which achieved the query sequence was 100% identical to hit sequence in the database. Thus, The BLAST result reveals that the most possible identification for the frog species was Rana catesbeina.

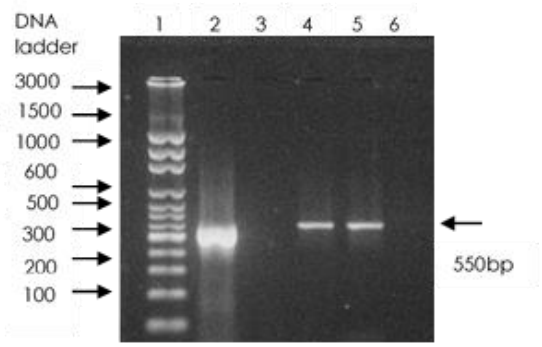


Figure 2 PCR products of DNA extracted from frog tissues. Lane 1: 100bp DNA ladder, lane 2: unpurified product, lane 3: negative control, lane 4 and lane 5: amplified products

Studies show that skin secretions of the frogs' genus Rana have shown to be plenty source of peptides with antibacterial and antifungus activity. For example the skin secretions of the Ranid family are known to have at least eight different families of AMP precursors [11]. The use of DNA molecular techniques to identify and track these amphibian species will provide more information on the diversity of these species and their bioactive compounds resource.

Table 1 BLAST results for frog species

Description	Max score	Total score	Query cover	E value	Ident	Accession
Rana catesbeiana mitochondrial DNA, complete genome	985	985	96%	0.0	100 %	AB761267.1
Rana catesbeiana mitochondrion, complete genome	985	985	96%	0.0	100 %	KF049927.1
Rana catesbeiana mitDNA for tRNA-Phe, tRNA-Leu, tRNA-Val, 12 rRNA and 16S rRNA	985	985	96%	0.0	100 %	X12841.1
Rana catesbeiana voucher NIBRAM0000100340 16S ribosomal RNA gene, partial sequence; mitochondrial	976	976	95%	0.0	99%	JQ815324.1
Rana catesbeiana voucher NIBRAM0000100407 16S ribosomal RNA gene, partial sequence; mitochondrial	976	976	95%	0.0	99%	JQ815323.1

4.0 CONCLUSIONS

The antimicrobial assay revealed that the bioactive compounds extracted from frog's skin may contain peptides with the ability to inhibit the growth of pathogenic microorganisms. However, further studies are needed to confirm the presence of the peptides and subsequent characterization to identify the peptides involved. The results in this preliminary study will contribute to our effort in search for new antimicrobial peptides (AMPs) profiles from Malaysian frogs that could be used as potential antimicrobial agents in therapeutic medicine.

In addition, the Molecular technique based on DNA analysis provides a rapid and reliable method for identification of frog species

Acknowledgment

This study was supported by University Teknologi MARA (UiTM) and Fundamental Research Grant Scheme (FRGS), Ministry of Higher Education (MOHE), Malaysia.

References

[1] Govender, T., Dawood, A., Esterhuyse, A. J., & Katerere, D. R. 2012. Antimicrobial Properties of The Skin Secretions of Frogs. *South African Journal Of Science*. 108(5-6): 1-6. http://doi.org/10.4102/sajs.v108i5/6.795.

[2] Leonardo de Azevedo Calderon, Alexandre de Almeid E.Silva, Pietro Ciancaglini, Rodrigo Guerino Sta´beli. 2010. Antimicrobial Peptides from Phyllomedusa frogs: from Biomolecular Diversity to Potential Nanotechnologic Medical Applications. *Amino Acids*. DOI 10.1007/s 00726 - 010-0622-3.

