

# Identification of cathelicidin gene from *Hoplobatrachus rugulosus* and the antioxidant capacity of PC29 peptide

Anupong Tankrathok (✉ [anupong2.ta@ksu.ac.th](mailto:anupong2.ta@ksu.ac.th))

Kalasin University <https://orcid.org/0000-0002-4636-6142>

**Chutima Kammongkol**

Kalasin University

**Arpaporn Punpad**

Kalasin University Faculty of Agricultural Technology

**Piyachat Wiriyaumpaiwong**

Kalasin University Faculty of Agricultural Technology

**Nattapong Srisamoot**

Kalasin University Faculty of Agricultural Technology

**Wutti Rattanavichai**

Kalasin University Faculty of Agricultural Technology

**Alongkod Tanomtong**

Khon Kaen University Faculty of Science

**Sakda Daduang**

Khon Kaen University Faculty of Pharmaceutical Sciences

**Sompong Klaynongsruang**

Khon Kaen University Faculty of Science

---

## Research Article

**Keywords:** antioxidant activity, cathelicidin, frog, peptide

**DOI:** <https://doi.org/10.21203/rs.3.rs-521559/v1>

**License:**   This work is licensed under a Creative Commons Attribution 4.0 International License.

[Read Full License](#)

---

# Abstract

Cathelicidins, a group of vertebrate multifunctional molecules, play a role in innate immunity. Cathelicidins are antimicrobial peptides (AMPs) that are involved in protection against microbial invasion. Presently, cathelicidin peptides have been identified from only 14 amphibian species. In the study, a novel cathelicidin was identified from the lungs of frogs, *Hoplobatrachus rugulosus*. A 474 base pairs (bp) complementary DNA (cDNA) sequence encoded a 157 amino acid residue prepropeptide of *H. rugulosus* cathelicidin (cathelicidin-HR), which consisting of a 20-residue signal peptide sequence, a 108-residue cathelin region, and a 29-residue cathelicidin peptide (PC29). Amino acid sequence alignment and cladogram analysis illustrated that cathelicidin-HR have a high degree of similarity to further amphibian cathelicidins. The PC29 peptide displays antimicrobial activity only against *Bacillus subtilis* and *Enterococcus faecalis*. However, the PC29 peptide performed dose-dependent antioxidant activity. This is the first cathelicidin antioxidant peptide identified from the lung which provided a template for the development of potent bi-functional peptide therapeutic agents.

## Introduction

Amphibians can survive in a broad range of environmental systems due to the containing pharmacological substances to combat environmental factors such as microbes and ultraviolet radiation [1]. They have abundant peptides that play a role in defense mechanisms [2]. Amphibian bioactive peptides have been proven a variety of biological functions, including antimicrobial, antioxidant, and immunomodulatory activities [3]. Many studies have demonstrated that amphibian skin peptides have the potential for drug development [4,5]. However, not only skin peptides play the role in a protective mechanism but other organ peptides, especially those found in amphibian lungs, also function in an important organ in the respiratory system which not well understood.

Cathelicidin is a class of multi-functional peptides found in almost all vertebrates [6]. The cathelicidins are synthesized as prepropeptides containing the conserve N-terminus cathelin domain and the variable C-terminus active peptide region [7]. Moreover, not only relates antimicrobial activity but cathelicidin is also associated in other biological roles including angiogenesis, induction of immune cytotoxicity, and immune cells chemotaxis [8].

Currently, more than 8,000 amphibian species have been reported on the AmphibiaWeb ([www.amphibiaweb.org](http://www.amphibiaweb.org)), but only 19 cathelicidins identified and characterized from 14 species. Among these cathelicidin genes that determined in the skin, such as cathelicidin-AL from *Amolops loloensis* [9], cathelicidin-PY from *Paa yunnanensis* [10], cathelicidin-TV from *Tylotriton verrucosus* [11], cathelicidin-PP from *Polypedates puerensis* [12], AdCath from *Andrias davidianus* [13], cathelicidin-OA1 from *Odorrana andersonii* [14], cathelicidin-NV from *Nanorana ventripunctata* [15], cathelicidin-DM from *Duttaphrynus melanostictus* [16], and recently PN-CATH1 and PN-CATH2 from *Pelophylax nigromaculata* [17]. Whereas, Lf-CATH1 and Lf-CATH2 were established from the spleen of *Limnonectes fragilis* [18], but cathelicidin-BG was investigated from an ear-side gland of *Bufo bufo gargarizans* [19]. Besides, only

cathelicidin-RC1 and cathelicidin-RC2 from *Rana catesbeiana* [20], FM-CATH1 and FM-CATH2 from *Fejervarya multistriata* [21], and OL-CATH1 and OL-CATH2 from *Odorrana livida* [22] were isolated from the lung. In this study, the cathelicidin gene was identified from the lung of *H. rugulosus* and the mature cathelicidin peptide function was as well characterized.

