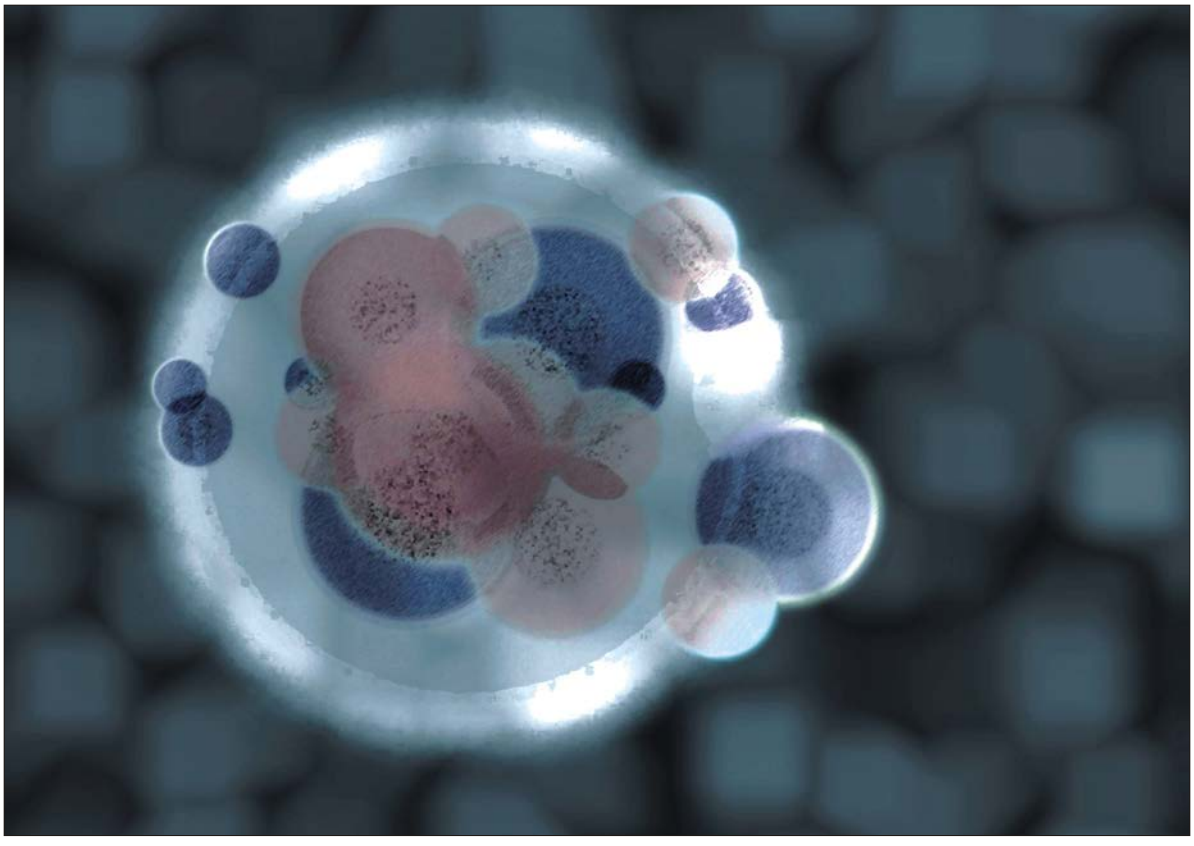

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Structure of *Duttaphrynus melanostictus* Frog Skin and Antifungal Potency of the Skin Extract

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Abstract: Frog skin histology has been largely explored, particularly in relation to the skin-derived secretions, among which a number of peptides have shown potential medicinal applications. *Duttaphrynus melanostictus* is a frog species ubiquitous in Indonesia; however, there is only limited information with regard to skin structural characteristics as well as its potential use. The present work explored the structure of the frog skin and further assessed antifungal activity of its extract. Structural studies were carried out by Scanning Electron Microscopic (SEM) assays while antifungal evaluations were performed by testing the activity of the lyophilisate of skin extract against the pathogenic fungi *Candida albicans*, *Microsporum gypseum* and *Trichophyton mentagrophytes*. Results of the structural studies revealed features common to frog species, in particular an extensive distribution of secretory glands. In addition, we observed a flattened, irregular-shaped structure, believed to be secretory 'vesicle' of the mucous gland, considered distinct to *D. melanostictus*. Antifungal studies demonstrated equipotent activity of the lyophilisate against all test fungi. Taken together, results of the present work shed some light on the structural characteristic of *D. melanostictus* frog skin and further open an opportunity for the development of alternative antifungal agents.

