

Original Research Paper

Antibacterial Activity of Skin Secretion of Bleeding Toad *Leptophryne cruentata* and Javan Tree Frog *Rhacophorus margaritifer*

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Abstract: Bacterial resistance towards antibiotics has increased morbidity, mortality and health-care costs. Therefore, it is important to search for new types of antibacterial agents. This study was aimed to test antibacterial activity of epinephrine-stimulated skin secretions derived from two species of Anura endemic in Indonesia, the bleeding toad *Leptophryne cruentata* and the javan tree frog *Rhacophorus margaritifer*. The filter-sterilized skin secretions were subjected to antibacterial assay against Gram-negative bacterium *Escherichia coli* and Gram-positive bacterium *Staphylococcus aureus*. Results showed that skin secretions of some toads and frogs have antibacterial activity against *Escherichia coli* and *Staphylococcus aureus*. We conclude that the toad and frog skin secretions have the potential to be used as a source of novel antibacterial agents. To the best of our knowledge, this is the first report on antibacterial activity of skin secretions of *Leptophryne cruentata* and *Rhacophorus margaritifer* from Indonesia.

Keywords: Antibacterial Agent, *Escherichia coli*, *Leptophryne cruentata*, *Staphylococcus aureus*, *Rhacophorus margaritifer*

Introduction

The discovery of antibiotics has been considered as the most significant finding in medicine (Govender *et al.*, 2012). However, the large scale production and use of antibiotics, estimated of 100,000 tons annually worldwide, for human therapy, farm animal and fish aquaculture, has resulted in the selection of pathogenic bacteria resistant to multiple drugs (Nikaido, 2009). The emergence of multidrug resistant bacteria constitutes a serious threat to public health and results in increased morbidity, mortality, as well as health-care costs. Therefore, it is necessary to search for novel types of antibacterial agents (Conlon and Sonnevend, 2011; Govender *et al.*, 2012).

Toads and frogs have skin glands as defense mechanism against predators and pathogens. The glands are usually activated by stress or injury and secrete a complex chemical cocktail which vary from species to species from being slightly noxious to extremely toxic (Gomes *et al.*, 2007; Siano *et al.*, 2014; Libério *et al.*,

2014). The skin glands of toads and frogs contain various bioactive molecules such as peptides, proteins, steroids, biogenic amines and alkaloids that possess antibacterial and other important biological activities. The compositions of the skin secretions differ among anuran groups according to their interactions with the environment (Gomes *et al.*, 2007; Libério *et al.*, 2014). Biomolecules derived from amphibian skin may provide potential clues towards development of new drugs to combat pathogenic bacteria (Gomes *et al.*, 2007).

Toads and frogs exist in every continent, except Antarctica. They are found in warm, wet tropical areas and some of them can even adapt to dry and cool climates. There are about 4100 species of Anura all over the world. Although Indonesia houses about eleven percent of the total anuran species (Wulandari *et al.*, 2013), reports on biological activities of Indonesian anuran skin secretions are limited. The present study was aimed to analyze antibacterial activity of skin secretions of *Leptophryne cruentata* and *Rhacophorus margaritifer* from West Java, Indonesia. This study

was conducted in attempt to search for new sources of antibacterial agents that are less likely to induce bacterial resistance.

Materials and Methods

Antibacterial Activity of Toad or Frog Skin Secretion

We have previously reported the collection of skin secretions from 7 toads *L. cruentata* (the specimens denoted LC1 to LC7) and 10 frogs *R. margaritifera* also known as *R. javanus* (the specimens denoted RJ1 to RJ10) (Artika *et al.*, 2015). All of the specimens were collected from Mount Gede Pangrango National Park, West Java, Indonesia. The filter-sterilized epinephrine-induced skin secretions were subjected to antibacterial activity test against *E. coli* and *S. aureus*. The antibacterial activity assay was carried out using a modified method of Afsar *et al.* (2011). As much as 50 μL of each bacterial culture was mixed with warm Mueller Hinton agar medium (30.0% beef infusion, 1.75% casein hydrolysate, 0.15% starch, 1.7% agar, pH 7.0) followed by incubation until the medium solidified.

Holes of 5 mm diameter were made into which the toad or frog skin secretion (40 μL) was applied. Chloramphenicol 0.4 mg mL^{-1} (40 μL) was used as a positive control and phosphate buffer solution (40 μL) of the same concentration as that used to liquify the freeze-dried skin secretions was employed as a negative control. The culture was then incubated for 24 h at 37°C. The antibacterial activity was measured from the formation of the clearing zone due to inhibition of bacterial growth around the treated area. Each assay was carried out in duplicate.

