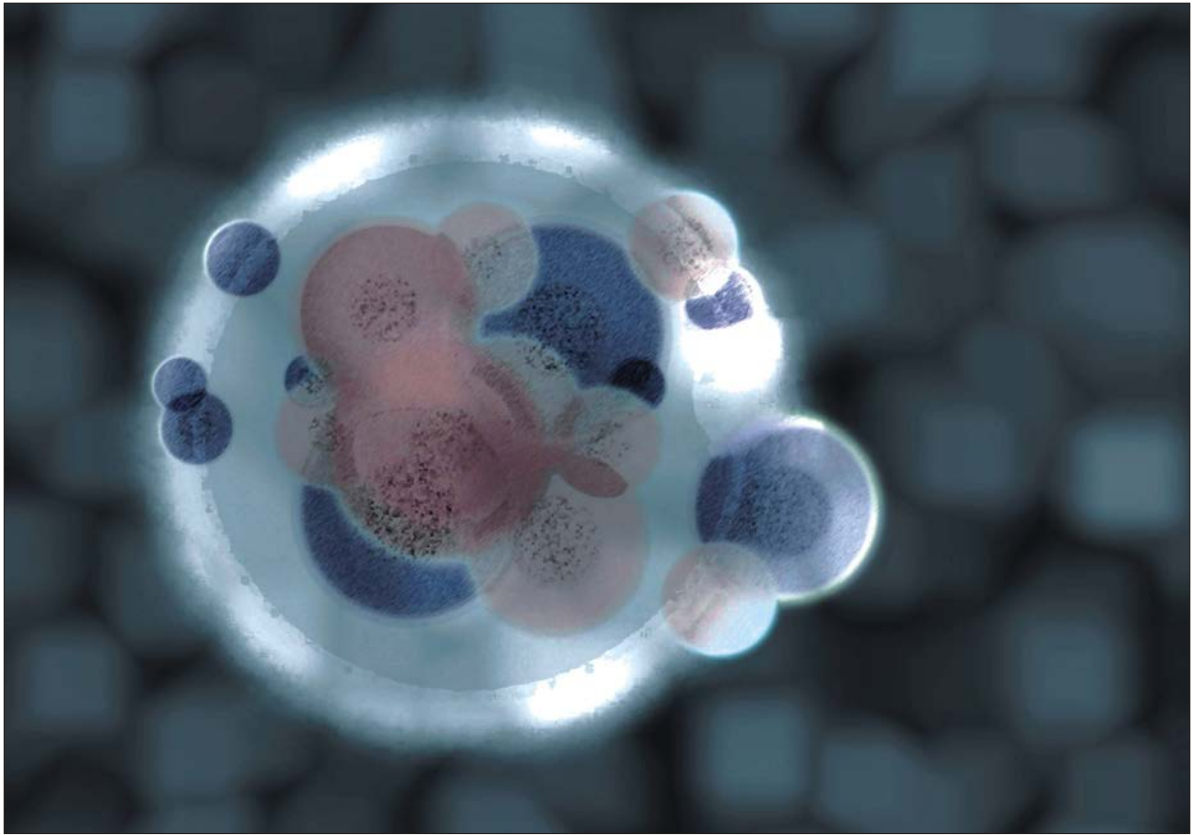




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Damage in Fungal Morphology Underlies the Antifungal Effect of Lyophilisate of Granular Gland Secretion from *Duttaphrynus melanostictus* Frog

¹Anggraini Barlian, ²Kusnandar Anggadiredja and ¹Astuti Kusumorini

¹School of Life Sciences and Technology, Institute of Technology Bandung

²School of Pharmacy, Institute of Technology Bandung,
Jl. Ganesa 10 Bandung 40132, Indonesia