Key words: Structure, *Duttaphrynus melanostictus*, skin, scanning electron microscope, antifungal

INTRODUCTION

The frog *Duttaphrynus melanostictus* is a species ubiquitous in Indonesian soil, easily found especially in the island of Java. The species has unique structure of distinguished wart patterns which have been used for individual identification in studies (Duellman and Trueb, 1994).

The skin has essential functions in a frog, among others, as mechanical barrier (Faquahar and Palade, 1965), as component of chemical defense mechanism (Brizzi *et al*., 2002), as sensor apparatus (Koyama *et al*., 2001), as media for ion transports and water regulation (Sullivan *et al*., 2000), as respiratory organ (Duellman and Trueb, 1994) and as sodium reservoir (Azevedo *et al*., 2007). These functions reflect the morphological complexity of frog skin (De Brito-Gitirana and Azevedo, 2005).

It has been of interesting focus of study to investigate the resistance of frog species against potentially harmful habitat in which they live. The outermost cornified layer of epidermis known as stratum corneum, for example, has been found to contain the substance alfakeratin, functions to prevent the loss of humidity, thus enables a frog to stand against superficial trauma and dryness. An important finding in this respect has recently been reported by Fenoglio *et al*. (2006). The authors revealed a kind of morphofunctional plasticity of the frog epidermis in response to environmental contamination. Thus, the morphological presentation of *Rana esculenta* grown in a contaminated environment, apart from high content of heavy metals, showed less keratinized superficial cells in epidermis and further changes in most of enzyme activities in keratinocytes cells that might be an important compensatory mechanism to counteract the exposure to oxidative and toxic stress.

One of the major interests in the application of frog skin-derived agents is the use of these agents for combating microbial infections. In this regard, a number of early studies have implicated the potency which has been expanded to, not only, the biosynthetic aspect, but also biotechnological development of the active agents (Rinaldi, 2002; Wang *et al*., 2007). Furthermore, a decline in population and even extinction of certain frog species has been associated with infection of hazardous biological agents, including the pathogenic fungus *Batrachochytrium dendrobatidis*. Indeed, frog species having effective antimicrobial peptides, mostly produced

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from the granular gland, have been prevented from population declines (Rollins-Smith and Conlon, 2005; Rollins-Smith, 2009). This evidence warrants a deeper inquiry into the development of potent alternative antimicrobial agents from frog skin.

While there has been fairly large body of information on the skin structural characteristics of many frog species, such data on those of *D. melanostictus* frog skin has only been scarce. In the current work, we explored the structure of the frog skin through Scanning Electron Microscopic (SEM) assay and further investigated the antifungal potency of the skin extract against pathogenic fungi.

MATERIALS AND METHODS

Examination of *D. melanostictus* skin structure: The *D. melanostictus* frogs used in this study were collected from a highly populated area in Eastern part of Bandung, West Java Province. The study was performed from January to December 2010 in School of Life Sciences and Technology and School of Pharmacy Institute of Technology Bandung and Marine Geological Research Center Bandung. Examinations were carried out on both gross and microscopic anatomy of dorsal as well as ventral skin of *D. melanostictus* frog. In SEM assay, a frog was firstly decapitated followed by peeling off the skin. The skin was soaked in cold phosphate buffer solution and was treated with Zenker fixative solution for 24 h. The procedure was continued with dehydration of skin in serial ethanol dilution (70, 80, 90 and 100%) in 24 h. The skin was subsequently dried at 70°C using a hot plate (JEOL JHP 1100). The dried skin was cut into section of 5 mm in diameter and mounted on the holder. The SEM assay, carried out on analytical SEM machine (JEOL JSM 6360 LA type), required a previous coating with a palladium:gold (80:20) mixture which was performed in a Fine Coat Sputter (JEOL Ion Sputter JFC 1100) at 1.2 kV of voltage and 7.5 mA of current for 4 min. Observations were done under 40-20000X magnification.

