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Antimicrobial Activity of *Fejervarya* Skin Secretions (Anura: Dicroglossidae) in West Sumatra, Indonesia

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Abstract

BACKGROUND: The emergence of pathogen microorganisms in recent years has developed antibiotic resistance. The resistance has increased morbidity, mortality, and the cost of medical care. Amphibian skin is rich in biologically active compounds. The compounds secreted from frog skin have the potential to be developed into new antimicrobial compounds to fight pathogenic bacteria and fungi.

AIM: This study aimed to analyze the ability of compounds secreted from the skin of F. cancrivora and F. limnocharis in West Sumatera to inhibit the growth of Gram-negative bacteria, Gram-positive bacteria, antibiotic resistant bacteria, and fungi.

METHODS: This study used the diffusion method with paper discs for antimicrobial test of frog skin secretions.

RESULTS: Result from this study showed that the skin secretions of F. cancrivora and F. limnocharis in West Sumatera, Indonesia did not show any antimicrobial properties.

CONCLUSION: Skin secretions of F. limnocharis and F. cancrivora do not show any antimicrobial activity.

Introduction

The genus *Fejervarya* is widely distributed in Asia and consisting of 13 species [1]. Morphological characteristics of *Fejervarya* are divided into four different groups, which are large, medium, small, and mangrove types [2]. Kurniawan *et al*. (2014) reported that there are five species of *Fejervarya* in Indonesia, namely, *Fejervarya* cf. *verruculosa*, *Fejervarya* sp., *F. iskandari*, *F. limnocharis*, and *F. cancrivora*. *F. limnocharis* and *F. cancrivora* are widely distributed and sympatric in Sumatera, Kalimantan, and Java [3]. This two frog species are commonly found in rice fields, thus also known as rice field frogs [4].

Research on *F. cancrivora* and *F. limnocharis* in Indonesia and several other countries has been conducted by several researchers. The previous research mostly concerning the morphological variations, reproductive isolation mechanisms, and molecular phylogenetic relationships of *F. limnocharis* complex in Indonesia, Malaysia, and Japan [5], genetic relationships and reproductive isolation mechanisms between *F. limnocharis* complex in Indonesia (Java) and other Asian countries [6], *Fejervarya* (Anura: Dicroglossidae) phylogenetic [7], genetic variation, and evolutionary relationships of *F. cancrivora* in Indonesia and other Asian countries based on the analysis of allozymes and mitochondrial DNA sequences [8], the taxonomic status of three types of *F. cancrivora* from Indonesia and other Asian countries based on morphological observations and crossing experiments [9], also about genetic divergence and geographic distribution of *Fejervarya* in Indonesia based on mitochondrial 16s rRNA gene analysis [3]. Recent research has shown that frog skin secretion is a potent antimicrobial.

The emergence of pathogen microorganisms in recent years has developed antibiotic resistance which is a serious threat to public health [10]. The World Health Organization (2014) reported there are 50% of *E. coli*, *Klebsiella*, and *Staphylococcus* have been resistant to antibacterial, while Candida has been resistant to fluconazole [11]. The resistance has increased morbidity, mortality, and the cost of medical care [12]. Therefore, it is important to find new types of antimicrobial agents, and one of the sources is frog skin secretions.

Gomes *et al.* (2007) reported that the compounds secreted from frog skin have the potential to be developed into new antimicrobial compounds to fight pathogenic bacteria and fungi [13]. Amphibian skin is rich in biologically active compounds [14]. Amphibians secrete chemical compounds from skin glands that are scattered throughout the skin surface in response to stress and to protect themselves from predators [13], [15]. The chemical compounds contained in the amphibian skin glands are peptides, biogenic amines, steroids, alkaloids, and proteins [15], [16]. Research on the potential of antimicrobial compounds from frog skin secretions has been carried out by Afsar *et al*., (2011) who reported that *Rana macrocnemis* frog skin secretions in Turkey have antimicrobial activity on *Bacillus cereus, B. subtilis, E. coli, Proteus vulgaris, Sarcina lutea, Enterobacter aerogenes*, *Salmonella typhimurium*, *S. aureus*, and *C. Albicans* [10]. Wang *et al*. (2012) reported that the peptides isolated from the skin of *Odorrana hainanensis* frogs in China were able to inhibit the growth of Gram-positive bacteria, Gram-negative bacteria, and fungi [16]. Katerere *et al*. (2013) also reported that compounds secreted from frogs (*Amietia fuscigula, Strongylopus grayi and Xenopus laevis*) and toads (*Amietophrynus pantherinus*) skins in South Africa have an antifungal activity which can inhibit fungal growth [17]. However, research on the skin secretions of *F. cancrivora* and *F. limnocharis* in West Sumatra which have the potential to produce antimicrobial compounds is still unknown. In contrast, antimicrobial activity will contribute as a basic data to the future studies in the monitoring and potential to produce novel antimicrobial. Therefore, antimicrobial activity of *F. cancrivora* and *F. limnocharis* skin secretion in West Sumatra is needed.