Abstract: The treatment of fungal infection poses particular challenge for the medical field. Thus, the development of antifungal agents has only been progressed slowly, partly due to the considerably high toxicity of the agents. We have previously demonstrated the antifungal effect of the skin extract from *Duttaphrynus melanostictus* frog. In the current study, the antifungal effect was particularly investigated in the lyophilisate of granular gland secretion of the frog skin against the pathogenic fungi *Candida albicans*, *Mycrosporium gypseum* and *Trychophyton mentagrophytes*. Antifungal test of the lyophilisate was performed by agar diffusion method, while the study of its mechanism of action was carried out under Scanning Electron Microscope (SEM). The lyophilisate was shown to be equipotent against all test fungi *in vitro*. Mean while, SEM assay revealed that the lyophilisate induced detrimental effect on cellular membrane integrity as indicated by the formation of pores in all test fungi and the shrinkage of hyphal and microconidial structures in *M. gypseum* and *T. mentagrophytes*, possibly due to the leakage of essential intracellular components. The results open an opportunity for developing effective alternative antifungal agents, particularly in the face of the emergence of drug-resistant fungi.

Key words: Antifungal, lyophilisate, granular gland, *Duttaphrynus melanostictus*, frog, fungal morphology

INTRODUCTION

Mycosis, the fungi-caused disease, has been considered of high clinical importance, whose prevalence was reported to be as high as 20-25% of the world's population (Havlickova *et al.*, 2008). Until recently, the treatment of mycosis still relies on several synthetic antifungal agents belonging to the class of azole, polyene and antimetabolite (Di Piro, 2003).

The development of antifungal agents has historically lagged behind compared to that of the antibacterial drugs (Pfaller and Yu, 2001). One of the obstacles in this area of medicine lies in the fact that fungi are eukaryotes. The resemblance of fungal cellular structure with that of the mammalian, including human, has been responsible for a number of toxic effects exerted by antifungal agents on the host. In addition, fungi grow slowly, can take multicellular form and under extreme condition they have the capacity to change into particular form, such as bud, spore, conidia or germ tube (Liu and Xiao, 2005).

The emergence of resistant pathogens is one of the reasons for the development of peptide-based antifungal

agents. These selective fungicides were originally the component of natural protection system of many living organisms, consisting of 12-50 amino acid with molecular weight of less than 10000 (Hancock, 2001). According to Theis and Stahl (2004), peptides are very good candidates to be developed as antifungal agents with expected superiority over conventional antibiotics.

During the last three decades, approximately 600 peptides with high antifungal potency have been isolated from amphibians (De Lucca, 2000; Theis and Stahl, 2004). One of these peptides is dermaseptin, isolated from *Phyllomedusa bicolor*, a tree frog from South America. This peptide has been demonstrated to inhibit the growth of *Candida albicans*, *Aspergillus flavus*, *A. fumigatus* and *Fusarium oxysporum* (De Lucca, 2000).

The frog *Duttaphrynus melanostictus* is a species ubiquitous in Indonesian soil. As commonly observed in amphibians, despite living in moist environment, there has not been any report on fungal infection in this species. This fact warrants inquiry into the antifungal potency of the secretion from the frog's secretory glands. Our previous work demonstrated the skin extract of the *D. melanostictus* frog had antifungal

activity (Barlian *et al.*, 2011). In the present work we tested the activity of the lyophilisate of granular gland secretion of *D. melanostictus* frog skin against *Candida albicans*, *Trichophyton mentagrophytes* and *Microsporum gypseum*. Growth inhibition observed with agar diffusion method was confirmed by electron microscopy to pin down the mechanism of the antifungal activity.

MATERIALS AND METHODS

Preparation of granular gland secretion: 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. Each frog was immobilized and electric shock of 220 V was delivered onto the surface the back skin for 1 second. Immediately after the shock a whitish milk-like liquid of the granular gland origin was released from the black-granulated part of the skin. Physiological saline was then flushed onto the skin for the isolation of the secretion. The solution thus obtained was collected and freeze dried at -80°C.

Antifungal activity test: The test was performed by agar diffusion method, as done in previous studies (Dulger and Gonuz, 2004a, b; Alanı and Mostahar, 2005; Musyimi and Ogur, 2008; Ajibesin *et al.*, 2008; Pieme *et al.*, 2008; Sittiwet *et al.*, 2009; 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 to make a final concentration of 7.5% v/v. 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.