Extraction of the frog skin: The procedure was done to extract the protein contained in the frog skin tissues using homogenization technique at 4°C. The skin was cut to small sections in PBS medium and then homogenized. The homogenate was subsequently centrifuged at 4350 G for 20 min at a temperature of 4°C. The supernatant was collected for lyophilization and the lyophilisate, a dry powder mass, was then collected for antifungal testing.

Antifungal activity test: The test was performed by agar diffusion method, as done in previous studies (Dulger and Gonuz, 2004a, b; Alam and Mostahar, 2005; Musyimi and Ogur, 2008; Sunilson *et al*., 2009). Fungi species used in the antifungal activity test were *Candida albicans*, *Trichophyton mentagrophytes* and *Microsporum gypseum*. All of these pathogenic fungi were clinical isolates, obtained from PT Bio Farma, Bandung, Indonesia. The fungi were cultured and maintained on Sabouraud's dextrose agar, SDA (Difco, USA) at 25°C. The lyophilisate was dissolved in sterile distilled water at various concentrations. Fifteen micro liter of the solution was then dropped onto a paper disc mounted on test fungi-inoculated agar plates in a Petri dish. Three replicates were used in this procedure. Incubation was carried out at 25°C for 72 h for all test fungi. The antifungal activity was determined by measuring the diameter of growth inhibition zone.

Minimum inhibitory concentration determination: The determination of MIC was performed by agar diffusion method using a series of concentrations of the lyophilisate solution. MIC Observations were performed after 72 h of incubation period at 25°C. The MIC was the lowest concentration that demonstrated inhibition zone.

Determination of equivalence to reference antifungal agents: The procedure was performed by agar diffusion method as that in antifungal activity test. A curve between the diameter of the inhibition as function of the logarithm of concentration was constructed. Ketoconazole was the reference agent for *C. albicans*, while Griseofulvin was used for *T. mentagrophytes* and *M. gypseum*. The equivalence was calculated from the equations of the curves, obtained by linear regression method.

RESULTS

Results of anatomical examinations on the dorsal skin of *D. melanostictus* frog are presented in Fig. 1 through 3. As shown in Fig. 1, gross anatomic observations revealed modified skin structures, such as tubercle and verrucea. A pair of parotid glands was seen posterior to the eyes.

Figure 2 depicts the results of SEM assay of the surface of dorsal skin of *D. melanostictus* frog. The scanning electron micrograph demonstrated grooves, the fold-like structures, on the surface of the skin (Fig. 2a). At the center of the tubercle, an opening, which seemed to be the outlet for glandular secretions, was observed (Fig. 2b).

More detailed observation through SEM assay on the cross section of dorsal skin unveiled clearer presentation of the mucus as well as granular gland, as