## Materials And Methods

### Rapid amplification of cDNA ends (RACE) reaction

Total RNA was extracted from frog lungs by using GF-1 Total RNA extraction Kit (Vivantis, USA). RNA concentration was measured by spectrophotometer. The experimental procedure was approved by the Institutional Animal Care and Use Committee of Khon Kaen University (Record number IACUC-KKU-10162). The first-strand cDNA was produced by M-MuLV reverse transcriptase reaction (Vivantis, USA) with oligonucleotide d(T). The cDNA template was magnified with the forward primer rcCATH-F (5'-ATGAAGATCTGGCAGTGTGTG-3') and the reverse primer rcCATH-R (5'-GGTCAGGCTGACGCACTTC-3') with designed based on the conserved signal peptide sequence and C-terminal cathelin domain of *R. catesbiana* cathelicidin (cathelicidin-RC) genes, respectively [20]. Furthermore, the 3'-RACE was synthesized using a 5'-specific forward primer (hrCATH34-F: 5'-GCAATCACATTGCAGTCAGC-3') and oligonucleotide d(T) primer. The polymerase chain reaction (PCR) was conducted by T100 Thermal Cycler (Bio-Rad, USA). The PCR product purification was carried out by gel electrophoresis with a GF-1 AmbiClean kit (Vivantis, USA). The purified PCR products were sequenced by Sanger sequencing (Macrogen, South Korea).

### Bioinformatic analysis

The amphibian cathelicidin amino acid sequences were obtained from NCBI database. The amphibian cathelicidin sequences were aligned using ClustalOmega (<http://www.ebi.ac.uk/Tools/msa/clustalw2/>). Besides, phylogenetic analysis was determined using the neighbor-joining method (Mega version X; [23]). The secondary structure was performed by PEP-FOLD servers [24]. The structure graphic was hence produced in *PyMol* (Schrödinger LLC). The prediction of antimicrobial region was calculated through Antimicrobial Sequence Scanning System (AMPA) server (<http://tcoffee.crg.cat/apps/ampa/>) [25]. The peptide sequences were computed for the physicochemical properties analysis via APD2 database (<http://aps.unmc.edu/AP/main.php>) [26].

### Peptide synthesis

PC29 peptide was produced by GenScript (NJ, USA). The amino acid sequencing was validated by electrospray ionization mass spectrometry while peptide purity was checked by reverse-phase high performance liquid chromatography (RP-HPLC).

### Antimicrobial activity

The antimicrobial activity assay of peptides was determined using the broth assay. Briefly, bacterial cells were cultured in nutrient broth until the mid-log phase at 37 °C. Then cultured cells were diluted to 10<sup>4</sup> CFU/ml. The 50 µl of diluted cells was aliquoted into microcentrifuge tubes then mixed with 50 µl of 4 mg/ml PC29 peptide, followed by the incubation at 37 °C for 16-18 h. the bacterial growth was observed by spectrophotometer. A decrease in optical density at 600 nm indicated the antimicrobial activity of the peptide. The melittin and double-distilled water (DW) were used as positive and negative controls, respectively. The percentage of bacterial growth inhibition was calculated the following formula:  $[(OD_{600} \text{ nm, control} - OD_{600} \text{ nm, peptide}) / OD_{600} \text{ nm, control}] \times 100$ .

### **Antioxidant activity assay**

A 2, 2-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) scavenging activity was determined as previously described [14]. Briefly, the ABTS radical solution was prepared by mixing 2.8 mM potassium persulfate with 7 mM ABTS in water, followed by incubating for 6 h in the dark. The ABTS solution was diluted 50-fold with DW. Samples dissolved in water were added to the diluted stock solution, and the same volume of solvent was used as the negative control. The reactions were kept from light for 30 min. A decrease in absorbance at 415 nm indicated the antioxidant activity of the samples. The rate of free radical scavenging (%) was calculated by  $[(A_{415} \text{ nm, blank} - A_{415} \text{ nm, sample}) / A_{415} \text{ nm, blank}] \times 100$ .

A 2, 2-diphenyl-1-picrylhydrazyl (DPPH) scavenging activity was assayed as previously described [14]. The reaction contained 190 µl of 50 µM DPPH radical dissolved in ethanol and 10 µl of 2-fold dilution peptide. The mixture was incubated at room temperature for 30 min in the dark. Then, the absorbance was measured against a blank at 517 nm. The percentage of DPPH free radical scavenging activity was calculated the following formula:  $[(A_{517} \text{ nm, blank} - A_{517} \text{ nm, sample}) / A_{517} \text{ nm, blank}] \times 100$ .

### **Hemolytic activity assay**

The hemolytic activity of the PC29 peptide was investigated against the defibrinated sheep red blood cells (shRBCs). Briefly, the shRBCs were washed with phosphate-buffered saline (PBS), pH 7.4, and then diluted to 0.5 % (v/v) in PBS. The 100 ml of shRBCs solution was divided into microcentrifuge tubes. The 10 ml of 2-fold serial dilution of PC29 peptide was added, followed by incubated at 37 °C for 1 h. The reactions were centrifuged at 1,000 g for 5 min. The 100 ml supernatants have measured absorbance at 415 nm with a spectrophotometer. The 1 % (v/v) Triton X-100 and DW were used as positive and negative controls, respectively. The percentage of hemolysis was computed as  $[(A_{415} \text{ nm, peptide}) / (A_{415} \text{ nm, 1 \% (v/v) Triton X-100})] \times 100$ .

## **Results**

### **Identification of cathelicidin-HR**

To identify the cathelicidin gene in *H. rugulosus*, the frog lung was collected then analyzed by RT-PCR with the conserved *R. catesbiena* cathelicidin gene primer. The 300 bp PCR product was obtained from

the reaction with 80% sequence similarity to the cathelicidin-PY1 precursor (AFX61592) from *N. yunnanensis* (Supplement Data Fig 1a). This nucleotide sequence was further used as a template to obtain the complete 3' ends of the cathelicidin-HR gene by RACE-PCR. The 800 bp 3' RACE-PCR product was amplified by using specific cathelicidin-HR primers combined with oligonucleotide d(T) primer (Supplement Data Fig 1b). The complete prepropeptide cathelicidin-HR cDNA sequence was presented in Fig. 1. The 474 bp prepro-cathelicidin-HR nucleotide encoded 157 amino acid residues.

