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Antifungal Activity of Skin Secretion of Bleeding Toad *Leptophryne Cruentata* and Javan Tree Frog *Rhacophorus Margaritifer*

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Abstract: Amphibian skin has been the source of a wide variety of biologically active compounds. This study was aimed to determine antifungal activity of skin secretion of two species of anurans endemic in Indonesia, the bleeding toad *Leptophryne cruentata* and the javan tree frog *Rhacophorus margaritifer*. We collected 7 adults of *L. cruentata* and 10 adults of *R. margaritifer* from the wild in Mount Gede Pangrango National Park, West Java. The toads and frogs were induced to release skin secretion using a nonlethal induction technique based on epinephrine injection. The collected skin secretion was filter-sterilized, freeze-dried and subjected to antifungal assay. Results showed that skin secretion of some toads and frogs has antifungal activity against the fungus *Trichophyton mentagrophytes*. Among the chemical compounds with potential bioactivity detected in the skin secretion include fatty acids, amines and steroids. Therefore, the toad and frog skin secretion has the potential to be developed as a source of antifungal agents.

Keywords: Antifungal Agent, *Leptophryne cruentata*, *Rhacophorus margaritifer*, *Trichophyton mentagrophytes*

Iintroduction

Fungi are an important cause of human, animal and plant disease. Fungal infections have been reported to increase dramatically in individuals with a reduced immune status. Treatment is limited due to a narrow range of effective antifungal agents which are available (Katerere *et al*., 2013). The development of antifungal agents has been slow compared to that of antibacterial drugs. This is partly due to the fact that fungi are eukaryotes with cellular structure similar to that of animals and humans so that the antifungal agents tend to be toxic to mammalian hosts (Barlian *et al*., 2011).

In response to stress or predator attack, amphibians secrete a complex chemical cocktail from highly specialized skin structures, namely the venom or granular glands, which are usually dispersed throughout the dorsal surface. The molecular diversity of active components in these glands is high and includes alkaloids, biogenic amines, proteins and peptides. These chemical defenses can be directed either against predators or against microorganisms (Siano *et al*., 2014). The secretions of frog skin might also be of benefit to human health with its antibacterial, antifungal, antiprotozoal, antidiabetic and other therapeutic properties (Gomes *et al*., 2007).

Conlon and Sonnevend (2011) reported that skin secretion of many anurans (toads and frogs) contain peptides with antifungal activity. These peptides are stored in a granular gland located mainly in the skin of the dorsal region. Similarly, Rollins-Smith *et al.* (2005) found that most amphibian species they tested have one or more peptides with potent activity against fungi.

Scientists are now exploring the therapeutic potential of various toad and frog skin secretions and extracts. Indonesia, being a tropical country with diverse geographical variations, is home to about 450 species of toads and frogs which account for about 11% of world toad and frog species. It is reported that in Java Island alone there are 42 species of toads and frogs (Wulandari *et al*., 2013). Studies on bioactivity and chemical composition of Indonesian toad and frog skin secretions are limited. In the present study we report the

antifungal properties and chemical compounds of skin secretions derived from the bleeding toad, *Leptophryne cruentata* and the Javan tree frog *Rhacophorus margaritifer* which is also named *Rhacophorus javanus*.

Materials and Methods

Collection of Toad or Frog Skin Secretion

In the present study we employed 7 toads *L. cruentata* (the specimens denoted LC1 to LC7) and 10 frogs *R. margaritifer* also known as *R. javanus* (the specimens denoted RJ1 to RJ10) collected from Mount Gede Pangrango National Park, West Java, Indonesia. Following collection, the Snout Vent Length (SVL) and weight of each specimen was measured.

Skin secretions were collected using a modified method of (Woodhams *et al*., 2006; Gibble *et al*., 2008). To induce skin secretion, we injected 0.01 mL g^{-1} body weight of epinephrine (1 mg mL^{-1}) under the skin of the toad or frog. Each injected toad or frog was put in a separate plastic container filled with 50 mL of acetate buffer (50 mM NaCl, 25 mM CH_3COONa, pH 7). After 15 min, the toads or frogs were then released back to the wild. The buffer containing each skin secretion was lyophilized. It was then liquified with Phosphate Buffer Saline (PBS, 10 mM Na_2HPO_4, 1.8 mM KH_2PO_4, 137 mM NaCl and 2.7 mM KCl, pH 7.4) for further use in experiments.