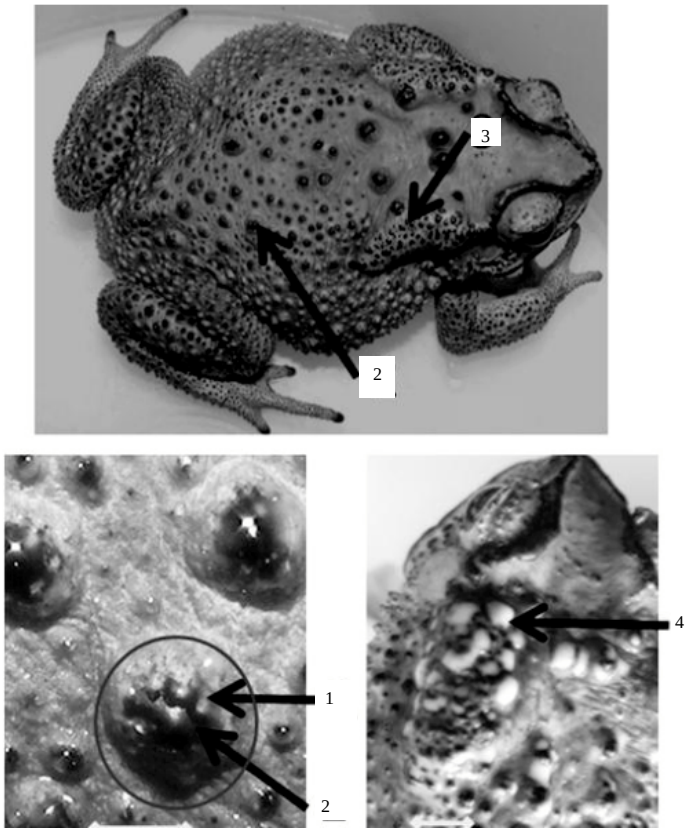

Fig. 1: Presentation of the frog *Duttaphrynus melanostictus*. Macroscopic examination shows: 1. Tubercles, 2. Verrucae, 3. Parotid gland, 4. Whitish milk-like secretion produced upon electric stimulation

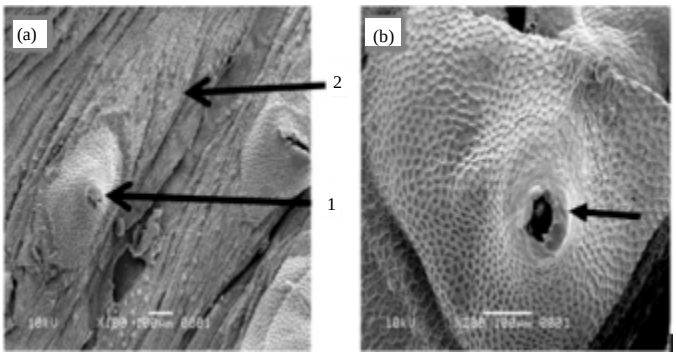

Fig. 2(a-b): Scanning electron micrograph of dorsal skin of *Duttaphrynus melanostictus* frog. (a): 1. Tubercle, 2. Groove (100 X); (b): Excretory opening on a tubercle (arrow) (200 X)

shown in Fig. 3. The micrograph showed that the mucous glands were scattered throughout the epidermal region of the skin, while the granular glands resided beneath the mucous gland within the dermal region (Fig. 3a). In terms of the shape of the respective gland, whereas the mucous gland seemed to be uniform, the granular glands in

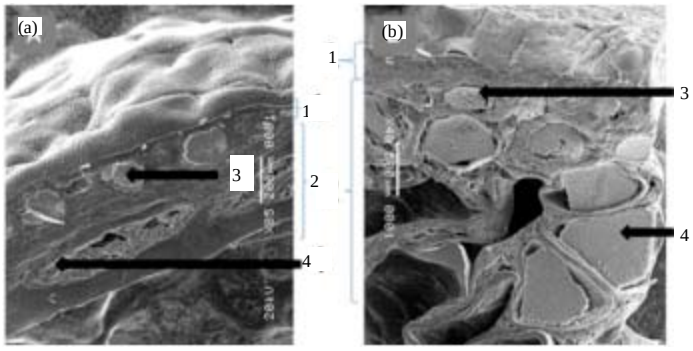

Fig. 3(a-b): Cross sectional scanning electron micrograph of dorsal skin of *Duttaphrynus melanostictus* frog. (a). Skin cross section (85 X), (b). cross sectional presentation of parotid gland (40 X); 1. Epidermis, 2. Dermis, 3. Mucous gland, 4. Granular gland

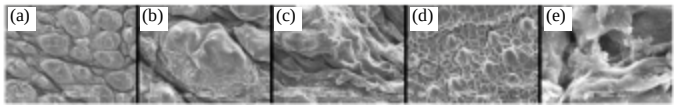

Fig. 4(a-e): Scanning electron micrograph of ventral skin of *Duttaphrynus melanostictus* frog, as observed under escalating magnification power, (a). 30 X, (b). 100 X, (c). 200 X, (d). 500 X, (e). 2500 X. Unlike structural feature of the dorsal skin, the ventral skin was lacked of secretory glandular structures

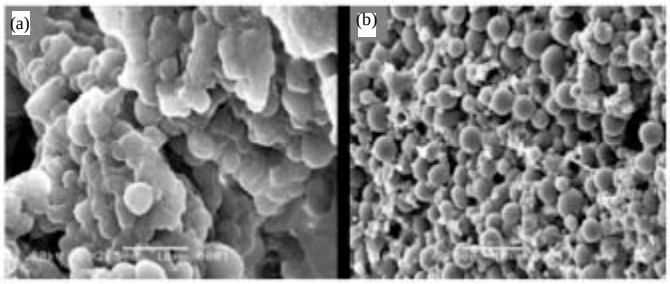

Fig. 5: Scanning electron micrograph of dermal secretory structures in *Duttaphrynus melanostictus* frog. A. flattened, irregular-shaped structures of the mucous gland and B. grape-like vesicles of the granular gland (2500 X)

parotid organ appeared larger and denser compared to that found in other part of the skin (Fig. 3b).