Methods

Sample preparation and collection of frog secretions

Samples of *F. limnocharis* were collected directly around the Limau Manis rice fields, Pauh District, Padang City and *F. cancrivora* was collected in the Koto Baru area, Kubung District, Solok Regency. Frog samples were captured using the visual night encounter technique. Research on the antimicrobial test of *Fejervarya* frog skin secretions (Anura: Dicroglossidae) in West Sumatera was carried out from April to September 2019.

The collection of frog skin secretions was carried out according to the method used by Grant and Land (2002) which has been modified. Electrical stimulation using an electric applied shock directly to the frog samples [18]. The electric shock instrument (Transcutaneous Amphibian Stimulator/TAS) was made according to a modified design by Grant and Land (2002) [18]. This instrument was placed on the dorsal part of the frog. The applied voltage was in accordance with the SVL (Snout Vent Length) of each frog and given for 30 s. The glandular secretions resulted then scraped using a spatula and put into micro tube. Frog skin secretions of *F. limnocharis* used a 10% concentration and *F. cancrivora* used a pure 100% concentration. Secretions of *F. limnocharis* and *F. cancrivora* secretions were tested in different concentrations due to the small number of individuals and the lack of *F. limnocharis* skin secretions.

Test microorganisms and growth conditions

This study used bacterial and fungal samples for the antimicrobial test. Bacterial samples used were *Escherichia coli* O157, *Pseudomonas aeruginosa* ATCC 27853, *Salmonella thypimurium* ATCC 14028, *Staphylococcus aureus* ATCC 25923, *Entrococcus faecalis* ATCC 29212, *Listeria monocytogenes* CFSAN004330, and Methycillin-Resistant *Staphylococcus aureus* (MRSA) while fungal sample used was *Candida albicans*. The microbial samples were obtained from Microbiology Laboratory, Biology Department, Andalas University, Padang.

The bacteria were cultured in Mueller-Hinton Agar (Oxoid) medium at 37°C for 24 h. Fungi were cultured in Sabouraud Dextrose Agar (Oxoid) medium at 30°C for 48 h.

Antimicrobial assay

An antimicrobial assay was carried out using agar diffusion method with paper disc based on the modified method of Afsar *et al*. (2011) [10], the modification was concerning the media and the concentration used. The microbial samples colonies were taken as much as 1–2 dose from the media, then it was suspended in a 0.85% NaCl physiological solution. Next, the solution was homogenized using vortex and the turbidity was adjusted with 0.5% Mc Farland solution which is equivalent to a cell density of 1.5×10^8 colony forming unit/ml. Then, 100 μL of bacterial suspension was swab-tested using a cotton bud in a sterile petri dish containing the media. Each paper disc was dripped with 30 μL of frog skin secretion solution.

Chloramphenicol 30 mg.mL^{-1} was used as a positive control for bacteria, 20% ketoconazole for fungi and DMSO 0.5% as a negative control. Bacterial cultures were incubated at 37°C for 20 h and fungal cultures were incubated at 30°C for 48 h. After incubation, the inhibition zone around the disc paper was observed. In this study, the whole testing was carried out with three replications.

Results

The antimicrobial assay on *F. limnocharis* skin secretions was done against five pathogenic bacteria including Gram-negative bacteria, Gram-positive bacteria, and antibiotic resistant bacteria. The test result did not show any antimicrobial ability. This was characterized by the absence of a clear zone/inhibition zone around the paper disc. The test result on positive control which were given chloramphenicol for bacteria and ketoconazole for fungi is shown in Figure 1.