Scanning electron microscopic (SEM) assay of antifungal activity: Antifungal effect of the lyophilisate was later confirmed under scanning electron microscope to clarify the antifungal mechanism of action. For electron in SDM

microscopic examination, two suspensions of each fungus medium were prepared, each with or without the lyophilisate (final concentration of the lyophilisate in the suspension was 7.5% w/v). All suspensions were then incubated in shaking incubator at 25°C for 72 h. After the incubation was completed, the fungal cells were harvested by centrifugation in an initial fixative solution (2% glutaraldehyde in sodium cacodylate buffer). The cells were then washed with phosphate buffer pH 7.4 and added final fixative solution (1% v/v OsO₄ in phosphate buffer pH 7.4). This procedure was continued with dehydration in serial ethanol dilution (50, 75, 88, 94 and 100%). The cells were then suspended in t-butanol. Subsequently, the suspension was dropped onto a cover slip and freeze dried at -50°C. 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 5000-20000X magnification.

RESULTS

Results of antifungal tests against test fungi are presented in Table 1. The potency of the test substance,

Table 1: Antifungal activity of the lyophilisate of granular gland secretion from *Duttaphrynus melanostictus* frog skin. Fifteen microliter of 7.5% w/v lyophilisate solution was dropped onto a paper disc mounted on agar plate pre-inoculated with the test fungi. Incubation was done at 25°C for 72 h

Test fungi	Diameter of growth inhibition zone (mm) ^a
<i>C. albicans</i>	20.3
<i>M. gypseum</i>	24.5
<i>T. mentagrophytes</i>	24.7

^aValues are averages of three replicates

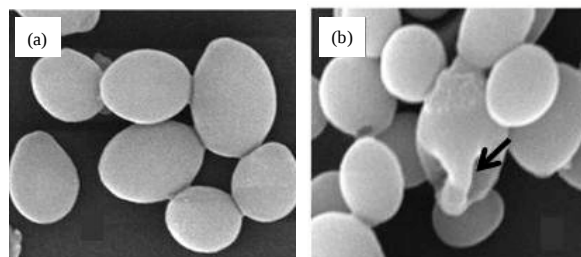


Fig. 1(a-b): Scanning electron micrograph of *Candida albicans* after exposure to the lyophilisate of granular secretion from *Duttaphrynus melanostictus* frog. *C. albicans* was suspended in culture media without (a) or with (b) the lyophilisate for 72 h. Exposure to the lyophilisate induced the formation of pores in cells, as indicated by arrow (10000X)

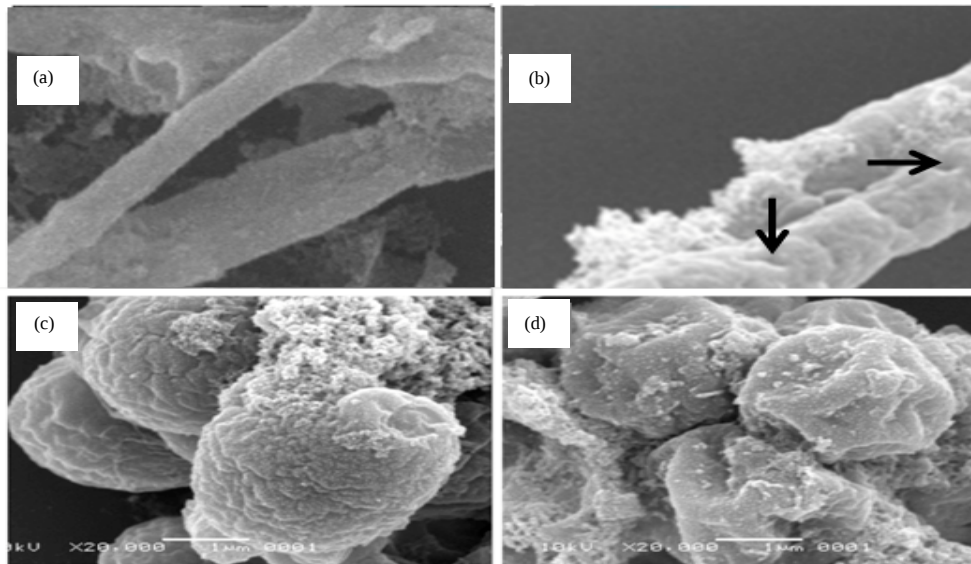


Fig. 2 (a-d): Scanning electron micrograph of *Microsporium gypseum* after exposure to the lyophilisate of granular gland secretion from *Duttaphrynus melanostictus* frog. *M. gypseum* was suspended in culture media without (a, c) or with (b, d) the lyophilisate for 72 h. Exposure to the lyophilisate induced deformation of cellular structure, as shown by hyphal shrinkage as well as formation of pores (arrows in b) and the shrunken conidia (d). Magnification: 5000X (a, b), 20000X (c, d)