Figure 4 demonstrates the scanning electron micrograph of the ventral skin of *D. melanostictus* frog. Present observation revealed that the structure was considerably different to that of the dorsal skin. Coarse superficial skin structures were noted, but even as we went through the higher magnification (30 X, 100 X, 200 X, 500 X, 2500 X), we could hardly find structures related to glandular secretory system.

Detailed presentations of dermal secretory structures are shown in Fig. 5. A flattened, irregular-shaped structure was observed (Fig. 5a), assumed to be the mucus secreting structure. Meanwhile, the granular gland (Fig. 5b), seen as grape-like structure, is a common structure among several frog species.

Results of antifungal tests using agar diffusion method showed inhibitory activity on the microbial culture media at concentrations of 60 and 100 mg mL^{-1} in all test fungi (Table 1).The test continued

Table 1: Antifungal activity of the lyophilisate of *Dnttaphrynus melanostictus* frog skin extract against pathogenic fungi

Test fungi	Concentration of extract (mg mL^{-1})	Inhibition zones (mm)[a]
C. albicans	60	17.13±15.45
	100	21.65±0.35
M. gypseum	60	19.85±0.25
	100	21.85±0.75
T. mentagrophytes	60	18.55±1.15
	100	20.25±0.45

[a]values are averages±SD of three replicates

Table 2: Minimum Inhibitory Concentration (MIC) of the lyophilisate of *Dnttaphrynus melanostictus* frog skin extract against pathogenic fungi

Test fungi	MIC (mg mL^{-1})	Diameter of inhibition zones (mm)[a]
C. albicans	0.5	10.04±0.25
M. gypseum	25.0	15.75±0.95
T. mentagrophytes	25.0	15.60±0.70

[a]values are averages±SD of three replicates

Table 3: Equivalence of antifungal activity of the lyophilisate of *Duttaphrynus melanostictus* frog skin extract to reference antifungal agents against pathogenic fungi

Test fungi	Reference agent	Quantity of reference agent (μg) equivalent to 100 mg lyophilisate
C. albicans	Ketoconazole	0.78
M. gypseum	Griseofulvin	93.24
T. mentagrophytes	Griseofulvin	50.38

with the sweeping through narrower ranged concentrations to determine the lowest concentration that produced inhibition. The lyophilisate of frog skin extract was most active against *C. albicans*, in which the lowest concentration to demonstrate inhibition was 0.5 mg mL^{-1} or 0.05% (w/v) with a diameter of inhibition of 10.04±0.25. Meanwhile, the lowest concentration to produce growth inhibition in *M. gypseum* and *T. mentagrophytes* was 25 mg mL^{-1}, equivalent to 2.5% (w/v) with diameter of inhibition of 15.75±0.95 and 15.6±0.7 mm, respectively (Table 2).

Results of the tests for equivalence in antifungal activity of the lyophilisate to reference antifungal agents are presented in Table 3. It was shown that 100 mg extract was equivalent to 0.78 μg of ketoconazole against *C. albicans* ($y = 7.666x+22.495$, $R^2 = 0.924$). Meanwhile, against *M. gypseum* and *T. mentagrophytes*, 100 mg extract was equivalent to the amount of griseofulvin of 93.24 ($y = 30.671x-38.560$, $R^2 = 0.974$) and 50.38 μg ($y = 17.694x-8.680$, $R^2 = 0.910$), respectively.