Results

Antibacterial Activity Against *E. coli*

Antibacterial activity against *E. coli* was shown by some skin secretions of *L. cruentata* and *R. margaritifera* (Fig. 1). The average diameter of the clear zone for skin secretions of toad *L. cruentata* against bacterium *E. coli* was 10.1 ± 1.7 mm, while that for frog *R. margaritifera* was 11.7 ± 1.9 mm (Fig. 2). These values were less than that shown by chloramphenicol 0.4 mg mL^{-1} (22.4 ± 1.3).



Fig. 1. Antibacterial activity of skin secretions of toad *Leptophryne cruentata* and the Javan tree frog *Rhacophorus margaritifera* against bacterium *E. coli*. The formation of clearing zone around the treated area indicated antibacterial activity. Selected plates are shown. LC3, LC4, LC5, LC6 and LC7 = skin secretions of *L. cruentata*; RJ4, RJ5, RJ6, RJ7, RJ8 and RJ9 = skin secretions of *R. margaritifera* (also known as *R. javanus*); a = chloramphenicol 0.4 mg mL^{-1} (positive control); b = phosphate buffer solution (negative control)

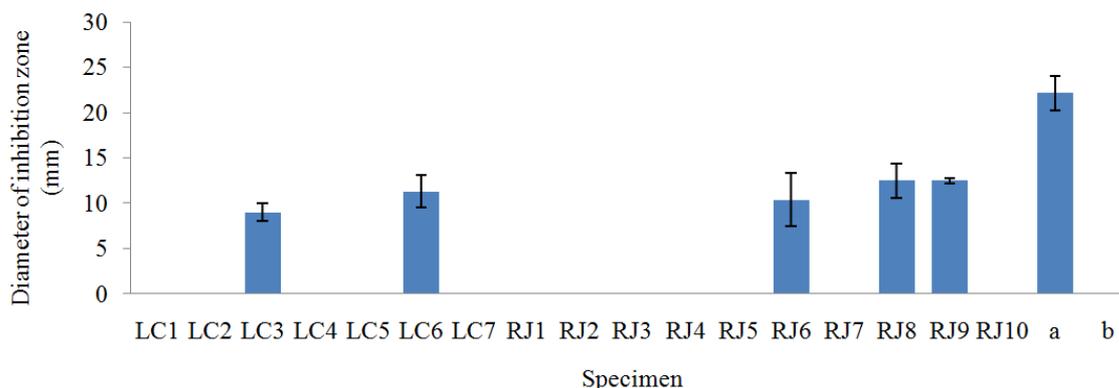


Fig. 2. Antibacterial activity of skin secretions of *L. cruentata* and *R. margaritifera* against bacterium *E. coli*. LC1 to LC7 = skin secretions of *L. cruentata*; RJ1 to RJ10 = skin secretions of *R. margaritifera* (*R. javanus*); a = chloramphenicol 0.4 mg mL^{-1} (positive control); b = phosphate buffer solution (negative control); I = standard deviation

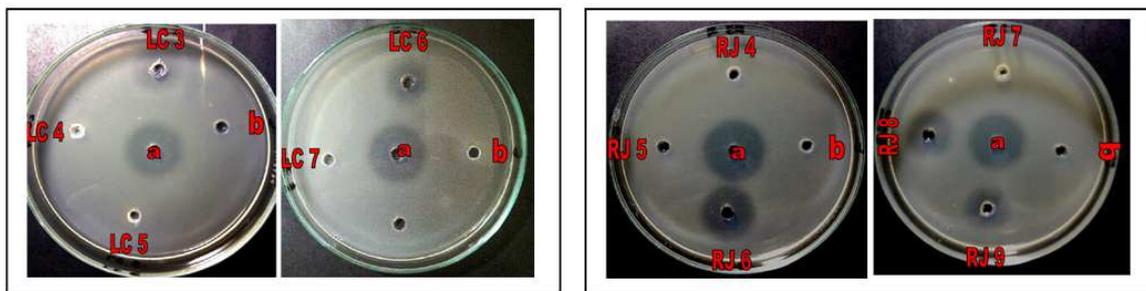


Fig. 3. Antibacterial activity of skin secretions of toad *Leptophryne cruentata* and the Javan tree frog *Rhacophorus margaritifera* against bacterium *Staphylococcus aureus*. The formation of clearing zone around the treated area indicated antibacterial activity. Selected plates are shown. LC3, LC4, LC5, LC6 and LC7 = skin secretions of *L. cruentata*; RJ4, RJ5, RJ6, RJ7, RJ8 and RJ9 = skin secretions of *R. margaritifera* (also known as *R. javanus*); a = chloramphenicol 0.4 mg mL⁻¹ (positive control); b = phosphate buffer solution (negative control)