### **Analysis of cathelicidin-HR amino acid sequence**

The amino acid sequence analysis with ExPASy showed that the molecular weight of prepro-cathelicidin-HR was 17.97 kDa with the 6.59 pI. Moreover, SignalP 4.0 sequence analysis indicated that the first 20 amino acid residues domain on the N-terminus site was the signal peptide region. The conserved cathelin region comprised 108 residues whereas the C-terminal end indicated the putative mature cathelicidin-HR (PC29) peptide comprised 29 amino acid residues with a molecular weight of 3.18 kDa and 10.86 of pI (Table 1). The amino acid sequence alignment illustrated that prepro-cathelicidin-HR showed 66 % similarity with Lf-CATH1 (*L. fragilis*) and performed 64% and 60% with the cathelicidin-NV precursor (*N. ventripunctata*) and OL-CATH1 from *O. livida*, respectively (Fig. 2a). Among amphibian species, the N-terminus cathelin region performed a highly conserved sequence. Whereas, the C-terminus mature cathelicidin peptide presented a peptide sequences variation. The phylogenetic tree analysis of amphibian cathelicidins was divided into three clusters (Fig. 2b). Cluster I was the largest group of amphibian cathelicidin which can split into two sub-groups (cluster I-a) and cluster I-b). Cluster I-a comprised cathelicidin-HR, Lf-CATH1, cathelicidin-NV, and OL-CATH1. In addition, cluster I-b included cathelicidin-RC1-2, OL-CATH 2, PN-CATH1-2, cathelicidin-PP, cathelicidin-PY1, and Lf-CATH2. Besides, cluster II had two cathelicidins from toads, cathelicidin-BG and cathelicidin-DM. Cluster III presented the diversity of amphibian cathelicidin, consisting of cathelicidin-OA1 and cathelicidin-AL.

### **Analysis of candidate cathelicidin-HR peptide**

The AMPA server analysis indicated that the region from Pro129 to Arg140 represented the bactericidal stretch with 13 % probability (data not shown) which is located on the mature cathelicidin peptide domain. Therefore, the candidate putative mature peptide denoted as PC29; NH<sub>2</sub>-PCRGIFCRTGSRSPIAKPSKDNLVRMSLS-COOH derived from the C-terminus site. The PC29 peptide comprised 29 amino acid residues and performed the cationic peptide property +5 net charge. However, PC29 peptide expressed a positive total net charge which presented a 34% of hydrophobic ratio with lower than at the level of a major group of AMPs. The predicted PC29 peptide secondary structure was showed in Supplement Data Fig. 3. The secondary structure modeling illustrated that the PC29 peptide exhibited the b-strand model containing one salt bridge between Arg12 and Asp21.

### **Antimicrobial activity of the cathelicidin-HR peptide**

The antimicrobial activity of the PC29 peptide was investigated by the broth assay (Table 2). The results performed that a high concentration of PC29 peptide could inhibit the growth of only *Bacillus subtilis*

TISTR124 and *Enterococcus faecalis* TISTR927. However, the minimum inhibition concentration of this peptide was performed at 1 mg/ml for both bacterial strains (data not shown). These results indicated that PC29 peptide exhibited low antimicrobial activity.

### **Antioxidant activity of PC29 peptide**

The antioxidant activity of the PC29 peptide was investigated by the ABTS and DPPH scavenging activity (Fig. 5). The results clearly showed that the PC29 peptide performs antioxidant capacity against both ABTS and DPPH scavenging activity. Wherewith, the PC29 peptide exerts the free radical scavenging activity of both ABTS and DPPH with the dose-dependent characteristic. These results illustrated that the PC29 peptide presented antioxidant properties.

### **Hemolysis of PC29 peptide against red blood cell**

The hemolytic of PC29 peptide on sheep erythrocyte presented that PC29 peptide expressed insignificant hemolysis activity between range 10 % to 18 % even at a concentration of 400 mg/ml (Fig. 6). Whereas, the negative control (DW) also showed hemolysis against red blood cells about 10 %. Besides, the positive control (1% Triton-X 100) destroyed red blood cells about 100 %. These results concluded that the PC29 peptide presented a low toxicity activity.

## **Discussion**

Amphibians were the first to evolve as land and water connectors. These animals are in direct contact and respond to a wide range of ecological and physical factors such as a microbe, parasite, and environment [1]. Therefore, it has a potent defense system consisting of a variety of gene expression and bioactive peptides [27]. Over the past decades, bioactive peptides from amphibian skin have been extensively studied [1], and more than a hundred peptides have been identified from amphibians [1]. Especially, the Ranidae frogs that contain large amounts of biological peptides in their skin. However, most previous studied peptides only presented peptides from the skin which containing antimicrobial activity. While frog peptide from other organs has not been considerably investigated. Cathelicidins are a class of antimicrobial peptides found extensively in vertebrates. Since their first discovery isolated from granules of bovine neutrophils [28]. Currently, over hundreds of cathelicidins have been identified from various vertebrates [29]. For amphibians, to date, a total of 19 cathelicidin sequences have been identified from 14 different species.