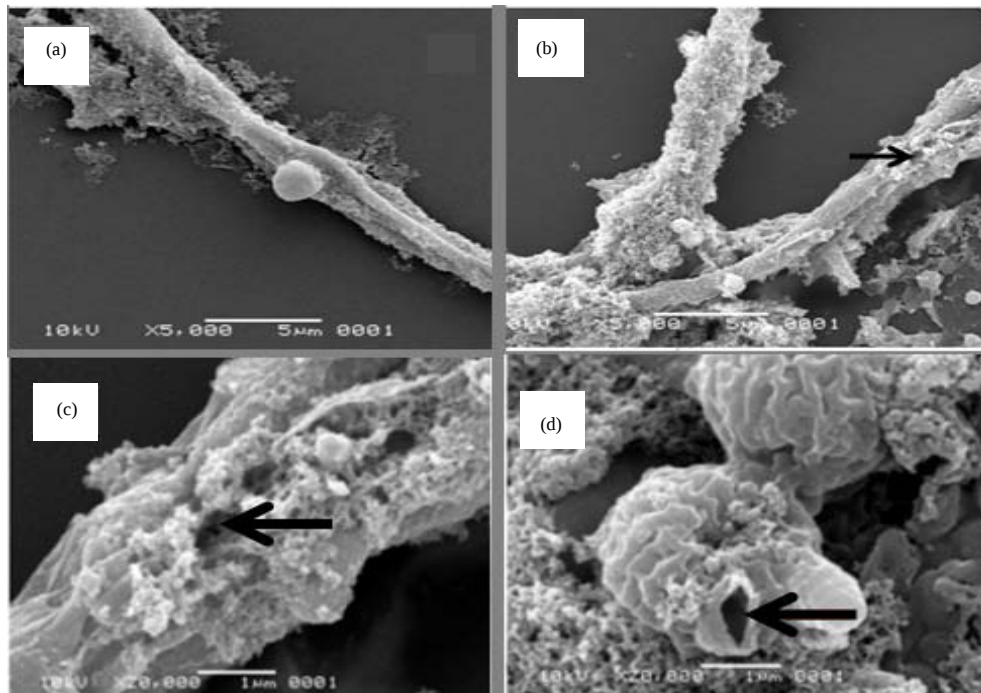


Fig. 3 (a-d): Scanning electron micrograph of *Trichophyton mentagrophytes* after exposure to the lyophilisate of granular gland secretion from *Duttaphrynus melanostictus* frog. *T. mentagrophytes* was suspended in culture media without (a) or with (b, c, d) the lyophilisate for 72 h. Exposure to the lyophilisate induced deformation of cellular (b, c) and microconidia (d). Magnification: 5000X (a, b), 20000X (c, d)

lyophilisate of the granular gland secretion, against all test fungi were comparable, as demonstrated by the similarity in diameter of growth inhibition zone observed (20.3, 24.5 and 24.7 mm, against *C. albicans*, *M. gypseum* and *T. mentagrophytes*, respectively).

Morphological changes induced by exposure to lyophilisate of the granular gland secretion are presented in Fig. 1 through Fig. 3. Figure 1a depicts the absence of structural changes in non-exposed candidal cells. On the other hand, as shown in Fig. 1b, exposure to the lyophilisate led to morphological changes in the fungal cells, characterized by cellular deformation and the formation of pores. In *M. gypseum*, unlike the normal structures depicted in Fig. 2a and c, exposure to the lyophilisate led to the shrinkage of the hyphae (Fig. 2b) as well as microconidia (Fig. 2d). Furthermore, under higher magnification pores were also noted on the surface the shrunken hyphae. As observed in *M. gypseum*, no damage was found in non-exposed *T. mentagrophytes* (Fig. 3a), while exposure to the lyophilisate produced shrinkage and pores in hyphal (Fig. 3b, c) as well as microconidial (Fig. 3d) structures of the fungus.