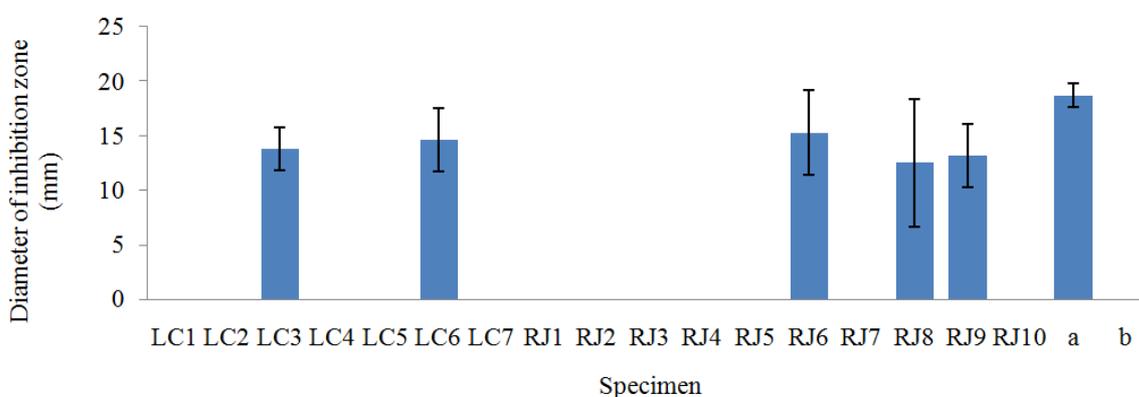


Fig. 4. Antibacterial activity of skin secretions of *L. cruentata* and *R. margaritifera* against bacterium *S. aureus*. LC1 to LC7 = skin secretions of *L. cruentata*; RJ1 to RJ10 = skin secretions of *R. margaritifera* (*R. javanus*); a = chloramphenicol 0.4 mg mL⁻¹ (positive control); b = phosphate buffer solution (negative control); I = standard deviation

Antibacterial Activity Against *S. aureus*

Antibacterial activity against *S. aureus* was shown by some skin secretions of *L. cruentata* and *R. margaritifera* (Fig. 3). The average diameter of the clear zone for skin secretions of toad *L. cruentata* against bacterium *S. aureus* was 14.2±2.1 mm, while that for frog *R. margaritifera* was 13.6±3.6 mm (Fig. 4). The average diameter of the inhibition zone generated by chloramphenicol 0.4 mg mL⁻¹ was 18.7±1.1 mm.

Discussion

We report that the skin secretion of the bleeding toad and javan tree frog of West Java, Indonesia, has the potential to be used as a source of antibacterial agents. In the Island of Java, live about 42 species of toads and frogs (Wulandari *et al.*, 2013). There have been limited studies carried out on antibacterial activity of skin secretions of anurans from Indonesia for the purposes of bioprospecting for potentially new antibacterial compounds. In the present study we showed that some of

the ephineprine induced-toad and frog skin secretions exhibit antibacterial activity.

This study employed *E. coli* and *S. aureus* as testing bacteria representing the gram-negative and gram-positive bacteria respectively. *E. coli* is the most abundant gram-negative bacterium of the intestinal microflora, naturally colonising the mucous layer of the colon. There are many different types of *E. coli* and while some live in the intestine quite harmlessly, others may cause a variety of diseases (Garmendia *et al.*, 2005). *S. aureus* is a gram-positive bacterium and is frequently found in the human respiratory tract and on the skin. *S. aureus* can be a versatile and virulent pathogen in humans and the rates of infections caused by this bacterium are increasing steadily (Corey, 2009). In addition, *S. aureus* is the main cause of infectious morbidity and mortality in hemodialysis patients (Vandecasteele *et al.*, 2009).

While some skin secretions were able to inhibit the growth of *E. coli* and *S. aureus*, some others, however, failed to show antibacterial activity (Fig. 2 and 4). The reasons for this remain unclear and this may reflect the

complexity of the biological system that causes characteristic diversity of the toad and frog skin secretions. In all cases, the activity of the skin secretions was less than that of the protein synthesis inhibitor, chloramphenicol, at a concentration of 0.4 mg mL⁻¹. In addition, the mode of bacterial growth inhibition by the skin secretions has yet to be elucidated.