Antifungal Assay

Antifungal activity assayed using the method of (Barlian *et al*., 2011) with slight modifications. As much as 50 µL of fungus *Trichophyton mentagrophytes* culture was grown on Sabouraud Dextrose Agar (SDA, 40 g L^{-1} dextrose, 10 g L^{-1} peptone, 20 g L^{-1} agar, pH 5.6) medium in a sterile petri dish. Holes of 5 mm diameter were made into which the toad or frog skin secretion (40 µL) was applied. Ketoconazole (2% w/v) (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 7 days at room temperature until the fungal growth in the petri dish was homogeneous. The antifungal activity was measured from the formation of clearing zone due to inhibition of mycelial growth around the treated area.

Determination of Chemical Compounds of Skin Secretion

Solution containing filtered skin secretions was analyzed using GC-MS. The number of compounds contained in the sample was indicated by the number of peaks in the chromatogram, while the types of compound were interpreted based on the data of the spectra of each peak using GC-MS database library.

Results

Toad and Frog Skin Secretion

We tested seven specimens of *L. cruentata* and 10 *R. margaritifer*. The average Snout Vent Length (SVL) of the *L. cruentata* was 28.3±5.8 mm, with average body mass of 5.9±0.6 g. The average SVL of the javan tree frog *R. margaritifer* was 44.7±1.5 mm and average body mass was 7.9±0.6 g.

The average dry weight of skin secretion of *L. cruentata* was 264±266 mg. On the other hand, the average dry weight of *R. margaritifer* skin secretion was 148±221 mg.

Antifungal Activity of Toad and Frog Skin Secretion

Antifungal activity was shown by some skin secretions of *L.cruentata* and *R. margaritifer* (Fig. 1). The average diameter of the clear zone of frog skin secretions of *L. cruentata* against fungus *T. mentagrophytes* was 14.5±2.9 mm, while that of *R. margaritifer* was 9.9±4.3 mm (Fig. 2).

Chemical Components of Toad and Frog Skin Secretion

The chemical components of skin secretion of *L. cruentata* (LC3) and *R. margaritifer* (RJ9) are shown in Table 1 and 2.

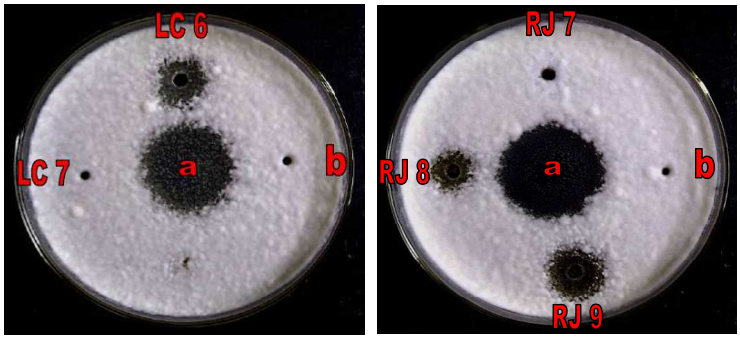

Fig. 1. Antifungal activity of toad *Leptophryne cruentata* and the Javan tree frog *Rhacophorus margaritifer*. The formation of clearing zone around the treated area indicated antifungal activity. Selected plates are shown. LC6 and LC7 = skin secretion of *L. cruentata*; RJ7, RJ8 and RJ9 = skin secretion of *R. margaritifer* (also known as *R. javanus*); a = ketoconazole 2% (positive control); b = phosphate buffer solution (negative control)