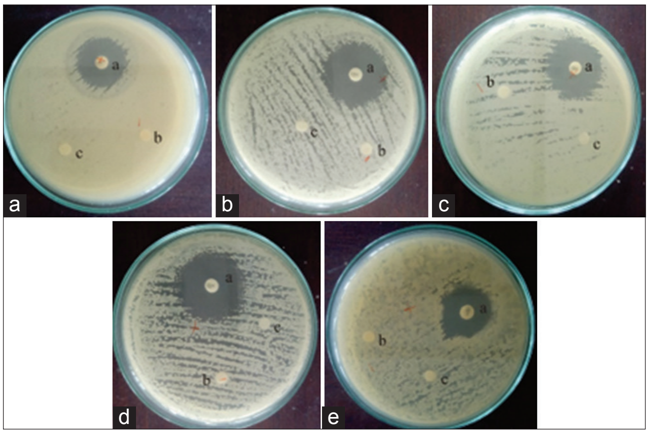

Figure 1: Antimicrobial test of F. limnocharis skin secretions. (a) Pseudomonas aeruginosa ATCC 27853; (b) Salmonella thypimurium ATCC 14028; (c) Staphylococcus aureus ATCC 25923; (d) Entrococcus faecalis ATCC 29212; (e) Methycillin-resistant Staphylococcus aureus (MRSA); a. control positive; b. control negative; c. skin secretion

The antimicrobial assay on *F. cancrivora* skin secretions used seven bacteria and one fungus consisting of Gram-negative bacteria, Gram-positive bacteria, antibiotics-resistant bacteria, and fungi. The test results did not show any antimicrobial ability. Figure 2 showed the absence of a clear zone around the paper disc.

Discussion

According to Figures 1 and 2, it can be concluded that skin secretions of *F. limnocharis* and *F. cancrivora* do not show any antimicrobial activity. Suhyana *et al.* (2015) reported that the skin secretions of *Fejervarya limnocharis* frogs in West Java showed relatively small antibacterial activity against *S. pneumoniae* multidrug resistant (MDR) SPN1307 [19]. Lu *et al.* (2007) also reported that cancrin peptides isolated from the skin secretions of *F. cancrivora* frogs living in mangrove swamps in Hainan, China had antimicrobial activity against *E. coli* ATCC25922, *B. dysenteriae, S. aureus* ATCC2592, and *C. albicans* ATCC2002 [20]. Song *et al*. (2009) reported that the peptides tigerinin-RC1 and tigerin-RC2 isolated from skin secretions of *F. cancrivora* frogs living in mangrove swamps in Hainan, China also exhibited antimicrobial activity against strain *S. aureus* ATCC25923, *S. aureus* ATCC43300, *B. subtilis*, *E. coli* ML-35P, *P. aeruginosa* PA01, *P. aeruginosa* ATCC27853, and *C. albicans* ATCC2002 [21]. The possibility of this result was due to the low concentration of protein contained in frog secretions compared to other bioactive compounds.