A number of studies have reported the antibacterial activity of various preparations derived from skin of toad and frog. Afsar *et al.* (2011) showed that extracts prepared from skin secretions of the frog *Rana macrocnemis* collected from north-eastern region of Turkey were active against *Bacillus cereus*, *Bacillus subtilis*, *E. coli*, *Proteus vulgaris*, *Sarcina lutea*, *Enterobacter aerogenes*, *Salmonella typhimurium* and *S. aureus*. Similarly, Abraham *et al.* (2014) found that electrically stimulated skin secretion of the Indian Ranid frog *Clinotarsus curtipes* of the Western Ghats, Kerala, India contains multiple peptides having antibacterial activity against diverse bacterial strains, including *V. cholerae* and Methicillin Resistant *S. Aureus* (MRSA). Dorsal skin secretions of Iranian frog, *Rana ridibanda*, was also shown to have a significant antibacterial activity against MRSA (Abbasi *et al.*, 2007).

The chemical components of the toad and frog skin secretions used in this study have been reported (Artika *et al.*, 2015). Compounds with potential antibacterial activity include: fatty acids, triacontane, nonadecane, heptadecane, pregnane, pentadecanone, nonacosane, palustrol and myrtenol. Zheng *et al.* (2005) reported that long-chain unsaturated fatty acids, such as linoleic acid, palmitoleic acid, oleic acid, linolenic acid and arachidonic acid show antibacterial activity by inhibiting bacterial enoyl-acyl carrier protein reductase (FabI), an essential component of bacterial fatty acid synthesis.

Volatile oils obtained from leaves of *Tamarix boveana* of Tunisia that contains triacontane, nonadecane and heptadecane as minor components were shown to have antibacterial activity against *S. aureus*, *Micrococcus luteus* and *Salmonella typhimurium* (Saïdana *et al.*, 2008). Similarly, essential oils distilled from leaves of the plant *Ageratum fastigiatum* of Minas Gerais, Brazil, which contains about 14.6% of nonadecane were shown to have antibacterial activity against *S. aureus*, *Streptococcus mutans*, *Streptococcus faecalis*, *E. coli* and *Salmonella typhosa* (Del-Vechio-Vieira *et al.*, 2009). Recently, Zhao *et al.* (2013) showed that a pregnane isolated from the marine organism *Carijoa sp.* exhibit promising antibacterial activity against *Pseudomonas putida* with minimum inhibitory concentration value about five-fold more potent than ciprofloxacin.

The skin secretions of specimen RJ9 contained about 2.4% of pentadecanone (Artika *et al.*, 2015). Volatile components of the plant *Minuartia meyeri* of

northeastern Turkey, which contains pentadecanone as one of the major components, was active in inhibiting growth of *Yersinia pseudotuberculosis*, *Enterococcus faecalis* and *S. aureus* (Yayli *et al.*, 2006). Similarly, essential oils of the herb plant *Leonurus japonicus* containing 0.9% of palustrol was shown to have antibacterial activity against *S. aureus*, methicillin-resistant *S. aureus*, *Staphylococcus epidermidis*, *E. faecalis* and *Macrocooccus caseolyticus* (Xiong *et al.*, 2013). Essential oils of the plant *Lavandula dentata* of eastern Maroco, that contains about 3% myrtenol was found to have antibacterial activity against *E. coli*, *Salmonella sp.*, *Neisseria meningitidis*, *S. aureus*, *Streptococcus pneumoniae*, *Listeria monocytogenes* (Imelouane *et al.*, 2009). Essential oils of *Commiphora ornifolia* bark collected from Soqotra Island in Yemen, that contains about 0.7% myrtenol was also found to inhibit growth of bacteria *S. aureus*, *B. subtilis*, *E. coli* and *Pseudomonas aeruginosa* (Mothana *et al.*, 2010).

Considering the potential use of skin secretions of *Leptophryne cruentata* and *Rhacophorus margaritifer* from Indonesia as source of new antibacterial agents, it is considered to be important to test the antibacterial activity of these secretions against multidrug resistant bacteria.

Conclusion

We conclude that epinephrine induced-skin secretions of the bleeding toad, *L. cruentata* and the javan tree frog *R. margaritifer* has the potential to be used as a source of antibacterial agents.

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Author's Contributions

I Made Artika: Conceived of the research, designed the study, drafted and revised the paper.

Sabrina Pinontoan: Involved in sample preparation and performed most of the laboratory work.

Mirza Dikari Kusri: Involved in study design, manuscript writing and scientific discussion.

Ethics

This article is original containing unpublished materials. All authors have read and approved the manuscript and no ethical issues involved.

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