DISCUSSION

Structurally *D. melanostictus* skin consisted of two layers, epidermis and dermis. Two types of glands were observed in the epidermal layer, the mucous and granular glands. In general, the granular gland such as that found in our study produces whitish milk-like secretion which is more poisonous than that of the mucus gland and contains peptides, amines and alkaloids. The secretion is released under stimulation of sympathetic nerve or hormones, which prevent frogs from predation and is effective against bacteria and fungi (Duellman and Trueb, 1994). In *D. melanostictus*, the species studied in present study, the gland producing milk-like secretion was probably that of the granular type. The fact which showed that this secretion was produced after delivery of electrical shock (Fig. 1) corroborates this notion. In addition, the shape of secretory vesicle of the granular gland, which was grape-like, found in the present study (Fig. 5) looked exactly the same as that of secretory granules found in *Phyllomedusa bicolor*, the storehouse of the microbe-active peptide dermaseptin (Lacombe *et al*., 2000).

The mucous gland produces clear secretion containing glycoprotein such as mucin, mucinogen, sialic acid and carbohydrate residue including galactose and fructose (Garg *et al*., 2008) secreted continuously to maintain skin moisture (Duellman and Trueb, 1994). As we found in our study, the mucous glands were located closer to the surface of the skin. This topography of the gland might be essential in enabling the mucus to reach the surface of the skin easily and prevent it from dryness. It is interesting to note that the mucous glands were mostly distributed throughout the dorsal skin and it seemed to be sparser in ventral skin area, as we could hardly notice any secretory outlet from the micrograph. Indeed, although, in general, the mucous glands are occurring over the entire body, these glands are especially denser dorsally (Zug *et al*., 2001). Such a distribution pattern of the glands might be considered vital in coping with much more intense exposure of dorsal skin to extreme environment, such as high temperature and high intensity light. Upon a more detailed examination on the mucous gland, as shown in Fig. 5a, we observed a flattened, irregular-shaped structure and this was probably the mucus secreting structure, comparable to secretory vesicle of the granular gland. As far as one can tell, this type of secretory structure was never reported before and was possibly a feature distinct to *D. melanostictus* species.

To explore the biological activity, we promptly embarked on the study of antifungal effect of the skin extract. This was based on cumulative previous results which have shown antimicrobial effects of peptides produced by certain species of frogs. Thus, Simmaco *et al*. (1993, 1996) have reported antimicrobial peptides from skin secretion of the European frogs *Rana esculenta* and *R. temporania*, the peptide from the latter frog has now been designated as temporin. Furthermore,

the peptides brevinin and alyteserin have been isolated from the frog *Lithobates septentrionalis* (Conlon *et al*., 2009) and *Alytes obstetricans* (Conlon *et al*., 2010), respectively. Present results demonstrated that the lyophilisate of the skin extract was active against *C. albicans*, *M. gypseum* and *T. mentagrophytes* to nearly the same degree. The data further showed that the most sensitive fungus was *C. albicans*, as shown by the lowest concentration to inhibit the fungal growth. These results indicated that the lyophilisate contained substances, most likely peptide, which may be used in infections caused by *C. albicans*, *M. gypseum* and *T. mentagrophytes*. Unfortunately, however, we noted that the lyophilisate were much weaker when its activity was compared to reference antifungal agents. This might be attributable to the complexity of substances contained in such a raw extract. Further isolation and refinement of the microbe-active ingredients are, therefore, essential. Yet, this evidence might be of importance from clinical standpoint since candidal infection remains a major cause of morbidity and mortality in the health care setting and the epidemiology of Candida infection is keep changing (Horn *et al*., 2009). Meanwhile *T. mentagrophytes* and *M. gypseum* are two species with the largest contribution to dermatophytes infections (Adefemi *et al*., 2010).

In the present work the structural characteristics of the skin of *D. melanostictus* frog were explored, followed by the preliminary study on antifungal activity of the skin extract. To the best of our knowledge, the present study was the first study on the characteristic of *D. melanostictus* skin and its antifungal activity.

CONCLUSION

Results of present study have shed some light on the structural characteristic of *D. melanostictus* frog skin, with the demonstration of several newly unveiled structures. To the best of our knowledge, our present data was the first presentation of a fairly detailed skin anatomy of the species. The results further showed antifungal activity of the lyophilisate of the skin extract against *C. albicans*, *M. gypseum* and *T. mentagrophytes*. This data can be an implication of some promising candidates to be developed as antifungal agents, which can be essential, particularly, in the face of the emergence of resistant microbes.

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