The cathelicidin-HR was identified from the lung as the previous reports of cathelicidin-RC1 and cathelicidin-RC2 from *R. catesbeiana*, FM-CATH1-2 from *F. multistriata*, and OL-CATH1-2 from *O. livida* [20-22]. The 157 amino acid residues of cathelicidin-HR contained the 20 amino acid residues signal peptide following the 108 residues conserved cathelin domain and the PC29 putative mature peptide end (Fig. 1). The sequence alignment elucidated that among the group of amphibian cathelicidin has a highly similar degree of cathelin sequence region which not only four conserved cysteine residues (Fig. 2a). However, except for cathelicidin-AL and cathelicidin-OA1 which exhibit sequence similarities to reptilian

cathelicidin [9,14]. The phylogenetic analysis illustrated that the cathelicidin-HR relate to other amphibian cathelicidins which mostly located in cluster I (Fig. 2b). On the other hand, cathelicidin-AL and cathelicidin-OA1 presented that both sequences are distinct to amphibian sequence clusters that might be the connecting link between the amphibian and reptilia cathelicidin [9,14] (Fig. 2b). However, the sequence similarity result indicated that the cathelicidin-HR sequence showed the closest sequence relates to Lf-CATH1 which was isolated from the spleen of *L. fragilis* (Fig. 2b).

Most amphibian cathelicidins exert direct antimicrobial activity against a broad range of bacteria, fungi, and drug-resistant pathogens [9,10,18,20,22]. Moreover, they also performed potent anti-inflammatory activity, such as cathelicidin-PY, OL-CATH1-2, and FM-CATH1-2 from *P. yunnanensis*, *O. livida* and *F. multistriata*, respectively [10,21,22]. Whereas, cathelicidin-NV from *N. ventripunctata*, and cathelicidin-OA1 and *O. andersonii* are lacking antimicrobial activity but they possess powerful wound healing and antioxidant activities [14,15].

In general, cathelicidin was essential enzymatically cleaved for proteolytic maturation. Enzymatic processing in most of the cathelicidins was mediated by elastase. This enzyme was typically sensitive to valine or an alanine residue. However, the proteolytic processing for amphibian cathelicidins is remaining unclear. In this study, based on antimicrobial peptide domain prediction and amino acid sequence alignment results, PC29 peptide (PCRGIFCRTGSRSPIAKPSKDNLVRMSLS) was estimated as the mature peptide released from cathelicidin-HR. The cationic PC29 peptide comprised 29 residues with +5 net charge and b-strand characteristic. As described earlier, some amphibian cathelicidin could not exhibit antimicrobial activity but we cannot refuse that this peptide lacks this property. Since the PC29 peptide performed antimicrobial activity against gram-positive bacteria *B. subtilis* TISTR124 and *E. faecalis* TISTR927 although at high concentration. The b-strand character of the PC29 peptide might be an uncommon AMPs structure. Although the PC29 peptide presented the positive total net charge this peptide expressed the hydrophobic ratio percentage at 34 % which lower than the range of a main group of AMPs which commonly hydrophobic ratio percentage between range 40 % to 50 % (APD). The optimum hydrophobicity of peptides may lead to the efficient action of the peptide on membranes especially penetration and disruption [30]. In cluster I, only Lf-CATH1 (40 % hydrophobic ratio) exhibits antimicrobial activity but OL-CATH1 and cathelicidin-NV with hydrophobic ratio percentage between range 30 % to 33 % were lacking antimicrobial activity although all peptides presented +5 net charge (Supplement Data Table 1).

PC29 peptide performed obvious ABTS+ and DPPH scavenging activity (Fig. 3). The two cysteine residues of PC29 peptide were estimated that might play a role in the free radicals scavenging activity. However, the antioxidant activity of cathelicidin peptide was disrupted by the formation of an intramolecular disulfide bridge [14]. Thus, it is assumed that two cysteine residues probably were free cysteine and performed the scavenging activity. Furthermore, the predicted PC29 peptide secondary structure also established that no disulfide bond formation (Supplement Data Fig. 3). According to the results, the conclusion can be made that the PC29 peptide expressed the bi-functional peptide with antimicrobial and antioxidant activities. Recently, PN-CATHs from *P. nigromaculata* also showed both

antimicrobial and antioxidant activities [17]. However, here is the first report of a non-skin cathelicidin antioxidant peptide identified from an amphibian.

## Conclusions

In summary, a cathelicidin peptide was identified from *H. rugulosus* that offers the diversity role of cathelicidin in amphibian. The amino acid sequence analysis revealed cathelicidin-HR shares the common conserved sequence among the amphibian cathelicidins. The PC29 peptide was predicted as mature peptide released from cathelicidin-HR exhibit low antimicrobial activity whereas performing the antioxidant activity in a dose-dependent manner. The results provide a new template peptide for the development of potent two modes peptide employing antimicrobial and antioxidant activities.

## Declarations

### Funding

This work was supported by Thailand Research Fund (Grant No. MRG6280081)

### Conflict of interest

The authors have no conflicts of interest to declare that are relevant to the content of this article.

### Informed consent

The authors declare that they consent to participate to this study.

CK, and AP performed experiments. AT and PW analyzed the data and wrote the manuscript. NS and AIT revised the manuscript. SD and SK supervised the study. All authors read the paper and approved the final manuscript.

### Ethical approval

This experiment was approved by T by the animal ethics committee of Khon Kaen University (Record number IACUC-KKU-10162).