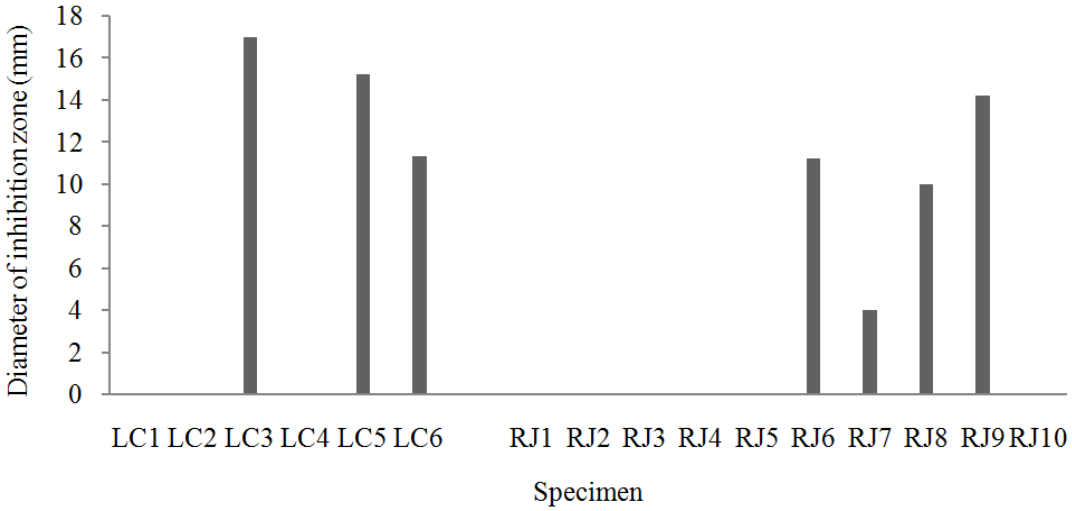

Fig. 2. Antifungal activity of skin secretions of *L.cruentata* and *R. margaritifer*. LC1 to LC6 = skin secretions of *L. cruentata;* RJ1 to RJ10 = skin secretions of *R. margaritifer* (*R. javanus*)

Table 1. Chemical components of skin secretion of *Leptophryne cruentata* (LC3)

Retention time (minute)	Area (%)	Name
4.029	16.00	Ammonium carbamate
4.295	22.43	Ethanol
4.597	37.76	Ethanol
7.515	10.13	Acetic acid
9.075	0.59	S-methylcysteamine
18.113	0.31	5-hydroxy-3-methylbutiric acid
24.603	1.08	Myrtenol
24.775	0.59	Isoadamantane
26.959	0.34	Methyl hexadecatrienoate
27.316	0.73	Pyrrolidine
30.371	0.66	Methyl palmitate
30.844	0.90	Palmitic acid
31.128	0.57	Ethyl palmitate
31.422	0.37	Palustrol
32.467	0.89	Methyl octadecenoate
33.253	0.52	Ethyl oleate
33.501	0.25	Oleoamide
33.660	0.26	Hexadecanamide
35.064	0.35	Palmitic acid
36.387	2.68	Oleoamide
36.688	0.46	Hexadecanamide
38.169	0.49	12-Nitro-15-hexadecanolide
38.528	0.62	Palmitic acid
39.817	0.60	1,2-Benzenedicarboxylic acid (phthalic acid)
45.348	0.42	Pregnane

The results were based on the detected mass spectrum compared to the WILLEY147 and NIST47 libraries available in the GC-MS software used. The chemical compounds detected include amines, fatty acids, organic acids, steroids, alkanes, benzoic acid vitamin E, alcohols and ketones.

Table 2. Chemical components of skin secretion of *Rhacophorus margaritifer* (RJ9)

Retention time (minute)	Area (%)	Name
3.915	8.17	Carbon dioxide
4.225	17.96	2-Butanamine, 3-phenoxy (amine)
4.345	35.79	Ammonium bicarbonate
6.165	17.14	Acetate Acid
27.693	0.20	2-Methyl-6-propyldodecane
28.875	1.17	n-Nonadecane
29.055	1.34	n-Triacontane
29.489	0.52	Peucelinendiol
29.705	0.45	9, 12-octadecadienoic acid
29.955	0.53	Glycine (amino acid)
30.373	1.01	Methyl palmitate
30.839	1.20	Palmitic acid
31.152	0.56	n-Tricosane
31.852	0.86	α-Tokopherol
32.468	0.90	Methyl oleate
32.692	0.38	Methyl stearate
33.030	0.77	Nonacosanol
33.209	1.77	oleic acid
33.514	1.34	n-Octadecane
33.972	0.58	Phyrimidinetrione
34.356	0.52	Pregnane
34.607	0.94	Allyl octadecanoate
34.805	0.61	Octadecane
35.063	0.24	palmitic acid
36.397	0.33	1-Heptadecanamine
37.552	0.25	Pregnane
38.162	0.17	8-Hexyl-8-pentyl-heksadecane
43.709	1.17	Hexadecanoic acid
48.026	2.37	2-Pentadecanone
51.135	0.75	25-homo-24-ketocholesterol (Steroid)

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 antifungal agents. In

the present study, we have developed a modified method for a nonlethal amphibian skin secretion collection based on epinephrine induction. In many classical methods, toad and frog specimens were sacrificed and their skin removed for extraction. For ethical and scientific reasons, this approach is now unacceptable. Sacrificing a large number of toads or frogs is unreasonable since amphibians are now in rapid global decline. In addition, only the components of the skin secretion are required for bioactivity assay and chemical analysis and not every component of toad or frog integument. Therefore, preparations generated from whole skin-tissue-solubilization is not representative of a glandular secretion (Clark, 2009).