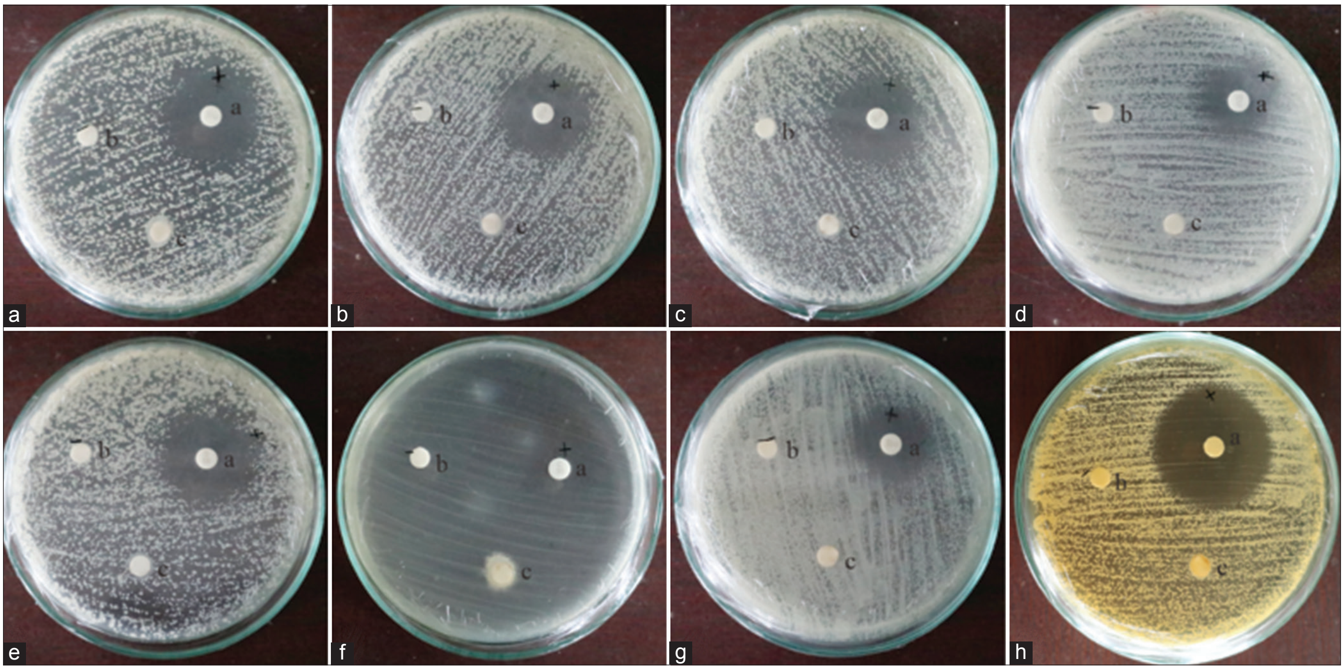

Figure 2: Antimicrobial test of F. cancrivora skin secretions. (a) Escherichia coli O157; (b) Pseudomonas aeruginosa ATCC 27853; (c) Salmonella thypimurium ATCC 14028; (d) Staphylococcus aureus ATCC 25923; (e) Entrococcus faecalis ATCC 29212; (f) Listeria monocytogenes CFSAN004330; (g) Methycillin-resistant Staphylococcus aureus (MRSA); (h) Candida albicans; a. control positive; b. control negative; c. skin secretion

The low protein concentration was probably caused by the use of pesticides in agricultural areas in the habitat of *F. cancrivora* and *F. limnocharis*.

The uses of pesticides also lead to the thickening of the epidermis, damage to the skin histology, and a decrease in the number of glands in frogs. Some of these negative impact of pesticides use on frogs conditions were reported by several studies. The first negative impact is thickening of the epidermis. Alina *et al*. (2010) reported that the skin of *R. ridibunda* injected with actara 25WG resulted in the thickening of epidermal layer [22] Musfar (2019) also reported that there was skin thickening on *F. limnocharis* frogs exposed to pesticides [23].

Second impact is the histological damage to the frog skin. Pasteris *et al*. (2006) reported that the skin of *R. castebaiana* experienced edema, enlarged blood vessels, distortion of serous, and glandular glands in connective tissue; the occurrence of orthokerosis (stratum corneum layer did not contain cell nuclei) in the epidermis; and the most of the connective tissue was separated in the stratum spongiosum [24]. In addition, Varga *et al*., (2019) also reported that short-term exposure to cadmium to the skin of Italian frogs (*Pelophylax bergeri*) resulted in the change in the structure of epidermis layer on the frog skin and induced cellular and molecular stress responses [25]. Furthermore, Musfar (2019) stated that there was skin damage in the form of edema in the compact stratum layer and hypertrophy in the epidermal layer of *F. limnocharis* frogs. The third impact is the decrease in the number of frog glands [23]. Musfar (2019) reported that the *F. limnocharis* frogs exposed to pesticides have less skin glands compared to those not exposed to pesticides [23].

Based on the previous researcher regarding the impact of pesticides, thus the absence of antimicrobial activity on the skin secretions of *F. cancrivora* and *F. limnocharis* frogs was suspected because the samples used were contaminated with pesticides. This is supported by Bruhl, Pieper and Weber (2011) that the permeable nature of frog skin can cause high absorption of pesticides through the skin. More than 83% of pesticides are absorbed through the dorsal and ventral skin [26].

Conclusion

Skin secretions of *F. limnocharis* and *F. cancrivora* do not show any antimicrobial activity.

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