### Acknowledgments

This work was supported by Thailand Research Fund (Grant No. MRG6280081), Kalasin University, and Protein and Proteomics Research Center for Commercial and Industrial Purposes (ProCCI), Khon Kaen University.

## References

1. Xu X, Lai R (2015) The chemistry and biological activities of peptides from amphibian skin secretions. *Chem Rev* 115(4):1760–1846
2. Yang X, Lee WH, Zhang Y (2012) Extremely abundant antimicrobial peptides existed in the skins of nine kinds of Chinese odorous frogs. *J Proteome Res* 11(1):306–319
3. Zhang Y (2015) Why do we study animal toxins? *Zoo Res* 36(4):183–222
4. Amiche M (2016) Peptides thérapeutiques à fleur de peau de grenouille [Amphibian skin as a source of therapeutic peptides]. *Biol Aujourd'hui* 210(2):101–117
5. Demori I, Rashed ZE, Corradino V, Catalano A, Rovegno L, Queirolo L, Salvidio S, Biggi E, Zanotti-Russo M, Canesi L, Catenazzi A, Grasselli E (2019) Peptides for Skin Protection and Healing in Amphibians. *Molecules* 24(2):347. doi:10.3390/molecules24020347
6. Avila EE (2017) Functions of Antimicrobial Peptides in Vertebrates. *Curr Protein Pept Sci* 18(11):1098–1119
7. Tomasinsig L, Zanetti M (2005) The cathelicidins—structure, function and evolution. *Curr Protein Pept Sci* 6(1):23–34
8. Kościuczuk EM, Lisowski P, Jarczak J, Strzałkowska N, Józwick A, Horbańczuk J, Krzyżewski J, Zwierzchowski L, Bagnicka E (2012) Cathelicidins: family of antimicrobial peptides. A review. *Mol Biol Rep* 39(12):10957–10970
9. Hao X, Yang H, Wei L, Yang S, Zhu W, Ma D, Yu H, Lai R (2012) Amphibian cathelicidin fills the evolutionary gap of cathelicidin in vertebrate. *Amino Acids* 43(2):677–685
10. Wei L, Yang J, He X, Mo G, Hong J, Yan X, Lin D, Lai R (2013) Structure and function of a potent lipopolysaccharide-binding antimicrobial and anti-inflammatory peptide. *J Med Chem* 56(9):3546–3556
11. Mu L, Tang J, Liu H, Shen C, Rong M, Zhang Z, Lai R (2014) A potential wound-healing-promoting peptide from salamander skin. *FASEB J* 28(9):3919–3929
12. Mu L, Zhou L, Yang J, Zhuang L, Tang J, Liu T, Wu J, Yang H (2017) The first identified cathelicidin from tree frogs possesses anti-inflammatory and partial LPS neutralization activities. *Amino Acids* 49(9):1571–1585
13. Yang H, Lu B, Zhou D, Zhao L, Song W, Wang L (2017) Identification of the first cathelicidin gene from skin of Chinese giant salamanders *Andrias davidianus* with its potent antimicrobial activity. *Dev Comp Immunol* 77:141–149
14. Cao X, Wang Y, Wu C, Li X, Fu Z, Yang M, Bian W, Wang S, Song Y, Tang J, Yang X (2018) Cathelicidin-OA1, a novel antioxidant peptide identified from an amphibian, accelerates skin wound healing. *Sci Rep* 8(1):943. doi:10.1038/s41598-018-19486-9
15. Wu J, Yang J, Wang X, Wei L, Mi K, Shen Y, Liu T, Yang H, Mu L (2018) A frog cathelicidin peptide effectively promotes cutaneous wound healing in mice. *Biochem J* 475(17):2785–2799
16. Shi Y, Li C, Wang M, Chen Z, Luo Y, Xia XS, Song Y, Sun Y, Zhang AM (2020) Cathelicidin-DM is an Antimicrobial Peptide from *Duttaphrynus melanostictus* and Has Wound-Healing Therapeutic