[3] Guilherme D. Brand, Raimunda C. Santos, Luisa Mayumi Arake, Valdelânia G. Silva, Leiz M. C. Veras, Vladimir Costa, Carlos Henrique N. Costa, Selma S. Kuckelhaus, José Guilherme Alexandre, Maria J. Feio and José Roberto S. A. Leite. 2013. The Skin Secretion of the Amphibian *Phyllomedusa nordestina*: A Source of Antimicrobial and Antiprotozoal Peptides. *Moleculaes*. 18: 2058-2070.

[4] Hailong Yang, Xu Wang, Xiuhong Liu, Jing Wu, Cunbao Liu, Weiming Gong, Zhiqiang Zhao, Jing Hong, Donghai Lin, Yizheng Wang, and Ren Lai. 2009. Antioxidant Peptidomics Reveals Novel Skin Antioxidant System. *Mol Cell Proteomics*. 8: 571-583.

[5] Che Q, Zhou Y, yang H, Li J, Xu X, Lai R. 2008. A Novel Antimicrobial Peptide From Amphibian Skin Secretions of Odorrana Grahami. *Peptides*. 2008.01.004, Pmud; 9790305.

[6] Channing. 2006. A. *Amphibians of Central and Southern Africa*. Pretoria: Protea Bookhouse

[7] Tao, J., Zhou, Mei, Wang, Lei, Chen, Tianbao, Shaw, Christopher. 2010. *Regulatory Peptides*. 164(09): 28-28.

[8] Ramlah Zainudin, M., & Rahman, Badr. M. M. Za. 2010. Mating Calls Description of Five Species of Frogs from the Genus *Hylarana tschudi* 1838 (Amphibia, Anura, Ranidae) from Sarawak, Malaysia. *Sains Malaysian*. 39(3): 363-369.

[9] Dehling, J. M. 2011. A New Karst-Dwelling Species Of Kalophrynus (Anura: Microhylidae) from Gunung Mulu National Park, Borneo, Malaysia. *Zootaxa* 2737(2011): 49-60.

[10] Mingming Zhang, Fan Zhou, Yuan Chu, Zhangwu Zhao, and Chunju An. 2013. Antimicrobial Peptide Diversity In The Skin Of The Torrent *Frog* Amolops Jingdongensis. *Amino Acids*. 44: 481-486.

[11] Guoxiang Wang, Ying Wang, Dongying Ma, Huan Liu, Jianxu Li, Keyun Zhang, Xiaolong Yang, Ren Lai, Jingze Liu. 2013. Five Novel Antimicrobial Peptides from the Kuhl's wart frog skin secretions. *Limnonectes kuhlii. Mol Biol Rep*. 40: 1097-1102.

[12] Mangoni, M. L., Papo, N., Barra, D., *et al*. 2004. Effects of The Antimicrobial Peptide Temporin L on Cell Morphology. *Membrane Permeability And Viability of Escherichia coli. Biochem J*. 380:859-865.

[13] Wiegand, I., Hilpert, K. & Hancock, R.E. 2008. Agar and Broth Dilution Methods To Determine The Minimal Inhibitory Concentration (MIC) of Antimicrobial Substances. *Nat Protoc*. 3: 163-175.

[14] Vences, M., Thomas, M., Meijden, A. van der, Chiari, Y., and Vieites, D. R. 2005. Comparative Performance of the 16S rRNA Gene in DNA Barcoding Of Amphibians. *Frontiers in Zoology*. 2(1): 5. doi: 10.1186/1742-9994-2-5.

[15] Andrew J. Crawford, Catalina Cruz, Edgardo Griffith, Heidi Ross, Roberto Ib Nez, Karen R Lips, Amy C. Driskell, Eldredge Bermingham and Paul Crump. 2012. DNA Barcoding Applied To Ex Situ Tropical Amphibian Conservation Programme Reveals Cryptic Diversity in Captive Populations. *Molecular Ecology Resources*. doi: 10.1111/1755-0998.12054.

[16] Conlon, J. M. & A. Sonnevend. 2011. Clinical Applications of Amphibian Antimicrobial Peptides. *J Med Sci*. 4: 62-72.