To accumulate *L. cruentata* and *R. margaritifer* skin secretion in this study, we applied ephinephrine hormone, a modification of the method by (Woodhams *et al*., 2006) which used norepinephrine hormone to trigger the increase of heart rate, sweating and shock. In other studies, to obtain the bioactive compounds from frog skin secretions (Woodhams *et al*., 2007) immersed frogs in norephinephrine bath for 15 min. Some other techniques that have been employed for releasing frog skin secretion include parotid gland squeezing (Garg *et al*., 2007), ether inhydrate exposure (Che *et al*., 2008) and electric shock (Barlian *et al*., 2011). This suggests that the accumulation of frog skin secretion can be carried out using different approaches depending on the purpose of the study. However, considering the conservation of amphibians as well as ethical reason, we developed a non-lethal method. Other reason of selecting the method used in this study was due to the fact that the toads and frogs employed belong to protected species.

Measurements of SVL and weight of each specimen was intended to obtain data on average size of *L. cruentata* and *R. margaritifer* in the area of Mount Gede Pangrango National Park. There was no clear correlation between specimen size and the amount of skin secretion as well as antifungal activity observed. Prior to antifungal assays, the skin secretions were dissolved in PBS. PBS was used because it is isotonic and non-toxic to most cells.

Not all of the secretions of *L. cruentata* and *R. margaritifer* specimens showed antifungal activity. The reasons for this have yet to be elucidated. Some skin secretions of *L.cruentata* and *R. margaritifer* showed antifungal activity. The ability of the bioactive compounds in these skin secretions to inhibit growth of fungus *T. mentagrophytes* is consistent with other studies (Garg *et al*., 2007; Afsar *et al*., 2011; Barlian *et al*., 2011) which found antifungal activity in toad and frog skin secretions. Katerere *et al*. (2013) also reported that skin secretions from frogs (*Amietia fuscigula, Strongylopus grayi* and *Xenopus laevis)* and a toad (*Amietophrynus pantherinus)* from South Africa showed antifungal activity against fungi *Candida albicans*, *Fusarium verticillioides* and *Aspergillus flavus.* This suggests that the frog skin secretions have the potential for the development of pharmacological compounds.

The chemical component analysis using GC-MS showed that the toad and frog skin secretions contain putative bioactive compounds such as fatty acids, amines and steroids. Previous studies also reported that bioactive compounds in skin secretions of frog and toad consist of peptides, amines, proteins, steroids and alkaloids (Garg *et al*., 2007; Gomes *et al*., 2007).

Fatty acids and their derivatives have been reported to possess antifungal activity (Pohl *et al*., 2011; Choi *et al*., 2010). The most important target of antifungal fatty acids is the cell membrane. Fatty acids cause an increase in membrane fluidity which results in a generalized disorganization of the cell membrane that leads to conformational changes in membrane proteins, the release of intracellular components, cytoplasmic disorder, cell disintegration and eventually cell death (Pohl *et al*., 2011). In addition to fatty acids, ethanamines (Thvedt *et al*., 2013), pregnane and its derivatives (Yoon *et al*., 2011; Subrahmanyam *et al*., 2005), pyrimidine and its derivatives (Sharma *et al*., 2014; Neumann *et al*., 2014), myrtenol (Tabassum and Vidyasagar, 2013) have also been reported to show antifungal activity.

In this analysis we failed to detect peptide and protein. This might be caused by the high temperature used in the GC-MS (280°C) thus damaging the structure of proteins and peptides. Other compounds which could be present but go undetected by GC-MS in the present study were alkaloids. The failure to detect alkaloid in the skin secretion of *L. cruentata* and *R. margaritifer* might be caused by the low concentration of alkaloid the specimen which might due to the type of prey that does not contain alkaloid or contains alkaloid at low level.

Conclusion

We conclude that the release of frog and toad skin secretion can be induced using injection of epinephrine. The skin secretion of the bleeding toad, *L. cruentata* and the javan tree frog *R. margaritifer* has the potential to develope as a source of antifungal egents.

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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 collection and performed most of the laboratory work.

Mirza Dikari Kusrini: Collected the samples, performed toad and frog identification and 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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