Potential. ACS Omega 5(16):9301–9310

17. Wang Y, Ouyang J, Luo X, Zhang M, Jiang Y, Zhang F, Zhou J, Wang Y (2021) Identification and characterization of novel bi-functional cathelicidins from the black-spotted frog (*Pelophylax nigromaculata*) with both anti-infective and antioxidant activities. Dev Comp Immunol 116:103928. doi:10.1016/j.dci.2020.103928
18. Yu H, Cai S, Gao J, Zhang S, Lu Y, Qiao X, Yang H, Wang Y (2013) Identification and polymorphism discovery of the cathelicidins, Lf-CATHs in ranid amphibian (*Limnonectes fragilis*). FEBS J 280(23):6022–6032
19. Gao F, Xu WF, Tang LP, Wang MM, Wang XJ, Qian YC (2016) Characteristics of cathelicidin-Bg, a novel gene expressed in the ear-side gland of *Bufo gargarizans*. Genet Mol Res 15(3). doi:10.4238/gmr.15038481
20. Ling G, Gao J, Zhang S, Xie Z, Wei L, Yu H, Wang Y (2014) Cathelicidins from the bullfrog *Rana catesbeiana* provides novel template for peptide antibiotic design. PLoS One 9(3):e93216
21. Wang AL, Chen Y, Yu HN, Wang YP (2017) Novel cathelicidins with potent antimicrobial, biofilm inhibitory, and anti-inflammatory activities from the frog *Fejervarya multistriata*. Asian Herpetol Res 8(3):199–212
22. Qi RH, Chen Y, Guo ZL, Zhang F, Fang Z, Huang K, Yu HN, Wang YP (2019) Identification and characterization of two novel cathelicidins from the frog *Odorrana livida*. Zool Res 40(2):94–101
23. Kumar S, Stecher G, Li M, Knyaz C, Tamura K (2018) MEGA X: Molecular Evolutionary Genetics Analysis across Computing Platforms. Mol Biol Evol 35(6):1547–1549
24. Thévenet P, Shen Y, Maupetit J, Guyon F, Derreumaux P, Tufféry P (2012) PEP-FOLD: an updated de novo structure prediction server for both linear and disulfide bonded cyclic peptides. Nucleic Acids Res 40:W288–W293
25. Torrent M, Di Tommaso P, Pulido D, Nogués MV, Notredame C, Boix E, Andreu D (2012) AMPA: an automated web server for prediction of protein antimicrobial regions. Bioinformatics 28(1):130–131
26. Wang G, Li X, Wang Z (2009) APD2: The updated antimicrobial peptide database and its application in peptide design. Nucleic Acids Res 37:933–937
27. Clarke BT (1997) The natural history of amphibian skin secretions, their normal functioning and potential medical applications. Biol Rev Camb Philos Soc 72(3):365–379
28. Gennaro R, Skerlavaj B, Romeo D (1989) Purification, composition, and activity of two bactenecins, antibacterial peptides of bovine neutrophils. Infect Immun 57(10):3142–3146
29. van Harten RM, Van Woudenberg E, Van Dijk A, Haagsman HP (2018) Cathelicidins: Immunomodulatory Antimicrobials. Vaccines (Basel) 6(3):63. doi:10.3390/vaccines6030063
30. Yin LM, Edwards MA, Li J, Yip CM, Deber CM (2012) Roles of hydrophobicity and charge distribution of cationic antimicrobial peptides in peptide-membrane interactions. J Biol Chem 287(10):7738–7745

# Tables

**Table 1** Physicochemical properties of PC29 mature peptide.

Peptide	Sequence	Length	Net charge	Theoretical pI	Mw	Hydrophobic ratio (%)
PC29 peptide	PCRGIFCRTGSRSPIAKPSKDNLVRMSLS	29	+5	10.86	3177.77	34

**Table 2** Antimicrobial activity of PC29 peptide.

Microorganism	PC29 peptide	Melittin
Gram-positive bacteria		
<i>Bacillus megaterium</i> TISTR067	-	-
<i>Bacillus subtilis</i> TISTR1248	+	+
<i>Bacillus cereus</i> TISTR1449	-	-
<i>Staphylococcus aureus</i> ATCC25923	-	+
<i>Enterococcus faecalis</i> TISTR927	+	-
Gram-negative bacteria		
<i>Escherichia coli</i> ATCC25922	-	+
<i>Pseudomonas aeruginosa</i> TISTR1287	-	+
<i>Salmonella thyphimurium</i> TISTR1472	-	-
Fungi		
<i>Candida albicans</i> TISTR5554	-	-
Aquatic pathogenic bacteria		
<i>Aeromonas hydrophila</i> TISTR1321	-	+
<i>Salmonella derby</i> DMST16881	-	+
<i>Edwardsiella tarda</i> DMST38217	-	-