DISCUSSION

Results of the antifungal testing showed that the lyophilisate of the granular gland secretion exerted growth inhibition activity against all test fungi to almost the same degree. It might be, thus, assumed that the lyophilisate contained ingredients which are potent against *T. mentagrophytes*, *M. gypseum* and *C. albicans* infections. This finding is beneficial due to the fact that candidal infection remains a major cause of morbidity and mortality in the health care setting and the epidemiology of *Candida* infection has been changing considerably (Horn *et al.*, 2009). Meanwhile *T. mentagrophytes* and *M. gypseum* are two species with the largest contribution to dermatophytes infections (Adefemi *et al.*, 2010).

Detailed examination on the antifungal activity of the lyophilisate through SEM assay showed that the exposure to the lyophilisate led to deteriorating structural changes in test fungi. In general, the cellular surface became shrunken and pores were formed throughout the cell. These cellular deformations have been implicated to lead to the loss of water, electrolytes and other vital intracellular components essential for the cell survival. Such a mechanism has been well documented in the action of several antifungal agents that interfere with cell wall integrity. Thus, by binding the fungal membrane sterol, the polyene fungicide nystatin has been shown to induce the increases in ionic permeability (Andreoli and

Monahan, 1968) and amphotericin B was demonstrated to increase nonelectrolyte permeability (Andreoli *et al.*, 1969). The present findings suggest that the fungal active ingredients in the lyophilisate also affected the integrity of fungal cell wall or membrane and the damages thus produced would eventually lead to a striking degree of detrimental changes in the trans-membrane transport of essential electrolytes and water.

Previous studies have revealed several peptide antifungal agents isolated from certain frog species (such as reported by Simmaco *et al.* (1993, 1996), Conlon *et al.* (2009), Subasinghage *et al.* (2010) and Conlon *et al.* (2010). These short-chained peptides, naturally the component of individual defense mechanism, have been shown to be very lethal against fungi (Theis and Stahl, 2004). In general, these low molecular weight peptides comprised no longer than 50 amino acid chains. Cecropin A, a naturally occurring peptide consisting of 37 amino acids, has been demonstrated to form a complex with lipopolysaccharide (Boman *et al.*, 1985; De Lucca *et al.*, 1995) and was later revealed to target ergosterol and cholesterol in exerting its antifungal actions against *Aspergillus* and *Fusarium* isolates (De Lucca *et al.*, 1997). In addition, a synthetic proteolytic-resistant peptide of 17-amino acid-long has been shown to bind cholesterol in the same fungal isolates (De Lucca *et al.*, 1998a). De Lucca *et al.* (1998b) also reported a physicochemical data indicating that cecropin B and dermaseptin bound to ergosterol and cholesterol, conidial wall constituents but not to chitin or beta-1,3-glucan. Taking into account our present results, it is intriguing to note the similarity in terms of the pattern of damage to our test fungi after exposure to lyophilisate of the granular gland secretion of *D. melanostictus* frog. Taken together, present results seem to be in favor with the previous works. The antifungal active ingredients contained in the lyophilisate of granular gland secretion from *D. melanostictus* frog might belong to antifungal peptides, which affect cellular structural integrity in exerting the antifungal activity. To delineate the exact component of the lyophilisate responsible for the antifungal effect, however, further structural elaborations are essential. This could be paralleled by follow up studies to assess the safety aspect.

CONCLUSION

The present results demonstrated antifungal activity of the lyophilisate of the granular gland secretion from *D. melanostictus* frog against the pathogenic fungi *C. albicans*, *M. gypseum* and *T. mentagrophytes*. We further showed for the first time the mechanism of action

of the lyophilisate in exerting its detrimental effect on the fungi. Our present findings can, thus, be a signpost for the development of potent antifungal agents.

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