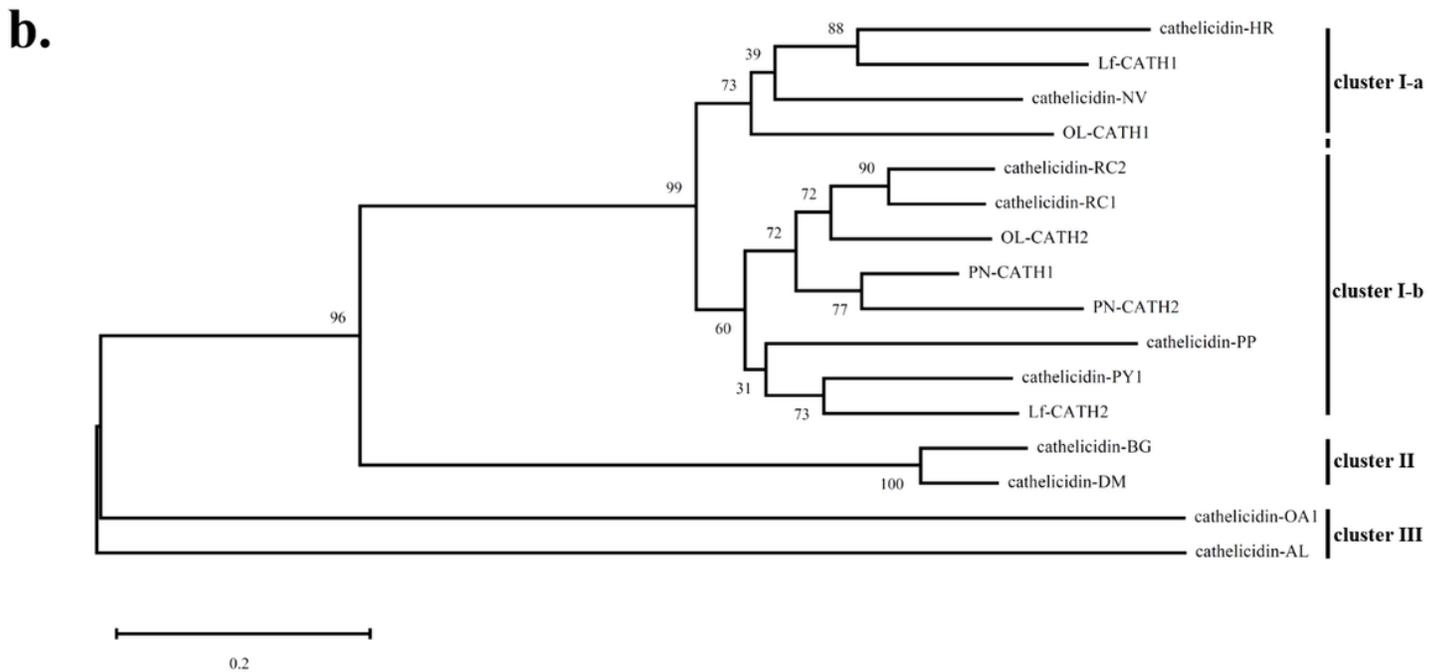
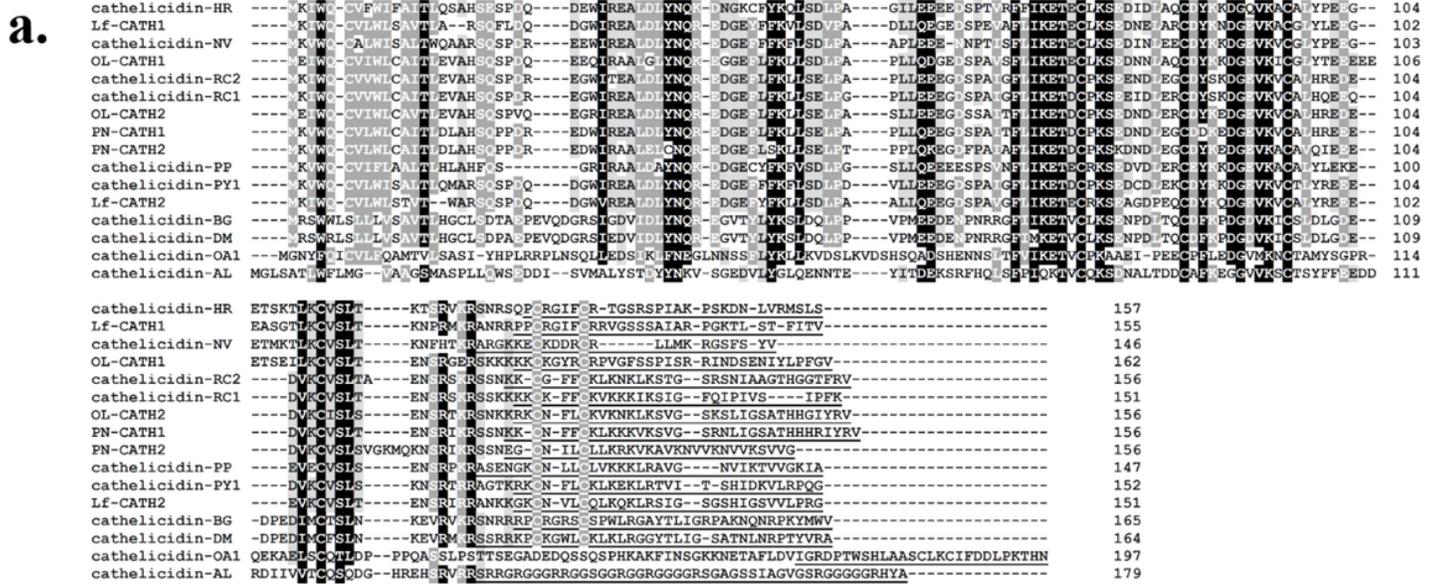
(+); Growth inhibition  $\geq$  50 % and (-); Growth inhibition < 50 %

# Figures

atgaagatctggcagtggtgtgttctggatcttcgcaatcacattgcagtcagctcactct	60
<u>M K I W Q C V F W I F A I T L Q S A H S</u>	20
gagtctccagatcaggatgagtgatcagagaggccttagatctctacaaccagaaggat	120
E S P D Q D E W I R E A L D L Y N Q K D	40
aatggaaagtgcttctataagcaactgtccgatctcccggctggcatcctggaggaagag	180
N G K C F Y K Q L S D L P A G I L E E E	60
gaagactctcccacagtcagggttctttataaaggagacagaatgcctcaagtctgaagat	240
E D S P T V R F F I K E T E C L K S E D	80
atagacttggcccagtggtgactacaagaaggatggggcaggtgaaggcctgtgcattgtac	300
I D L A Q C D Y K K D G Q V K A C A L Y	100
ccggaggagggggagacctcaaagactctgaaatgcgtcagcttgaccaagacctcccgt	360
P E E G E T S K T L K C V S L T K T S R	120
gttaaacgatcaaatagatcacaaccctgcaggggtatcttttgtagaactggatccaga	420
V K R S N R S Q <b>P C R G I F C R T G S R</b>	140
agtccgattgccaagccaagcaaagacaatttagttcggatgtcactgtcttaa	474
<b>S P I A K P S K D N L V R M S L S *</b>	157

Figure 1

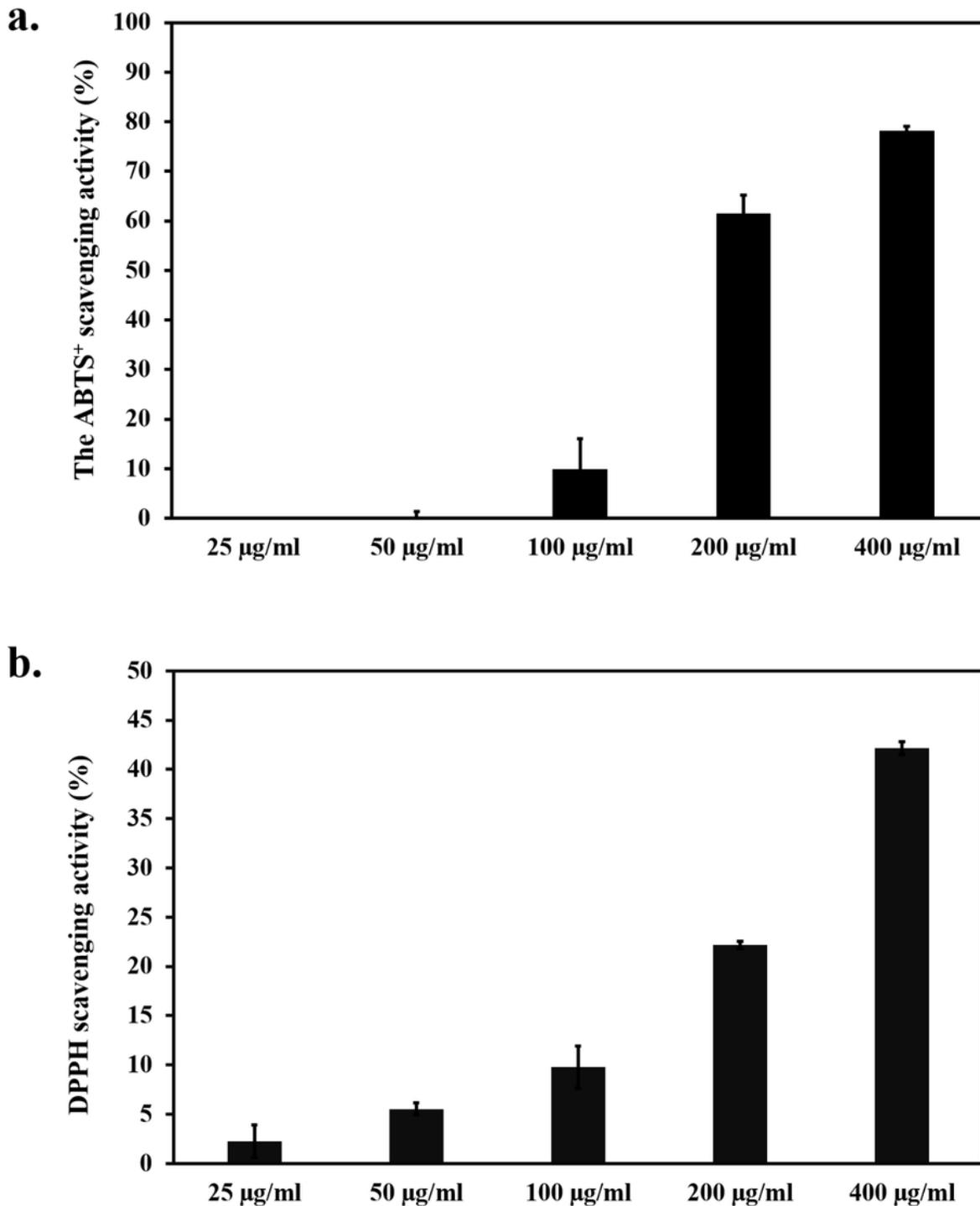
The cDNA sequence and the predicted prepropeptide sequence of cathelicidin-HR. The predicted mature peptide is displayed in bold. The predicted signal peptides are underlined. An asterisk (\*) indicates the stop codon.



**Figure 2**

The cathelicidin-HR sequence analysis. The amino acid sequence alignment (a.) and phylogenetic analysis (b.) of cathelicidin-HR complete peptide compared with other amphibian cathelicidins. The highly conserved amino acid are shaded. The mature peptides are underlined. A. loloensis cathelicidin (cathelicidin-AL<AEI69698>), B. gargarizans cathelicidin (cathelicidin-BG<ANV28414>), D. melanostictus cathelicidin (cathelicidin-DM<AJQ20790>), H. rugulosus cathelicidin (cathelicidin-HR<MW725232>), L. fragilis cathelicidins (Lf-CATH1 and Lf-CATH2 <without accession No.>), N. ventripunctata cathelicidin (cathelicidin-NV<AZW10343>), O. andersonii cathelicidin (cathelicidin-OA1<AWO14611>), O. livida cathelicidins (OL-CATH1<AXR75913> and OL-CATH2 <AXR75914>), P. nigromaculata cathelicidins (PN-

CATH1<QPL18198> and PN-CATH2 <QPL18199>), *P. puerensis* cathelicidin (cathelicidin-PP<ASU44943>), *P. yunnanensis* cathelicidin (cathelicidin-PY1<AFX61592>), and *R. catesbeiana* cathelicidins (cathelicidin-RC1< AHW58220> and cathelicidin-RC2 < AHW58221>).



**Figure 3**

The antioxidant activity of PC29 peptide. The ABTS<sup>+</sup> (a.) and DPPH (b.) scavenging activity of PC29 peptide.

## Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- [SupplementalData.docx](#)